ViVac Final Report

- Publishable Summary

Executive summary

Safe and efficient prophylactic vaccines are still lacking for a number of infectious diseases. Moreover therapeutic vaccines to e.g. cancer, autoimmune diseases or allergy, constitute an emerging area of interest. The effect of a vaccine can be enhanced by adjuvants, which are also able to direct the type of immune response elicited. Thus novel adjuvants have the potential to improve prophylactic and therapeutic vaccines.

In the ViVac project the overall objective was to develop and to show safety and efficacy for a new innovative carbohydrate (chitosan) based adjuvant - ViscoGel - to be used both in prophylactic and therapeutic vaccination. For prophylactic vaccination a model vaccine to *Heamophilus influenzae* type b (causing bacterial meningitis), Act-Hib, was used to show preclinical and clinical proof of concept (POC). For therapeutic vaccination the target was allergy vaccination using the major birch pollen allergen Bet v1. In addition an immune- and permeation- enhancing peptide, LTX-315, was evaluated, since mucosal allergy vaccination through the sublingual route was specifically addressed. Three SMEs (Viscogel AB, SE; Lytix Biopharma AS, NO; Inmunotek SL, ES) provided their background technologies to the project: ViscoGel manufacturing, LTX-315 and recombinant as well as natural purified Bet v 1.

Research and technical development (RTD) activities were performed to reach the project goals. Extensive formulation work was carried out including design, development and characterization of chitosan and ViscoGel formulations. Chitosan analysis methods were developed and careful analysis of chitosan and ViscoGels performed. A number of activities focused on the development of a clinical ViscoGel product for prophylactic vaccination. Preclinical characterization of ViscoGel-Act-Hib led to the identification of a ViscoGel clinical product candidate that was subjected to extensive characterization, process development and GMP production. Toxicity evaluation of ViscoGel and ViscoGel-Act-Hib was carried out in two species. The compiled preclinical documentation on ViscoGel formed the basis for regulatory and ethical approval to perform a clinical Phase I/IIa trial to show POC for prophylactic vaccination. The clinical trial evaluated safety and efficacy of one intramuscular injection of ViscoGel alone and as adjuvant for Act-Hib. RTD on ViscoGel formulations for allergy vaccination followed two lines, development of vaccines for subcutaneous (SCIT) and for sublingual (SLIT) allergen specific immunotherapy/vaccination. The target was birch pollen allergy and the major allergen Bet v 1. Mouse experiments were performed to find promising Bet v 1 SCIT candidates formulated with ViscoGel. A mouse model for birch pollen allergy and SLIT was established to obtain preclinical POC for ViscoGel as an adjuvant in SLIT. Experiments to identify SLIT candidates were performed with a model antigen, OVA, evaluating formulations containing OVA in chitosan solutions, with ViscoGel and with LTX-315. Finally a Bet v 1 SLIT candidate was tested in the SLIT model.

The most important result of ViVac was the development of a clinical ViscoGel adjuvant product that was applied in a Phase I/IIa trial. Intramuscularly administered ViscoGel was shown to be safe and well tolerated in man. Subgroup efficacy analyses revealed a positive adjuvant effect of ViscoGel. These results are of critical importance for the exploitation of ViscoGel as adjuvant in human vaccines. For therapeutic vaccination useful data were obtained on formulation of ViscoGel based allergy vaccines intended both for SCIT and for mucosal vaccination, which will support further developmental work.

In conclusion, ViVac has provided an innovative platform for development of ViscoGel as adjuvant in prophylactic and therapeutic vaccines. The project has generated new foreground and potential new collaborations for the benefit of the participating SMEs. In ViVac, ViscoGel was proven to constitute a simple, safe and versatile adjuvant system applicable for human use. In a wider perspective, such an adjuvant system may provide solutions to current challenges in the development of therapeutic vaccines to e.g. cancer, autoimmune diseases and allergy, as well as for the development of vaccines to infectious diseases to which efficient protection cannot be provided with present vaccines.

Summary description of project context and main objectives

There is a huge global demand for new vaccines able to elicit efficient and appropriate immune responses to infectious agents (prophylactic vaccines) as well as vaccines capable to modify pathogenic immune responses (therapeutic vaccines). Suboptimal vaccines may be improved by adjuvants, i.e. enhancers of the immune response. Adjuvants also provide means to modify an immune response and direct it towards a specific functional response. The route of administration is known to affect the immune response stimulated and, in addition, adjuvants can be designed to act as vehicles or delivery systems for vaccine administration, e.g. for mucosal vaccines. Only four adjuvants in total have been licensed in the EU and US. There is therefore a significant opportunity for new adjuvants and vaccine technologies, as vaccine design has focused on highly purified antigens with limited efficacy. In addition to increasing the efficacy and duration of a vaccine, an efficient adjuvant can reduce the vaccine costs and improve the supply in a pandemic, which is of interest to governments and global health organisations. Moreover, new vaccination strategies are sought for therapeutic indications, e.g. cancer and allergy, where specialised adjuvants constitute an emerging target for development in order to obtain immune modulation.

In the ViVac project the **overall objective** was to develop and to show safety and efficacy for a new innovative carbohydrate (chitosan) based adjuvant - ViscoGel® - to be used both in prophylactic and therapeutic vaccination. In the project ViscoGel has been applied as adjuvant for a model prophylactic vaccine, the commercially available *Haemophilus influenzae* type b (Hib) glycoconjugate vaccine, with the aim to show proof of concept (POC) in man. The target for therapeutic vaccine development has been allergen-specific immunotherapy, i.e. therapeutic vaccination for treating allergic disease. The objective for therapeutic vaccination was to show pre-clinical POC for treatment of allergy with a novel efficacious vaccine formulation composed of the main birch pollen allergen Bet v 1 and ViscoGel acting as adjuvant and vehicle for administration over the sublingual mucosa. Finally the peptide LTX-315 was evaluated for its immune potentiating effects.

Specific objectives set up for ViVac:

1. to develop analytic tools for quality assessment of ViscoGel preparations and provide building blocks for formulations
2. to characterize and optimize chitosan gel systems with controllable biodegradation and mucoadhesive properties
3. to provide GMP-produced ViscoGel formulated with Act-Hib for clinical application
4. to investigate the nature of the immune response to antigens administered with ViscoGel and if the co-formulation with LTX-315 can enhance the adjuvant and epithelial penetrating capacity
5. to provide formulations of ViscoGel with Bet v 1, with LTX-315 and with both Bet v 1 and LTX-315 intended for preclinical application
6. to show POC for prophylactic vaccination using ViscoGel formulated with Hib and to demonstrate preclinical and clinical safety.
7. to show POC for therapeutic vaccination using ViscoGel formulated with Bet v 1 in relevant preclinical sub-lingual immunotherapy (SLIT) and subcutaneous immunotherapy (SCIT) models for treatment of birch pollen allergy
8. to develop new and increased IP protection for candidate ViscoGel formulations
9. to obtain the documentation needed for clinical development and commercialisation

Three SMEs participated in the ViVac project and contributed with their expert competences:

- Viscogel AB, Sweden, provider of the ViscoGel technology – a chitosan based gel with demonstrated immunostimulatory capability and unique properties making it suitable for application as vaccine adjuvant. Viscogel AB manufactures medical grade chitosan (Viscosan®) that is soluble at physiological pH, from which ViscoGel is processed by crosslinking the chitosan carbohydrate chains, generating a viscoelastic gel (ViscoGel®)
- Lytix Biopharma AS, Norway, provider of peptide technology and LTX-315 – a cationic/mucoadhesive peptide with cell penetrating (cytolytic) capability. Although the application area for LTX-315 so far has mainly been cancer treatment, it was hypothesised that the peptide could increase the immunomodulating and epithelial penetrating capacity when formulated with ViscoGel+antigen.
- Inmunotek SL, Spain, provider of Bet v 1 **-** recombinant allergen and enriched natural allergen extracts technology. The company's focus is on the development and commercialization of innovative products for allergy treatment using proprietary technologies. Purified enriched natural allergens from this platform have successfully passed phase II clinical trials for allergy vaccines and diagnostics. The major birch pollen allergen Bet v 1 is an excellent model allergen for showing POC for allergy vaccination.

In order to introduce a new adjuvant on the vaccine market a number of requirements have to be fulfilled. Primarily the adjuvant should enhance the immunological response to the antigen in question (e.g. Hib), without being toxic or eliciting a response towards itself. Both a strong and sustained humoral response with elevated antibody titers as well as a cellular response with memory cells to the antigen should be induced. For therapeutic vaccination an established pathological immune response must be replaced by a beneficial immune response activated by the adjuvant. Thus immune regulation and facilitated antigen presentation should be promoted. Novel administration routes may improve the effect of vaccines and mucosal vaccination via the sublingual route has attained specific interest for allergy vaccination.

In the ViVac project, research and technical development (RTD) activities aimed to provide pre-clinical and clinical POC for prophylactic vaccination using a model vaccine against Hib (Act-HIB, Sanofi-Pasteur, MSD). For therapeutic vaccination the objective was to provide pre-clinical POC for allergy vaccination, SCIT and SLIT, with Bet v 1. Seven RTD consortium partners with specific key competences have together with the participating SME partners performed research to support technical development and new IP opportunities for the SMEs.

Competence and role of RTD performer partners in ViVac (Partner; Competence; Role in ViVac):

NOBIPOL, Norwegian University of Science and Technology (NTNU), Norway; Chitosan technology; Characterisation of chitosan and chitosan gels

Hacettepe University, Turkey; Formulation characterisation; Mucoadhesion and permeation studies

Huntingdon Life Sciences, UK; CRO performing toxicology studies; Toxicology evaluation of clinical material

Karolinska Institutet (KI), Sweden; Immunological research competence; Preclinical immunological characterisation

Stallergenes SA, France; Leading company in allergy immunotherapy and sublingual immunotherapy (SLIT); Assessment of chitosan-, ViscoGel- and LTX-315 SLIT formulations *in vitro* and *in vivo*

Stockholm county council, Karolinska Trial Alliance (KTA), Sweden; Phase I/II clinical trial unit at Karolinska Hospital, Stockholm, Sweden; Clinical trial performance

Pharma Consulting Group in Uppsala AB (PCG), Sweden; CRO providing services for clinical trials; Trial monitoring, documentation and data management

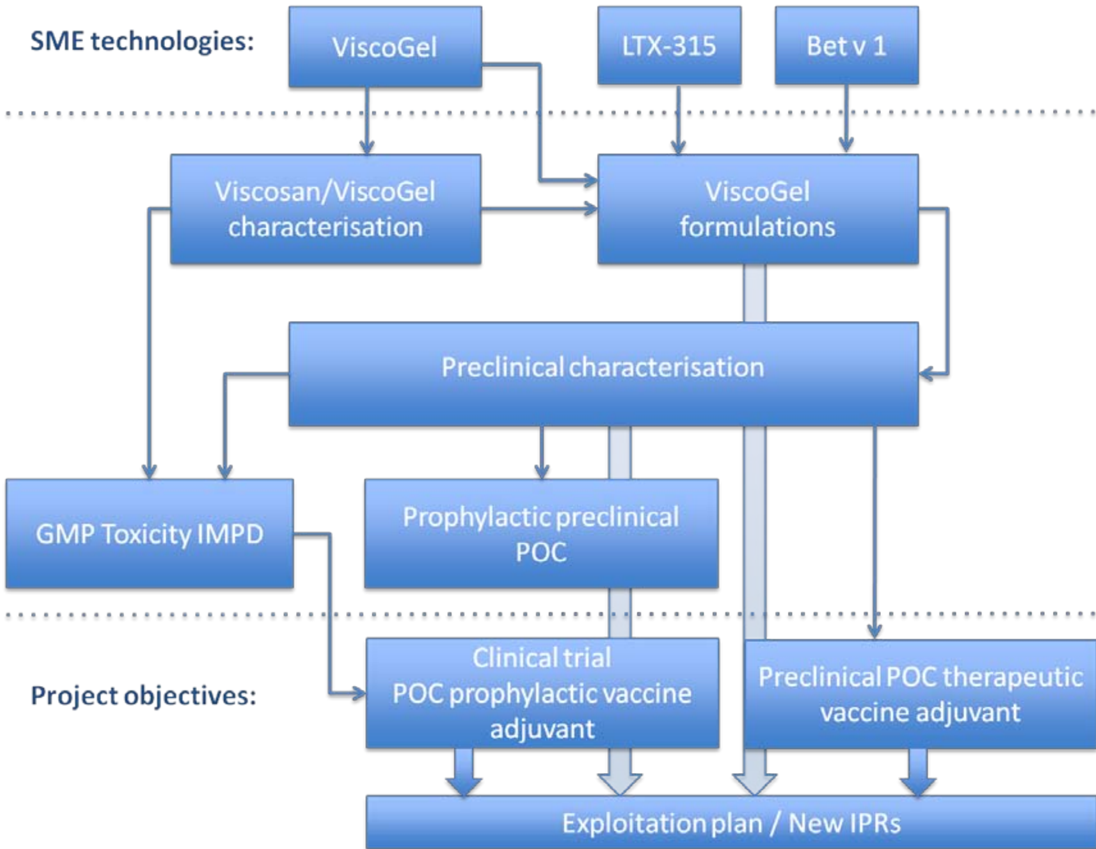
To achieve the ViVac project goals, the following activities have been performed:

1) Development and production of ViscoGel formulations, including development of a ViscoGel manufacturing process according to GMP
2) Characterisation of chitosan and gels; assessment of physicochemical, mucoadhesion and permeation properties
3) Preclinical characterisation and POC of ViscoGel as adjuvant for prophylactic vaccination to Hib
4) Toxicology evaluation and clinical trial to obtain safety data and show POC for ViscoGel in man
5) *In vitro* and *in vivo* characterization of ViscoGel preparations for SLIT application, establishment of a mouse model for sensitization to birch pollen allergen, and evaluation of candidate vaccines
6) *In vivo* characterization of ViscoGel preparations with recombinant and natural Bet v 1 for SCIT application
7) Management and dissemination of project results

The activities were organised in ten work packages (WPs):

WP1 - Production of Bet v 1
WP2 - Production of LTX-315
WP3 - Development and production of Viscogel formulations
WP4 - Characterisation and stability of chitosan / chitosan gel systems
WP5 - Formulation studies for mucosal delivery
WP6 - Toxicology studies of ViscoGel-Hib
WP7 - Proof-of-concept for prophylactic vaccination
WP8 - Pre-clinical proof-of-concept for therapeutic vaccination
WP9 - IP protection, knowledge management, training and dissemination
WP10 – Management

PROJECT OVERVIEW



Description of main S&T results/foregrounds

Introduction

The overall objective of ViVac was to develop and show proof of concept (POC) for ViscoGel as a novel adjuvant for prophylactic and therapeutic vaccination. An overview of the results of ViVac shows that the objective has been fulfilled for prophylactic vaccination, showing safety and efficacy for ViscoGel as adjuvant in man. For therapeutic vaccination useful data have been obtained for the use of ViscoGel as adjuvant, but POC was not obtained for this application. The project results will here be presented as reports of the individual milestones set up for ViVac, followed by a summary description of the main S&T results/foregrounds of the project.

List of Milestones:

1. Recombinant and natural Bet v 1 for formulation with ViscoGel
2. LTX-315 peptide for formulation with ViscoGel
3. ViscoGel formulations with Hib, Bet v 1 and LTX-315 for preclinical applications
4. Description of chitosan and gel properties, as well as stability and antigen release data
5. Characterisation of ViscoGel-formulation for SLIT
6. Preclinical characterisation and POC for prophylactic vaccination (ViscoGel-Hib)
7. ViscoGel-Hib for clinical use
8. Toxicology study completed
9. POC in man for ViscoGel as adjuvant (for Hib)
10. Bet v 1-formulations in ViscoGel w and w/o LTX-315 for SLIT and SCIT
11. POC for SCIT vaccination
12. Preclinical characterisation and POC for therapeutic vaccination –SLIT
13. Protection of IPR developed in RTD activities
14. Exploitation plan and plan for the use and dissemination of knowledge

Results, Milestone reports

Milestone 1: Recombinant and natural Bet v 1 for formulation with ViscoGel

The objective of Milestone 1 was to provide recombinant and natural Bet v 1 for formulation with ViscoGel early in the project. One hundred mg of recombinant (r)Bet v 1 and 200 mg of natural (n)Bet v 1 were delivered on time from partner Partner 3, Inmunotek SL, to Partner 1, Viscogel AB. It was important to show that both the rBet v 1 and the nBet v 1 purified from birch pollen extract were of high quality and, most importantly, that they possessed retained allergenicity. This was shown by analysis of binding to patients' IgE.

Recombinant (r)Bet v 1 (Bet v 1.0101 isotype) was produced in *E. coli* and affinity purified using standard molecular biological techniques. The allergenicity (i.e. IgE reactivity) of rBet v 1 was characterised by Western blot and direct ELISA binding assays using different concentrations of rBet v 1 on the solid phase. These analyses showed that the rBet v 1 preparation retained IgE binding.

Purified enriched nBet v 1 was produced from birch pollen extract. A total of five different preparations were produced at Inmunotek. The manufacturing process included combined chromatography and solubility fractionation to obtain allergen enriched fractions. The enriched fraction and a hypoallergenic fraction were analysed for Bet v 1 content by monoclonal antibodies and scanning densitometry, showing that the enriched fraction was indeed significantly enriched for nBet v 1.

The IgE-reactivity of rBet v 1 and nBet v 1 (enriched fraction) were compared by ELISA analysing binding to sera from 65 birch pollen allergic patients. A positive correlation between IgE reactivity to rBet v 1 and nBet v 1 was obtained.

To further characterise the IgE binding capacity of rBet v 1 compared to nBet v 1 in birch pollen extract, inhibition ELISAs were performed using serum pools from different geographical origins (Spain: n = 8, Sweden: n = 6, USA: n = 4). The results show that rBet v 1 inhibits approximately 80% of the IgE binding to the birch pollen extract, while the extract inhibits 100% of IgE binding to rBet v 1. These results are consistent with the presence of additional birch pollen allergens in the extract and they confirm that the recombinant allergen possesses allergenic properties equivalent to the natural Bet v 1.

Milestone 1 was connected to the activities in WP1, where Partner 3, Inmunotek, was the lead beneficiary.

Milestone 2: LTX peptide for formulation with ViscoGel

LTX-315 was synthesised by Bachem AG, Bubendorf, Switzerland, on commission of Partner 2, Lytix Biopharma AS. One gram of the synthetic LTX-315 peptide, LTX-315 Acetate batch 1015058, was delivered on time to Partner 3, Viscogel AB, together with a declaration on physico-chemical characteristics of the product.

The objective of Milestone 2 was to provide LTX-315 to the project. This was also the objective of WP2, with Partner 2, Lytix Biopharma, as lead beneficiary.

Milestone 3: ViscoGel formulations with Hib, Bet v 1 and LTX-315 for preclinical applications

Milestone 3 served as a checkpoint for providing formulations for prophylactic and therapeutic vaccine design and preclinical testing. The development and production of chitosan, Viscosan and ViscoGel preparations with different properties was carried out by Partner 3, Viscogel, from the start of the project and continued after the delivery date of Milestone 3 at month 9.

Manufacturing of Viscosan and ViscoGel for chitosan and gel characterisation

Viscosan and Chitosan were manufactured from the project start for different project tasks.

Viscosan was delivered to Partner 4, NTNU, for characterization of degree of deacetylation (DD), viscosity and molecular weight (MW), and for production of ViscoGel samples with varying crosslinker concentrations. A technology transfer of the ViscoGel manufacturing process to NTNU, beneficiary 4, was successfully accomplished. Subsequently ViscoGel could be prepared for analyses on site at NTNU.

Chitosan and Viscosan preparations with different DD and viscosity were prepared for analysis of mucoadhesion and formulated with the model compounds chicken albumin (OVA) or theophylline (TEO) for analysis of buccal mucosa permeability. The model substances were used instead of Bet v 1 as planned in the DoW, since the experimental set-up was shown to require large quantities of material and pose special demands on the nature of the material. Test formulations were delivered to Partner 5, Hacettepe.

Formulation development for prophylactic vaccination:

ViscoGel can be processed into gel blocks or particles of different predefined sizes. ViscoGel particles of different sizes were formulated with the model vaccine Act-Hib, i.e. a commercial glycoconjugate vaccine to *Heamophilus influenzae* type b from Sanofi Pasteur MSD, for preclinical studies to be performed by Partner 7, KI. In accordance with preclinical test results, ViscoGel formulations with Act-Hib were optimized regarding concentrations and ViscoGel characteristics identified as suitable for clinical development.

Formulation development for therapeutic vaccination:

Stability and compatibility studies were carried out for ViscoGel together with rBet v 1 and LTX-315. The compounds were shown to be compatible and formulations of good stability were possible to make.

ViscoGel formulated with rBet v 1 and nBet v 1 was produced together with control test material and was delivered to Partner 3, Inmunotek, for application in mouse SCIT experiments (WP8). For the last experiment rBet v 1 was also covalently linked to ViscoGel in order to assess if physical linkage between antigen and ViscoGel enhanced, or possibly modified, the immune response induced.

ViscoGel was formulated with LTX-315 and the model antigen OVA for a mouse experiment designed to evaluate if ViscoGel's adjuvant capacity could be enhanced by LTX-315 and if LTX-315 exerted immunostimulatory effects with less cytotoxic side effects if used in combination with ViscoGel. The experiment primarily aimed for dose-finding in relation to side effects. In summary the results showed that compared to OVA alone, both ViscoGel and LTX-315 improved the humoral response to a similar extent. The combination of ViscoGel with LTX-315 did not result in any added or synergistic effect on the humoral response (IgG1 levels). No dose-response effect was seen for LTX-315, indicating the lowest dose used (25 µg) to be sufficient to induce the effect seen. The highest dose (100 µg) resulted in most local reactions at the injection site, already one day post injection. No systemic effect in terms of weight loss or visual signs was seen for any of the experimental groups. Thus this dose-finding study revealed that 25 µg or possibly lower doses of LTX-315 can be used to achieve

immunopotentiation in mice. Whether formulation with ViscoGel can improve the effect at lower doses remains to be addressed in future experiments.

A continuous developmental program of a mucosal adjuvant /delivery system for SLIT was carried out in collaboration between Partner 1 (Viscogel) and Partner 8 (Stallergenes). Two sets of formulations were provided to Stallergenes for evaluation in dendritic cell (DC) capture *in vitro* and *in vivo* assays, cervical lymph node T-cell activation *in vivo* assay and finally for application in a SLIT model in mouse. For the screening experiments to find a vaccine candidate (DC capture and T-cell activation experiments), the model antigen OVA was formulated with different Viscosan and ViscoGel preparations. LTX-315 was also added to some formulations to evaluate the effect of adding the peptide to the mucosal vaccine candidates. Several formulation strategies were investigated: covalent linkage or just mixing the antigen to ViscoGel, different particle sizes of ViscoGel, chitosan (Viscosan) solution or ViscoGel, different acetylation degrees of the chitosan, different concentrations of Viscosan/ViscoGel and the addition of different amounts of LTX-315. Based on the results from the evaluation of these formulations using OVA, a candidate Bet v 1 vaccine was designed, where rBet v 1 was formulated by mixing the allergen in a Viscosan solution. Results of screening experiments for SLIT, as well as the POC-experiment in the Bet v 1 SLIT model are described below in the Milestone 12 report.

Milestone 4: Description of chitosan and gel properties, as well as stability and antigen release data

The major results of WP4 are given here as a report on Milestone 4. The aim of this milestone was to investigate the suitability of ViscoGel as a vehicle for antigens in vaccine design. The method development and results obtained in WP4 were of importance for compiling an Investigational medical product dossier (IMPD) for Viscosan/ViscoGel. Partner 4, NTNU, was the lead beneficiary in WP4.

Characterization of chitosans

1. The chitosans were characterized with respect to their monomer composition, i.e. degree of acetylation (F_A), and F_A–values were in the range from 0.36 to 0.58 (36 to 58% acetylated).
2. The intrinsic viscosities were in the range from 520 to 1620 ml/g, with weight-average molecular weights from 156 000 to 686 000.
3. The block length distribution was determined for the Viscosan samples with the lowest and the highest F_A-values, and was found to be very similar to the block length distributions in chitosans with known random degree of acetylation.

Characterisation of gel properties

Long-term storage experiment – Compression measurements

1. The ViscoGel formulations, independent upon F_A, M_W and degree of cross-linking, exhibited excellent stability over the 1 year period both with respect to the elastic modulus and force/deformation at failure.
2. As expected, the strongest gels were obtained using a high concentration of cross-linker and Viscosans with high molecular weight.

Kinetic measurements

1. An increased average molecular weight and degree of deacetylation increased the mechanical response (gel strength).
2. The setting curves (gelling kinetics) for the different systems exhibited a steady increase in both the elasticity modulus and the viscosity during the time of measurement.
3. Equilibrium values for the elasticity modulus and viscosity were reached after approximately 5-6 days.
4. Increasing the pH also increased the gel strength, because of larger degree of linkage formation between diethyl squarate and the preferred uncharged primary amino groups of chitosan.

Enzymatic stability of chitosan gels

1. Inclusions of highly de-acetylated chitosans prolonged the half-life of the cross-linked particles.
2. Particles with an average size of 200 µm containing 10% highly de-acetylated chitosan had an almost tripled half-life compared to standard ViscoGel particles of the same average size.

Overall conclusions

Characterisation of Viscosans has successfully been carried out applying proton NMR spectroscopy, intrinsic viscosity measurements and SEC-MALLS. These methods seem to be very well suited as they provide complimentary information with respect to chemical composition and sequence, average molecular weight and molecular weight distribution. The degrees of acetylation of the provided samples as well as their average molecular weights reported here correspond well with the values put forth by ViscoGel. The block length distribution was found to correspond very well with the distribution found in chitosans with known random degree of acetylation.

Even though the characterisation of ViscoGel systems, both in terms of bulk properties and particulate gel stability, was found to be unexpectedly challenging, the methods used for their characterization in this report seem to provide the necessary information. Small strain dynamic oscillatory rheological measurements give information on the gelling kinetics applying Viscosan samples and DES as cross-linker. All gels exhibited a classical sol/gel

setting curve, which leveled off after 5-6 days. As expected, these apparent equilibrium values increased with increasing molecular weight of the Viscosan, with increased content of acetylated units and with increased concentration of cross-linker. Equilibrium moduli were also dependent on pH around the pKa-value of the deacetylated units.

Long term (12 months) controlled storage of bulk ViscoGels revealed a very good stability; both in terms of moduli as well as for force and deformation at failure. These values were obtained applying well geometrically defined ViscoGels in a large deformation regime using a texture analyser with controlled speed of deformation. Stability of particulate ViscoGel formulations was studied applying a standard pharmaceutical dissolution unit and relevant concentration of lysozyme. Even though the absolute values from these experiments will differ from *in situ* vales due to a considerable more dynamic environment, the relative differences observed will still be valid. By incorporating highly deacetylated chitosans (up to 10% of the total chitosan concentration) the half-life of the particles could be extended by a factor of 3.

The results obtained in this WP regarding the Viscogel properties confirm the appropriateness of ViscoGel as an administration form for the presentation of antigens. The gels are very stable under sterile conditions suggesting that antigen-containing products can be manufactured and stored for a prolonged period of time. Furthermore, the obtained results also suggest that ViscoGels can be tailored to meet specific demands with respect to biodegradability and hence also specific antigen properties.

Milestone 5: Characterisation of Viscogel-formulation for SLIT

This Milestone was dedicated to studies on mucoadhesion and permeability characteristics of Viscosan and ViscoGel preparations. The aim was to gain information that could guide the design of allergen-ViscoGel/Viscosan formulations for SLIT application. The activities linked to Milestone 5 were performed by Partner 5, Hacettepe.

<u>Mucoadhesion</u>

Experimental system: The mucoadhesion studies were performed on bovine buccal tissue freshly obtained from the slaughterhouse. The epithelial tissue was separated from the connective tissue. Mucoadhesion was assessed with a Texture Analyser using a mucoadhesive rig. The force needed to detach the test formulations was recorded as a function of elongation and both maximum strength and area under the force/time curve was obtained. The results were converted into work of adhesion (mJ).

Test formulations: Viscosan and highly deacetylated (80%) chitosan were obtained from Viscogel. Commercially available chitosans investigated were Protosan (Novamatrix, Norway, 79-90% DD) and Chitopharm M (Cognis, Germany, 75% DD). The chitosan/Viscosan solutions were prepared with two levels of viscosity (high and low viscosity) and ViscoGel particles were incorporated in 80% DD chitosan samples. The model

drugs, anhydrous teophylline (TEO) and ovalbumin (OVA, lyophilized powder), were incorporated into the formulations at 0.02g /mL concentration.

Summary of results: The adhesion was found to be significantly increased with increased contact time. Increasing the contact time provides interdiffusion and chain entanglement between polymer and mucin chain in mucus membrane. Extending the contact time between mucoadhesive polymer and mucosa causes secondary bond formation, increasing the mucoadhesive strength.

Mucoadhesion was found to be affected by the viscosity of the test preparations, showing a positive correlation between viscosity and mucoadhesion (i.e. increased viscosity corresponded to increased mucoadhesion.

The degree of deacetylation of the viscosans was found to have no significant effect on mucoadhesion, indicating that viscosans with different DD may be applied in terms of mucoadhesive properties.

A summary of the results on how viscosity, DD, concentration and presence of ViscoGel particle affects mucoadhesion is shown in the Table 1. Only viscosity was found to have a significant effect on mucoadhesion, which increased with the increased viscosity.

Factor	**Effect**
Viscosity	(+)
Deacetylation Degree	(–)
Concentration	(–)
Particle presence	(–)

Table 1. A summary of the results on how viscosity, DD, concentration and presence of ViscoGel particle affects mucoadhesion is shown in the table. Only viscosity was found to have a significant effect on mucoadhesion, which increased with the increased viscosity.

The presence of TEO or OVA in the chitosan formulations was found to affect the mucoadhesive properties, generally exhibiting lower values of mucoadhesion (work of mucoadhesion, mJ) when the model compounds were formulated in the chitosan preparation, compared to the chitosan preparation alone. The addition of drug (low molecular weight or macromolecular) was also shown to affect the viscosity of the chitosan formulation.

Conclusion: Evaluation of the mucoadhesion of Viscosan with different viscosity, DD and concentrations, with or without ViscoGel particles, revealed that only the viscosity had a significant impact on mucoadhesion. The presence of TEO or OVA affected the mucoadhesive properties, indicating that the physicochemical properties of a formulated compound, such as solubility, molecular weight etc., have to be taken into account when evaluating mucoadhesion of a Viscosan/ViscoGel formulation.

Mucosal permeability

Experimental system: Freshly obtained bovine buccal mucosa from the local slaughterhouse was used as a model for the non-keratinized human buccal mucosa. The underlying tissue was

removed from the mucosa, and then the epithelium was separated from most of the connective tissue with the help of scalpel.

The permeability studies were performed in Franz diffusion cells with 2.01 cm² diffusion area and 20 mL receptor volume at 37ºC. PBS was used as receptor medium (pH 7.4), which was under constant mixing by magnetic stirring. Test chitosan formulations or solution was placed into the donor side. Samples were taken from the receptor medium at certain time intervals and the content of TEO and OVA assayed spectrophotometrically at 272 and 278 nm, respectively. Permeability coefficients were calculated from the steady state part of the permeation curves.

Test substances: Viscosan and chitosan samples with different viscosity, w/wo ViscoGel particles present, were provided by Viscogel. Two commercially available chitosans were included for comparison: a water soluble chitosan (Protosan UP CL 213) and a base chitosan (Chitopharm-M).

TEO and OVA were incorporated into Viscosan/chitosan samples at 0.02g mL concentration. Control solutions of TEO and OVA were prepared in phosphate saline buffer (PBS).

Results: Effect of viscosity, deacetylation degree, ViscoGel particle presence, and concentration of the chitosan on *permeation of TEO* across the buccal mucosa is summarized in Table 2. An effect was detected for viscosity (increased permeation with decreased viscosity), DD (decreased permeation with decreasing DD) and presence of ViscoGel particles (lower permeation for Viscosan with ViscoGel particles, compared to without particles), while concentration had no effect on permeation.

Table 2

Factor	Effect	Comment
Viscosity	+	increased permeation with decreased viscosity
Deacetylation Degree (DD)	+	Decreased permeation with decreasing DD
Particle presence	+	lower permeation for Viscosan with ViscoGel particles, compared to without particles
Concentration	–	No significant effect on permeation

With the commercially available chitosans, lower permeation was obtained compared to that of the Viscosans.

The permeation profiles obtained for OVA were affected by DD, whereas no significant effect was obtained with viscosity and concentration (Table 3). Presence of ViscoGel particles in the chitosan preparation was also found to affect the permeation of OVA.

Table 3

Factor	Effect	Comment
Viscosity	-	No effect
Deacetylation Degree	+	Increased permeation with increasing DD
Concentration	-	No effect
Particle presence	+	Increased permeation in presence of ViscoGel particles

The permeability coefficients were calculated from the steady state of the permeation curves and comparison of the permeability coefficients of TEO and OVA revealed that permeability of TEO formulations was higher than that of OVA formulations. Higher permeability coefficients were obtained with high viscosity samples.

Conclusion: Permeability studies of different chitosan and viscosan preparations, with and without ViscoGel particles, formulated with the small drug theophylline (TEO) or the high molecular weight OVA revealed different patterns for the two model compounds. Generally permeation was lower for OVA than for TEO.

General conclusion:

The mucoadhesion and permeation studies were performed with chitosan solutions with different physicochemical characteristics. Viscosity was the only property that affected mucoadhesion, but viscosity had opposite effects on permeation of TEO and no effect on permeation of OVA. It was found that incorporation of drug into chitosan preparations (model compounds TEO or OVA) affects mucoadhesion and permeation. Model compounds with similar characteristics to a test compound (e.g. protein nature, MW) could potentially generate useful data in this kind of studies, but the actual compound to be tested has to be evaluated. In ViVac the mucoadhesion and permeation studies were meant to be performed for guidance of formulation design for SLIT studies with Bet v 1. Due to initial difficulties to apply the chitosan formulations in the mucoadhesion- and permeation assay systems and the demand for huge amounts of test substances, it was not feasible to perform these experiments with Bet v 1 within the given time frame. Thus the results obtained in WP5 could not be used for guidance when designing SLIT formulations for WP8.

Milestone 6: Preclinical characterisation and POC for prophylactic vaccination (ViscoGel-Hib)

The objective of Milestone 6 was to conclude the preclinical characterisation of ViscoGel as an adjuvant for prophylactic vaccination. Moreover preclinical data were obtained in mice to guide the selection of ViscoGel formulation for the clinical POC study. In the clinical trial of ViscoGel as adjuvant a model vaccine to *Haemophilus influenzae* type b, Act-HIB, was

planned to be used and thus most preclinical evaluations were performed with Act-Hib. The results also formed the basis for the documentation in order to obtain all regulatory permissions to ensure that the clinical trials could be conducted. Here the results leading to selection of the formulation to be used in the clinical trial are briefly described. A report has been published based on the studies related to Milestone 6 (Neimert-Andersson et al. (2011) Vaccine 29:8965). The activities were linked to WP7 and Karolinska Institutet was the lead beneficiary.

First the *minimal amount of ViscoGel* needed for obtaining a robust immune response was sought. For this purpose ViscoGel was tested together with a protein antigen, the cat allergen Fel d 1, in addition to Act-HIB. In the first experiment where Fel d 1 was used, 25 mg ViscoGel resulted in antibody titers equal to those generated by higher doses of ViscoGel. Lower doses than 25 mg were technically difficult to inject, and it cannot be excluded that doses below 25 mg may result in the same robust immune response. In a second experiment we showed that 28 mg ViscoGel together with Act-Hib generated the strongest immune response in terms of anti-Act-HIB antibodies. A lower dose (5mg) did not generate a response of the same magnitude. It was decided that 25 mg ViscoGel should be used as the lowest dose together with Act-Hib in the upcoming clinical study.

The next aim was to *characterize the immune response to Act-Hib* when administered to mice together with ViscoGel via the subcutaneous (s.c.) or the intramuscular (i.m.) route, with the focus to provide data for the selection of ViscoGel formulation for the clinical POC study. We could show that ViscoGel particles having a size between 30 and 200 µm resulted in the strongest humoral and cellular response to HIB, and 200 µm particles were selected to be used in the clinical trial. The effect of ViscoGel on the immune response was not dependent on the administration route (s.c. or i.m.) since both routes resulted in enhanced responses compared to the vaccine alone. This was important to show, as the ViscoGel formulated vaccine should be administered by the i.m. route in the planned clinical trial.

The *local immune response* to ViscoGel was investigated. ViscoGel was injected s.c. and the infiltrating cells were phenotyped. In histological sections from the injection site, a complete infiltration of cells could be demonstrated after 24-48 hours. Morphologically, these cells were identified as mainly neutrophils and some eosinophils. The subcutaneously injected ViscoGel could still be found in terms of infiltrating cells, one week after injection. Three weeks post injection only normal tissue was found in the area where the injection had been administered, indicating complete degradation.

The quadriceps muscles were removed after i.m. injection of ViscoGel and single cell suspensions prepared to phenotype the recruited cell types by flow cytometry. Already 4 hours post injection the percentage of cells identified as neutrophils, had markedly increased in comparison with the contralateral muscle where PBS had been injected. The neutrophil infiltration peaked after 12-24 hours post injection. In addition to neutrophils, eosinophils were detected post injection in the ViscoGel injected muscle and continued to increase during the analysis (up to 72 hrs), in contrast to the contralateral PBS-injected muscle. An increase in percentage of cells possibly constituting antigen presenting dendritic cells or mature eosinophils was seen 24 hrs post injection.

When characterizing a novel adjuvant, it is of interest to compare it to a well-known adjuvant. The most widely used adjuvant is aluminium salts (alum). Alum is used both in experimental settings and until recently it was the only adjuvant approved for human vaccines. Therefore *ViscoGel was compared to alum.* It is not possible to adsorb the glycoconjugate vaccine Act-Hib to alum. Instead mice were immunized either with Fel d 1 or the model protein OVA together with ViscoGel, and the immune response was compared to mice immunized with the same antigen adsorbed to alum. Within WP8 mice were immunized with the birch pollen allergen Bet v 1 together with ViscoGel and compared to a control group receiving Bet v 1 adsorbed to alum. We could show that, for all these three antigens, ViscoGel as an adjuvant resulted in higher antibody levels compared to if alum was used as adjuvant. For the cat allergen Fel d 1, both the IgG1 and IgG2a levels were higher compared to alum, while for the birch pollen allergen Bet v 1, the strongest response was seen for the Th1-associated IgG2a levels. This indicates ViscoGel to be a superior immunostimulant compared to alum.

Conclusion

From the preclinical studies performed in WP7, we can conclude that ViscoGel is a potent adjuvant, superior to the established adjuvant alum, resulting in enhanced humoral and cellular responses when administered both subcutaneously and intramuscularly. The best effect was obtained with larger ViscoGel particles (200 µm) used together with Act-HIB. The 200 µm particles were used to characterize the cellular infiltration when administered both subcutaneously and intramuscularly. The results indicate that the primary action taking place locally is the rapid infiltration of granulocytes (neutrophils and eosinophils), which then can induce rapid, within three weeks complete, degradation of ViscoGel. The recruited neutrophils may interact with dendritic cells for priming of the adaptive branch of the immune system, resulting in a strong immune response.

The preclinical characterization of ViscoGel in WP7 supported the design of a ViscoGel formulation to be used in the clinical trial: ViscoGel prepared from 50% DD Viscosan, particle size 200 µm, start dose 25 mg ViscoGel.

A report presenting preclinical POC for ViscoGel as adjuvant for Act-Hib has been published:

Neimert-Andersson T., *et al.* “Improved immune responses in mice using the novel chitosan adjuvant ViscoGel, with a Haemophilus influenzae type b glycoconjugate vaccine” *Vaccine*, (2011) 29:8965-73

Milestone 7: ViscoGel-Hib of GMP grade for clinical use

Based on the preclinical characterisation of ViscoGel as an adjuvant for the model vaccine Act-Hib, a ViscoGel to be formulated with Act-Hib was GMP produced for the clinical trial. A summary of the GMP production of ViscoGel, as well as a description of the ready to use kit with Act-Hib for the clinical trial is given here. Partner 1, Viscogel, was responsible for these WP3 activities.

GMP-produced ViscoGel

The entire process, spanning from manufacturing of chitosan (Viscosan) to release of a final test article for clinical use involved many steps, the most important being:

a. Establishment of product specifications for raw materials and products.
b. Implementation of a quality system for manufacturing of Viscosan (GMP compliant)
c. Manufacturing of Viscosan (50% DD)
d. Technology transfer and manufacturing of ViscoGel and ViscoGel particles at a GMP-approved Contract manufacturing Organisation (CMO).
e. Selection of packing materials and labelling
f. Release of test material for clinical study.
g. Regulatory approval for clinical plan from the Swedish Medical Product agency (MPA) and from the local ethical committee

In brief, early and very basic observations made us focus on the manufacturing of chitosan with a DD of 50%. Based on a large amount of experimental work, protocols for an efficient removal of endotoxins and protein impurities were identified. These results and information in European Pharmacopeia was the basis for setting limits for impurities in Viscosan and lead to a tentative product specification for Viscosan. The next step was to establish a quality system for the manufacturing of a starting material for the pharmaceutical industry, that could meet the industrial standards. In brief, we implemented a GMP-compliant quality system for our manufacturing of Viscosan. A chemical process could then be developed for Viscosan, which meets the common goals normally used for industrial processes, i.e. a process that gives a consistent batch quality, has a low cost of goods (COGS), is scalable and that produces waste streams of low toxicity and a low environmental impact. Manufacturing of approved Viscosan batches for toxicology-, stability and clinical studies went according to plan and these Viscosan batches all met the targets set.

With secured supply of approved material for pivotal studies, a technology transfer was successfully performed to a GMP certified Contract Manufacturing Organization (CMO), Apoteksbolaget (APL) in Umeå, Sweden. The product intended for clinical studies consisted of a suspension of ViscoGel particles in Ringers acetate pH 5.0 and a total volume of 1.00 ml. A siliconized glass vial was chosen to avoid surface interactions with the cationic ViscoGel particles. This vial could, after filling with ViscoGel, be sterilized in an autoclave without getting losses due to unwanted surface interactions. The manufacturing of the ViscoGel batch intended for clinical studies started in February, 2012. After setting of the gel, crushing to 200 µm particles, filling into vials and sterilization in an autoclave the batch (300 vials) was visually inspected and finally labelled. Ten vials were then randomly chosen and analysed. The analysis revealed that the samples met the criteria in the product specification. With analytical data at hand and all documents generated during manufacturing, the batch was released by APL's QP (quality person) on April 3, 2012.

ViscoGel-Hib for clinical use

The product for the clinical trial consisted of ViscoGel in combination with the commercial vaccine, Act-Hib from Sanofi Pasteur MSD. The vaccine was designed to be intramuscularly injected after a bedside mixing procedure in which the vaccine, dissolved in a buffer, was added to a sterile ampoule of ViscoGel particles suspended in Ringers acetate.

APL delivered one type of ViscoGel vial in a kit containing up to three buffer vials and one vial of lyophilized Act-Hib. This kit was specially designed to enable the clinical personnel to mix these vials in to one of eight different formulations through a randomization procedure. The final formulation is a “bedside preparation” that is prepared just before injection. Eight different kits were prepared in advance for the eight groups planned to be included in the clinical trial (clinical trial design described in Milestone 9). Careful measures were taken to assure exact and reproducible handling by the clinical trial personnel, in order to keep the doses exact and to avoid adherence to the walls of the glass vial.

Conclusion

A fully GMP compliant batch of ViscoGel was produced and a patient kit including all necessary vials packaged and delivered to the clinical trial unit on April 5, 2012.

Milestone 8: Toxicology study completed, toxicology evaluation report

The toxicity of ViscoGel, as well as for the formulated vaccine consisting of Act-HIB mixed with ViscoGel, was evaluated in mice and rabbits. Local and systemic toxic effects were carefully investigated after three intramuscular administrations of ViscoGel. A full toxicology report was filed by Partner 6, Huntingdon.

Summary of the toxicity study in rabbits

The licensed *Haemophilus influenza* type b vaccine Act-Hib (Sanofi Pasteur MSD) combined with the adjuvant ViscoGel and the ViscoGel adjuvant alone were evaluated for potential local and/or systemic toxic effects induced by three intramuscular administrations in rabbits; recovery from any effects was then evaluated during a six week recovery period. A group comprising four male and four female New Zealand White rabbits received ViscoGel alone at a dose of 200 mg/occasion and a similarly constituted group received ViscoGel combined Act-Hib vaccine at a dose of 200 mg/10µg; a further control group received Ringers Acetate at the same volume-dose. An addition, two males and two females were assigned to each group; these animals completed a further six weeks without treatment to assess recovery from any treatment related effects. Intramuscular injections were given on Days 1, 15 and 29 of the study. Main study animals were sacrificed on Day 32 of study and Recovery phase animals on Day 71 of study. During the study, clinical condition, bodyweight, food consumption, body temperature, ophthalmic examination, haematology, blood chemistry, immunology, organ weight, macroscopic and microscopic pathology investigations were undertaken.

Results: There was neither treatment-related death, nor clinical sign attributable to treatment. Bodyweight and food consumption was considered to have been unaffected by treatment and there was no effect of treatment on body temperature or ophthalmoscopy. Haematology investigation and analysis of the plasma during Week 5 revealed no treatment related abnormality.

All post-treatment serum samples analysed from animals dosed with the vaccine were confirmed positive for anti-*Haemophilus Influenzae* type b IgG antibodies with 89% of these samples having values above the level of quantification (1200 units/mL).

Necropsy examination on Day 32 or 71 revealed dark or pale areas within the muscle at the sites of administration. Histopathological examination of the injection sites revealed findings that were an exacerbation of findings seen in the Controls with full recovery after six weeks and were, therefore, not considered as being adverse.

In conclusion, it is considered that intramuscular injection of either ViscoGel alone or ViscoGel combined Act-Hib vaccine to New Zealand White rabbits was well tolerated producing minor changes at the site of administration that were not considered adverse. Act-Hib vaccine take was confirmed by a positive antibody response.

Summary of the toxicity study in mice

The objective of this study was to evaluate the licensed *Haemophilus Influenza* type b vaccine Act-Hib combined with the adjuvant ViscoGel and the ViscoGel alone, for potential local and/or systemic toxic effects induced by three intramuscular administrations in Balb/c mice over a five week period. Recovery from any effects or potential delayed systemic toxic or local effects was evaluated during a six week recovery period. One group, comprising ten male and ten female mice received the ViscoGel/Act-Hib vaccine at a dose of 20 mg/1µg. A similarly constituted group received only the ViscoGel (adjuvant) at 20 mg. A further group received Ringers Acetate at the same volume-dose and acted as a Control. Intramuscular injections were administered on Days 1, 15 and 29 of study. A further five male and five female mice were assigned to each group. These animals were treated for five weeks, followed by a six week period without treatment to assess recovery from any treatment related effect. During the study, clinical condition, bodyweight, food consumption, body temperature, ophthalmic examination, haematology, blood chemistry, humoral and cellular immune response, organ weight, macropathology and histopathology investigations were undertaken.

Results: 90% of the animals dosed with Act-Hib were confirmed positive for anti-Act-Hib IgG antibodies; 8 of these samples had values above the upper limit of quantification (25000 units/mL). Generally no significant change in IFNγ production was demonstrated upon challenge of splenocytes *ex vivo* with Act-Hib from Balb/c mice immunised with the Act-Hib vaccine, over the vehicle or adjuvant controls in both the main study group and the recovery group. A general dose-related (in relation to Act-Hib) response to Act-HIB was seen in all groups. Treatment of splenocytes with Concanavalin A (Con A) *ex vivo* resulted in an increased response over the untreated controls in all animal groups. Therefore it is concluded

from this phase of the study that immunisation with Act-Hib causes no notable cell mediated immunity (CMI) response of the type characterised by IFNγ release in the ELISpot assay.

There was no clear effect of treatment upon bodyweight, food consumption, ophthalmic findings, macropathology findings or organ weights for animals given ViscoGel or ViscoGel/Act-Hib vaccine when compared to controls. Signs observed during the treatment phase, in animals given ViscoGel or ViscoGel/Act-Hib, were limited to very slight erythema, oedema and/or bruising at the injection sites following the first and second dose administration only. The incidence and severity were broadly similar in both groups.

After five weeks of treatment histopathological changes related to treatment with ViscoGel alone or with combined ViscoGel/Act-Hib vaccine were seen in the spleen (extramedullary haemopoiesis), popliteal lymph node (increased incidence of germinal centre development and apoptosis within the germinal centres) and treated sites 1 and 2 (minimal/slight) inflammatory, degenerative and regenerative changes. The changes in the spleen and popliteal lymph nodes were considered to indicate immunogenic stimulation following administration of ViscoGel alone or ViscoGel/Act-Hib vaccine.

After 6 weeks of recovery no changes associated with previous treatment with ViscoGel alone or combined ViscoGel/Act-Hib vaccine were seen in treated site 1 or 2 of males and females or in the spleen of both sexes. Evidence of recovery was seen in the popliteal lymph nodes.

In addition to the histopathological findings, haematological findings of significantly high total white blood cell count, due to the high lymphocyte count, identify an immune response in males given ViscoGel after 5 weeks of treatment. These haematological findings were not evident following the 6-week recovery period, supporting the histopathological findings. Body temperatures recorded on Day 29 of the treatment phase for animals given ViscoGel only were slightly increased at six hours post dose when compared to pre-treatment values, this is thought to be a sign of an immune response to the ViscoGel.

Conclusion: It is concluded that the three intramuscular injections of either ViscoGel or ViscoGel/Act-Hib vaccine, when compared to controls, did not elicit any systemic toxic effects. Treatment related changes were largely confined to the dose sites, with histopathological changes in the spleen and popliteal lymph nodes indicative of immunogenic stimulation. These findings indicated the presence of a local reaction at the injection sites and immunogenic stimulation, as expected from the pharmacological activity of the test articles, and not toxicity. Act-Hib vaccine take was confirmed by a positive antibody response but there was no CMI response.

<u>Conclusion Milestone 8:</u>

The results of the toxicity studies performed in mice and rabbits did not reveal any systemic toxic effects caused by ViscoGel. The local reactions observed were linked to the immune stimulation induced by vaccination. An eight times higher dose than the start dose planned to be used in the clinical trial was considered to be safe in rabbits. The toxicity reports supported

the application to the Swedish MPA and local ethics committee for permission to perform a clinical trial with ViscoGel and the ViscoGel/Act-Hib vaccine.

Milestone 9: POC in man for ViscoGel as adjuvant (for Hib), clinical trial result

Milestone 9 is dedicated to a Phase I/II POC clinical trial performed at a single centre, Karolinska Clinical Trial Alliance (KTA), Partner 9. The primary study outcome was safety of ViscoGel and secondary the adjuvant effect of ViscoGel mixed with Act-Hib for intramuscular vaccination was evaluated. The study was monitored and data processed and recorded by Partner 10, Pharma consulting Group (PCG).

Study design

Phase A: Initial safety with three dose levels of ViscoGel (25, 50 and 75 mg in a dose escalating design) in 3x10 subjects, i.e. healthy volunteers.

Phase B: Five groups with 20 subjects/group, dosing in a single-blind randomized design

Group	Act-Hib dose (μg)	ViscoGel (dose determined from Phase A)
1	0.2	+
2	0.2	-
3	2	+
4	2	-
5	10 (=standard dose)	-

An independent Drug Safety Monitoring Board provided oversight in Phase A and recommended the ViscoGel dose for Phase B. Efficacy in phase B was measured as HIB antibody titers in serum at baseline, and post-injection at 4, 7, 14, 28 and 180 days with the 28 day measurement as the primary efficacy outcome variable.

Top-line results - safety

Safety was assessed by evaluation of the frequency and types of adverse events (AE), by injection site inspection, subject diary, vital signs, physical examination and laboratory tests in both phases of the trial.

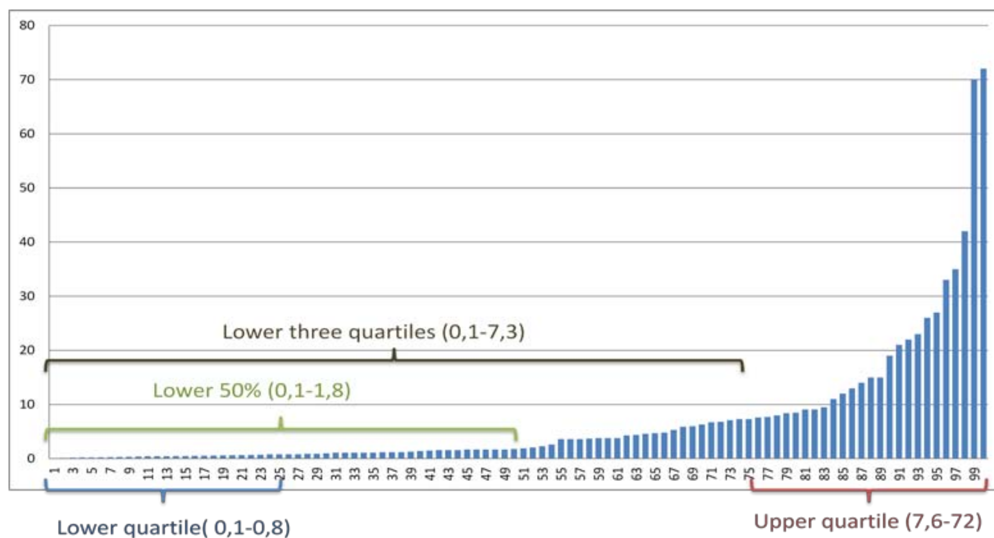
Overall ViscoGel was well tolerated by the subjects in the study and no safety concerns were identified. In Phase A, AEs were reported in 73% of the subjects, of which a majority (65%) were local site injection reactions. A dose-response relationship was observed for local reactions. The independent DSMB recommended the middle ViscoGel dose (50 mg) for Phase B, which was safe and well tolerated. In Phase B the selected 50 mg dose of ViscoGel was shown to be safe and well tolerated in combination with Act-HIB. Sixty-five % of the subjects receiving ViscoGel in combination with Act-HIB exhibited local injection site reactions (of which induration and injection site pain were the most common) as did 30% of the subjects receiving act-HIB alone.Systemic reactions were reported by 33% of subjects receiving ViscoGel + Act-HIB and by 30% of the subjects receiving Act-HIB alone. A

majority of all injection site reactions had a duration of <3 days. Physical examination and active questioning regarding symptoms relating to the injection site were used.

Top-line results - efficacy

There was a large variation in baseline anti-HIB antibody titers between individuals, in spite of the inclusion criteria of no previous infection or exposure to HIB (Figure 1).

Figure 1. Basal anti-HIB antibody titers in the study population



In the mixed population of subjects with low and high basal anti-HIB titers (n=100), ViscoGel had no statistically significant effect on the immune response 4, 7, 14 or 28 days after vaccination as shown by an ANCOVA model. To evaluate the data without the obstructing effect of high baseline anti-HIB titers, subgroup analyses were performed. First the subjects belonging to the lower half of base-line titers (i.e. 0.1-1.8 mg/L, Figure 1) were analysed separately. An increase (not statistically significant) in anti-HIB titers for subjects treated with Act-HIB +ViscoGel compared to subjects receiving the corresponding Act-HIB dose alone was observed. In subjects with baseline anti-HIB antibody titers within the three lower quartiles (i.e. 0.1-7.3 mg/L, Figure 1), a ViscoGel-mediated enhancement of the immune response was seen for the 2 µg Act-HIB dose. The data will be submitted to a scientific journal and are not shown in the present report due to publication policies concerning original data. Further sub-grouping was not feasible because of small study groups and variations in group sizes.

Preliminary data on cell response, secondary efficacy outcome

Heparinized blood was collected on day 0 (pre-immunisation) and day 7 (post-immunisation) from all subjects and peripheral blood mononuclear cells (PBMC) were prepared and frozen. To evaluate the cellular response to vaccination with Act-HIB+ViscoGel compared to Act-HIB alone, PBMCs from all subjects will be thawed and subjected to *in vitro* stimulation with

Act-HIB. Proliferation measured by ^{3}H-thymidine incorporation and IFN-γ response (ELISpot analysis) will be assessed. Preliminary ELISpot data have been obtained from 14 subjects in each of the 2 μg Act-HIB groups (with and without ViscoGel). The data reveal that the number of IFN-γ producing cells increase between day 0 and day 7 for the Act-HIB +ViscoGel group (p=0.017) and decrease for the Act-HIB group (p=0.019).

Conclusion

The clinical trial performed in ViVac has provided "first in man" data on ViscoGel. Safety objectives were fully accomplished, while efficacy objectives could not be fully addressed due to unexpectedly high incidence of HIB exposure in the study population, leading to high base-line anti HIB antibody titers. However, subgroup analyses excluding subjects with the highest baseline anti-HIB titers (either the top half or top quartile) indicated a higher increase of anti-HIB antibody production in groups treated with ViscoGel. Importantly, preliminary data on the cellular response induced by ViscoGel point to a Th1-biased cell mediated response to Act-HIB with ViscoGel, while vaccination with Act-HIB alone seems to have an opposite effect on the cell-mediated response. The safety data and the positive data on efficacy provide a platform for further development of ViscoGel as an adjuvant for parenteral administration of human vaccines.

Milestone 10: Bet v 1-formulations in ViscoGel w and w/o LTX-315 for sublingual (SLIT) and subcutaneous (SCIT) allergen specific immunotherapy

The objective of Milestone 10 was to provide therapeutic Bet v 1 vaccine test formulations for preclinical characterisation and SLIT and SCIT POC studies. The formulations were provided by Partner 1, Viscogel, for activities in WP8 to be carried out by Partner 3, Inmunotek (SCIT), and Partner 8, Stallergenes (SLIT).

Test formulations for SCIT application

An initial experiment was performed at Viscogel (Partner 1) where ViscoGel formulations with covalently linked or mixed rBet v 1 were stored at +4°C for up to three weeks and then used for immunisation of mice. The immune response was compared to the response to rBet v 1 alone and to freshly prepared rBet v 1-ViscoGel. It was demonstrated that the rBet v 1-ViscoGel formulations were stable in terms of antigenicity when stored at +4°C for three weeks, irrespective to if rBet v 1 was coupled to or mixed with ViscoGel. Formulations for experiments aiming at characterising the immune response to subcutaneously administered rBet v 1 and nBet v 1 were then designed and produced. For the first experiments rBet v 1 and nBet v 1 (Bet v 1 enriched birch pollen extract fraction) were formulated with ViscoGel. For the last experiment rBet v 1 was also covalently linked to ViscoGel. The objectives of the investigations planned for the formulations were:

- to ensure that rBet v 1 and nBet v 1 could be stably formulated with ViscoGel with preserved immunogenicity
- to investigate the adjuvant capacity of ViscoGel (30 µm particles) for s.c. administrated rBet v 1 and nBet v 1 (and compare it to alum and i.p. administered antigen)
- to compare the immune response to s.c. administered rBet v 1 and nBet v 1 formulated with ViscoGel
- to investigate the effect of covalently linking Bet v 1 to ViscoGel

Test formulations for SLIT application

A main objective of ViVac was to develop a ViscoGel-based allergy vaccine for SLIT with rBet v 1 and birch pollen allergy as target. The immunological mechanisms for successful SLIT are only partially understood and therefore the only way to confirm a functional SLIT vaccine formulation is to test it in an allergy model, where the candidate SLIT formulation is administered sublingually to sensitised mice. However, allergy models and SLIT protocol are time- and resource demanding experiments. Therefore prescreening of SLIT candidate formulations have to be done, identifying formulations with characteristics considered to be essential for a successful candidate. In WP8 screening was performed in three systems:

1) an *in vitro* dendritic cell system assaying antigen capture
2) an *in vivo* system measuring uptake of sublingually administered labelled antigen in cervical lymph node cells
3) an *in vivo* system measuring antigen specific activation of cervical lymph node T-cells after sublingual administration of formulated antigen

The screening experiments were for technical reasons performed with the model antigen OVA instead of Bet v 1. Two sets of formulations were prepared. For the antigen uptake assays the formulations were prepared with fluorescently labelled OVA (OVA-Alexa Fluor 488) and for the T-cell activation studies the corresponding formulations were prepared with OVA.

The following SLIT test formulations with OVA-Alexa Fluor 488/OVA were prepared:

Formulations for first set of experiments: ViscoGel particles of mean sizes of 2 µm and 30 µm with OVA covalently linked were either formulated in a buffer or a 0.5% Viscosan solution. Free OVA was also formulated in the Viscosan solution and in buffer.

Second sets of experiments:

Viscosan – Viscosan (50% DD) and a highly deacetylated chitosan (80-85% DD). Three Viscosan concentrations, 0.5%, 0.2% and 0.05%.

ViscoGel - Two Doses (e.g. 10 and 20% of formulation (w/w)), 30 µm particle size

LTX-315 - Two different concentrations, alone or in combination with Viscosan or ViscoGel

After evaluation of the screening experiments (see Milestone 12 below), a formulation of rBet v 1 in 0.2% Viscosan (50%DD) was prepared for a SLIT POC experiment in a mouse SLIT model.

Milestone 11: Preclinical POC for SCIT vaccination

The aim of Milestone 11 was to obtain data to support application of ViscoGel as adjuvant in SCIT with rBet v 1 and/or nBet v 1. The experiments focused on characterisation of the humoral and cellular immune response to parenterally administered Bet v 1 formulated with various ViscoGel preparations in order to find an optimal ViscoGel formulation for SCIT application. The activities were performed within WP8 by Partner 3, Inmunotek.

First intraperitoneal (i.p.) immunisation with either rBet v 1 or nBet v 1 (Bet v 1 enriched birch pollen extract) formulated with ViscoGel (30 µm particles) was compared to immunisation with rBet v 1 in buffer, nBet v 1 in buffer, ViscoGel alone, rBet v 1 adsorbed to alum, nBet v 1-alum and alum alone. Activation of the cellular immune response was analysed by proliferation measured by CFSE dilution in nBet v 1 *in vitro* stimulated splenocytes. nBet v 1-ViscoGel treatment led to higher proliferation than ViscoGel alone and nBet v 1-alum to higher proliferation than alum alone. The humoral response to Bet v 1 was analysed by ELISA measuring IgG1 and IgG2a antibodies to Bet v 1. The results revealed that nBet v 1/rBet v 1-ViscoGel and nBet v 1/rBet v 1-alum both generated higher IgG1 and IgG2a responses compared to corresponding controls. Interestingly, the ViscoGel formulations predominantly stimulated an IgG2a response indicative of a Th1 type of response, while alum formulated Bet v 1 mainly stimulated IgG1, linked to Th2 type responses.

In the next experiment subcutaneous administration was used (two groups were given i.p. administration of either nBet v 1 or nBet v 1/ViscoGel) for immunisation of mice. It was also investigated if covalent linkage of the antigen (rBet v 1) promoted stronger immune responses than formulations where the antigen was just mixed with ViscoGel. The reason was that we have previously found that covalent linkage of antigens to ViscoGel gives more efficient immune responses than when there is no physical linkage between adjuvant and antigen. Finally comparison was made to immunisation with nBet v 1-Alum. The only significantly enhanced splenocyte proliferation response compared to controls, was obtained for mice immunised with nBet v 1-alum. For the humoral response, the highest IgG1 and IgG2a levels were detected in mice immunised with rBet v 1 covalently linked to ViscoGel, higher than for rBet v 1-alum.

In conclusion, the immunisation with rBet v 1 and nBet v 1 showed that the immune response to both the recombinant Bet v 1 and the natural enriched allergen was enhanced by formulation with ViscoGel. Furthermore some interesting observations were made on subtle differences between the immune response generated by different administration routes (i.p. or s.c.), between responses to nBet v 1 and rBet v 1 and there were indications of a more Th1-skewed response generated by ViscoGel- than by alum-adjuvanted antigen. Taken together,

the results support application of ViscoGel as a promising adjuvant for SCIT. The data suggest that the allergen, which could be rBet v 1 or nBet v 1, should be covalently linked to ViscoGel. However, the experiments performed within ViVac do not provide a definitive POC for ViscoGel as adjuvant in SCIT. For that, treatment experiments have to be performed in an allergy model to investigate if the strong immune response elicited by Bet v 1-ViscoGel is able to counteract the pathological allergic immune response and ultimately lead to allergen tolerance.

Milestone 12: Preclinical characterisation and POC for therapeutic vaccination –SLIT

The activities linked to Milestone 12 comprised both characterization and screening of candidate SLIT formulations and experiments dedicated to show preclinical POC for a candidate Bet v 1 SLIT vaccine formulation. They were performed by Partner 8, Stallergenes, in WP8.

<u>Characterisation and screening to identify candidate SLIT formulations</u>

In the first set of experiments ViscoGel particles (2 and 30 μm size) with covalently linked fluorescently labeled OVA (OVA-Alexa Fluor 488) were tested for *in vivo* uptake in cervical lymph node (LN) cells and for activation of cervical lymph node T-cells. ViscoGel particles with covalently linked OVA did not, irrespective of particle size, enhance the uptake of fluorescently labeled antigen by cervical LN cells 12 hours after sublingual administration. Similar to the Viscosan formulated OVA it resulted in less uptake compared to free OVA. The test formulations were then applied to an *in vitro* system assessing antigen capture by human monocyte derived dendritic cells (DC). In this system the OVA formulated in Viscosan generated similar uptake as the OVA-alone control, while the ViscoGel-formulations resulted in slightly less uptake after 0.5 and 1 hour. The capacity of the test formulations to enhance the activation of T-cells *in vivo* after sublingual application was then analysed. The *in vivo* system used for measuring OVA specific T-cell activation in cervical lymph nodes consisted of mice that had received adoptive transfer of CFSE *in vitro* labelled CD4 positive T-cells from DO11.10 mice, i.e. BALB/c mice having OVA specific T-cells that can be analysed for activation by measuring proliferation by CFSE dilution. These mice received the test formulations via the sublingual route. After five days the cervical lymph nodes were removed and the proliferation of OVA-specific T-cells could be assessed as a measure of *in vivo* activation of OVA-specific T-cells. The results showed that the formulations containing OVA covalently linked to ViscoGel induced less activation of cervical LN T-cells than OVA alone, while OVA in Viscosan induced similar T-cell activation as the OVA-alone control. None of the test formulations were able to significantly enhance the *in vitro* or *in vivo* uptake by antigen presenting cells or the activation of cervical LN T-cells, in contrast to a positive control (OVA-PSC, i.e. maltodextrin nanoparticle formulated OVA (Razafindratsita et al. 2013, JACI 120:278)). We concluded that covalently linked antigen is not advantageous in this system and set out to design a new set of formulations based on the first results.

In the second set of experiments, test formulations with Alexa Fluor 488 labeled OVA were first screened for antigen capture by human monocyte derived DC:s. The uptake of OVA (not covalently liked) in the different Viscosan-, ViscoGel- and LTX-315 containing formulations (see Milestone 10 above) was compared to the positive control OVA-PSC, OVA alone and buffer (negative control). The results showed that none of the test formulations were captured by DCs to the same extent as OVA-PSC. A small enhancement of OVA-uptake compared to OVA alone was detected for OVA formulated with the 0.2% Viscosan formulations and the formulations with LTX-315. The capacity of OVA test formulations to enhance the activation of cervical LN T-cells after sublingual application was analysed using the above described *in vivo* system. The mice received sublingual administrations of OVA test formulations (containing 0.2% Viscosan, LTX-315 at two concentrations or combinations of Viscosan and LTX-315), PSC-OVA or OVA alone. The formulations with 0.2% Viscosan and with the lower dose of LTX-315 both were shown to enhance the LN T-cell proliferation compared to OVA alone, but not to the same extent as PSC-OVA.

Taken together, the screening experiments did not lead to the identification of an obvious SLIT candidate formulation. As there are no absolute experimental parameters defining formulations that will give a successful outcome in SLIT, it was decided to formulate rBet v 1 in 0.2% Viscosan for application in a SLIT model for birch pollen allergy.

Preclinical POC SLIT experiment

The first step for showing POC for therapeutic SLIT vaccination was to set up a birch pollen allergy model in mice. This objective was fully accomplished. In the established model, mice sensitized to birch pollen (BP) extracts exhibit a strong airway hyperresponsiveness (AHR), high numbers of eosinophils in bronchoalveolar lavages (BAL), and mount cellular allergen-specific Th2-biased responses. Groups of BP-sensitized mice received a SLIT treatment with rBet v 1 or BV twice a week for eight weeks. In a dose-response experiment (using a 5-500µg rBet v 1a/ dose range), SLIT with rBet v 1 (50µg/dose) was as efficacious as SLIT with BP extracts containing 50µg of Bet v 1 as a reference. Both vaccines led to significant reduction in AHR, eosinophils in BAL and decreased Th2 responses in lung. Thus a model for evaluating the efficacy of ViscoGel-Bet v 1 formulation via the sublingual route was established by Partner 8, Stallergenes.

In the SLIT POC experiment mice sensitized with BP extract were treated sublingually with a suboptimal dose of rBet v 1 (5 µg) either alone or formulated in 0.2% Viscosan, with BP as a positive control, or with Viscosan alone or PBS as negative controls. In addition non-sensitised and non treated naïve mice were included as controls. The treatment effect was evaluated by analyzing the AHR response and airway eosinophilia (eosinophils in BAL). The result showed that Viscosan had a positive effect on AHR and a slight effect on AW eosinophilia compared to treatment with 5 µg Bet v 1 and the Viscosan control (Figure 2). The effect was however not significantly different from the PBS control group, in contrast to BP treatment.

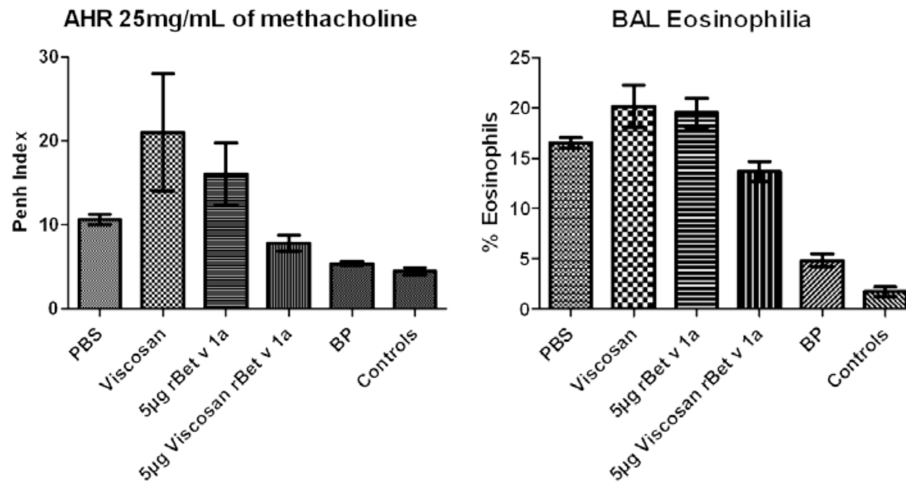


Figure 2: POC SLIT experiment. A suboptimal SLIT treatment dose of Bet v 1 compared to the same dose formulated in 0.2% Viscosan and to control treatments (BP, Viscosan and PBS). A naive control group was also included. AHR measured by pletysmography at 25 mg/ml methacoline challenge (left) and BAL eosinophilia measured be differential counting of BAL eosinophils (right). Only the BP treatment generated significantly lower values for AHR and eosinophils compared to the PBS control.

Conclusion

The characterization of a number of different Viscosan and ViscoGel formulations with the model antigen OVA for antigen presenting cell (APC) capture *in vitro* and *in vivo* did not lead to the identification of any formulation able to significantly enhance the antigen uptake by APCs, a characteristic that is considered to be of importance for efficient immune stimulation. A slight effect on *in vivo* activation of cervical LN T-cells after sublingual administration of OVA formulated with 0.2% Viscosan or with LTX-315 was observed. We conclude that covalent linkage of the antigen to chitosan has a negative effect on the APC uptake and T-cell stimulation in sublingual antigen administration. Moreover, in the present experiments ViscoGel particles, either of larger size (mean 30 µm) or small size (mean 2 µm), did not have a positive effect on antigen capture by APCs or on T-cell activation. In the final SLIT POC study a tendency to a positive treatment effect was detected for Bet v 1 formulated in 0.2% Viscosan. Since the effect was not statistically significant, we could however not show preclinical POC for Viscosan as adjuvant in therapeutic vaccination.

Milestone 13: Protection of IPR developed in RTD activities

The activities linked to Milestone 13 were dedicated to the management of IP:s emanating from ViVac RTD activities and to ensure proper IPR protection of project results. These WP9 activities are described in the "Potential impact, Dissemination and Exploitation" section below.

Milestone 14: Exploitation plan and plan for the use and dissemination of knowledge

An exploitation plan handling the ViVac results has been prepared. It is briefly presented in the "Potential impact, Dissemination and Exploitation" section below, together with a description on dissemination activities. Milestone 10 was linked to the tasks of WP10.

OVERALL SUMMARY OF S&T RESULTS/FOREGROUNDS

The ViVac project has, with a few exceptions, been performed according to plan. The S&T project activities have led to new foregrounds that will form the basis for exploitation of IPs and generate further scientific development.

Key findings, new foregrounds

Characterisation of Viscosan and ViscoGel: A thorough characterisation of Viscosan revealed important physicochemical properties. Method development provided means for analysing gel properties of ViscoGel and gel stability data were obtained. The data have been included in the regulatory documentation on Viscosan/ViscoGel.

ViscoGel manufacturing: Process development resulting in a GMP manufacturing process. Having such a process in place is of critical importance for future drug product development and clinical testing.

Formulation development: ViscoGel could be stably formulated with rBet v 1, nBet v 1 and LTX-315. rBet v 1 was also covalently linked to ViscoGel. A vaccine designed for clinical trial use as a bedside mix of ViscoGel and Act-Hib (Sanofi-Pasteur) that met all regulatory guidelines was successfully manufactured.

Toxicity data obtained in mice and rabbits: Clean toxicity reports on ViscoGel and ViscoGel-Act-Hib was obtained after evaluation of local and/or systemic effects induced by three intramuscular administrations over a five week period, as well as recovery from any effects or potential delayed systemic toxic or local effects after a six week recovery period.

Preclinical POC for ViscoGel as adjuvant: Preclinical POC for ViscoGel as adjuvant was obtained with the model vaccine Act-Hib (Sanofi-Pasteur). Data obtained on the innate and adaptive immune response induced by ViscoGel will be of importance for further development of ViscoGel based vaccines.

POC data in man for ViscoGel as adjuvant: Safety and efficacy data from a Phase I/IIa trial was obtained for ViscoGel alone (safety) and as adjuvant for the model vaccine Act-Hib (safety and efficacy). Safety objectives were fully accomplished, while the primary efficacy objectives could not be fully addressed due to unexpectedly high variations in base-line anti-Hib antibody titers. In subgroup analyses where subjects with the highest base-line anti-Hib titers were excluded a positive effect of ViscoGel could be detected. The clinical trial results are of critical importance for the exploitation of ViscoGel as adjuvant for human vaccines.

Additional exploitable findings

LTX-315 in combination with ViscoGel: ViscoGel could be formulated with LTX-315 and a tolerable dose for further preclinical development was identified. –Discussions were initiated between Lytix and Viscogel on possible applications beyond ViVac (subject not disclosed).

Enriched natural Bet v 1 pollen fraction and Bet v 1 formulated with ViscoGel for SCIT application: Formulation of both rBet v 1 and nBet v 1 with ViscoGel led to enhanced immune responses after s.c. administration to mice. The highest IgG levels (IgG1 and IgG2a) were obtained when rBet v 1 was covalently linked to ViscoGel. The immune response to the ViscoGel-Bet v 1 formulations should be better characterised, but the present data strongly suggest ViscoGel as a promising adjuvant for SCIT and that formulations with allergens covalently linked to ViscoGel should be further explored.

ViscoGel as mucosal adjuvant: Chitosan possesses mucoadhesive and permeation enhancing characteristics. Chitosan and chitosan derivatives have previously been evaluated particularly for intranasal vaccination preclinically and in clinical trials. Even though we could not identify a ViscoGel formulation suitable to apply for allergy treatment in SLIT, the data obtained in ViVac will form the basis for future development of ViscoGel/Viscosan for mucosal prophylactic and/or therapeutic vaccination.

CONCLUDING REMARKS ON S&T RESULTS

The ViVac project has mainly been performed according to the plan. An amendment to the Description of Work (DoW) was approved in March 2012. The main change was an extension of the project until the end of December 2012. The extra four months added to the project time were needed to be able to include results from a clinical trial on ViscoGel in the final report. In addition, two new partners, KTA and PCG, joined ViVac. KTA had been involved in ViVac from the project start but not as an individual partner. PCG was engaged in the project to ensure that the clinical trial could be successfully performed within the given time frame.

The overall objective of ViVac to develop and show POC for ViscoGel as a novel adjuvant was fulfilled for prophylactic vaccination, showing safety and efficacy as adjuvant in man. For therapeutic vaccination useful data have been obtained for the use of ViscoGel as adjuvant for Bet v 1 in SCIT application. The data obtained from SLIT formulations will support further development of ViscoGel for mucosal vaccination. The new foregrounds obtained from the ViVac data are of critical importance for the further development and exploitation of ViscoGel as an adjuvant for human prophylactic and therapeutic vaccines. The project has led to new foregrounds and to potential new collaborations for the benefit of the participating SMEs. In ViVac, ViscoGel was proven to constitute a simple, safe and versatile adjuvant system applicable for human use. In a wider perspective, such an adjuvant system may provide solutions to current challenges in the development of therapeutic vaccines to e.g.

cancer, autoimmune diseases and allergy, as well as for the development of vaccines to infectious diseases to which efficient protection cannot be provided with present vaccines.

ETHICAL CONSIDERATIONS

When studying vaccines and adjuvants, examination of immune responses generated *in vivo* is necessary. Thus animal experiments were essential for the successful performance of ViVac. Furthermore toxicity studies are compulsory for medical application products. When developing therapeutic vaccines, relevant disease models are needed. In ViVac a mouse model for birch pollen allergy was developed. The animal experiments in ViVac have been carried out according to the fundamental principle of the 3Rs (Replace, Reduce and Refine) for work with animals. Approvals for animal experiments were obtained from the local ethics committees for animal welfare in France, Spain, Sweden and UK.

Implementation of the 3Rs

Replacement: various *in vitro* methodologies used when possible, e.g. SLIT formulations were screened in human monocyte derived DC cultures.

Refinement**:** mice anesthesized appropriately before sacrificing for collection of tissues; animal accommodations meeting the needs of the animals including nesting, 12-/12-hour light/dark cycle and with food and water ad libitum.

Reduction**:** the number of animals kept to a minimum by use of inbred strains and standardized procedures for treatment and analysis.

The clinical trial was conducted in accordance with the ethical principles originating in the Declaration of Helsinki in its latest version (the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000), and in consistence with GCP and applicable regulatory requirements.

Swedish laws (in compliance with the Declaration of Helsinki) were followed concerning the collection, storage and experimental use of human material and for electronic data confidentiality, security and integrity. All study subjects gave their informed consent to participate in the study. Data and safety monitoring was conducted by a data safety monitoring board (DSMB). The trial protocol and amendments to the protocol, the Investigator's Brochure, the study subject information and informed consent form and subject recruitment procedures were evaluated and approved by the local Ethics Committee at Karolinska Institutet in Stockholm. Approval from the medical product agency was required and was obtained from the Swedish MPA.

Potential impact (incl socioeconomic impact and wider societal implications so far) and dissemination activities and exploitation of results

POTENTIAL IMPACT OF PROJECT RESULTS

Despite the undisputable success of vaccines and vaccine programs in the battle against infectious diseases several challenges still remain. The unmet needs include both therapeutic vaccines to e.g. cancer, autoimmune diseases and allergy, and prophylactic vaccines to several infectious diseases. Examples of diseases to which efficient vaccines are still lacking include malaria, tuberculosis and HIV- infection/AIDS, collectively responsible for millions of deaths every year. A group of patients with a particular need for efficient vaccines is the worldwide growing elderly population. Individuals over the age of 65 often get insufficient protection from current vaccination to e.g. seasonal influenza and pneumococcal infections. Thus there is a need for vaccines able to elicit efficient and appropriate immune responses.

New vaccines developed today are often based on subunits that do not have a strong effect on the immune system. Moreover, in the situation of a pandemic, shortage of vaccine doses is an actual threat. In the case of therapeutic vaccination, modulation of an established pathological immune response is required. Depending on the indication, the vaccine has to break immune tolerance (cancer vaccines), induce self-tolerance (autoimmune diseases) or elicit appropriate nonpathogenic responses or tolerance (allergy). Thus safe and efficient adjuvants able to promote strong and appropriate immune responses are urgently needed. Some basic features are of critical importance for the successful development of new innovative adjuvants:

- *safety* is an absolute requirement, the adjuvant must be non-toxic and should not elicit responses to itself or unspecific immune responses that may potentially increase the risk for autoimmune and inflammatory disease
- *characterized mechanism of action*, to be able to create vaccines with defined and appropriate effects, e.g. stimulation of a cellular versus a humoral immune response
- *manufacturing capacity*, i.e. manufacturing process of GMP material to low cost of goods available
- *general applicability*, offering a versatile technology platform for formulation of a large variety of vaccines
- *regulatory demands met*, proper analysis methods supporting documentation on e.g. purity and stability are required. Complex adjuvants are more difficult to characterize than chemically simple and well defined adjuvants
- *biocompatibility,* adjuvants from natural sources may be advantageous compared to artificial compounds

Technology platforms for adjuvants fulfilling the requirements listed above have the potential to provide solutions to the unmet needs for novel prophylactic and therapeutic vaccines. This would influence health and quality of life for hundreds of millions of people worldwide, having a great socio-economic impact. Novel innovative adjuvant platforms also offer new opportunities for the vaccine market. Supporting such technology development at the

European level will increase Europe's strength and competitiveness in this highly interesting and expanding market.

In ViVac, the ViscoGel technology was proven to constitute a simple, safe, versatile and clinically applicable adjuvant system matching the requirements for a novel promising adjuvant platform. If successfully implemented the project results may thus contribute to:

- preventing shortage of vaccine supply, by reducing antigen needed per dose
- improved protection against infectious diseases for which available vaccines have poor efficacy
- development of therapeutic vaccines leading to improved quality of life, treatment and clinical outcome for patients with allergy or cancer
- decreasing the societal costs in Europe and world-wide
- expand the vaccine/adjuvant market in Europe, leading to new opportunities for employment

IMPACT FOR THE PARTICIPATING SMES

Viscogel AB (Partner 1)

The most advanced results in ViVac were obtained for the prophylactic vaccine application, where Partner 1, Viscogel AB, has gained substantial benefits in terms of new exploitable IPs. Important advancements have been made on chitosan characterisation, formulation development and chitosan/ViscoGel GMP manufacturing. The preclinical characterisation and the toxicity evaluation of ViscoGel as adjuvant supported the regulatory documentation on ViscoGel. Approval for conducting a Phase I/IIa clinical trial evaluating safety and efficacy of ViscoGel alone and as adjuvant for the model vaccine Act-Hib could then be obtained from the Swedish MPA and from the local ethics committee. The successful development of ViscoGel as a medical product approved for clinical use, together with the positive outcome of the clinical trial will facilitate future applications of ViscoGel as adjuvant in human vaccines. In addition, through the preclinical studies performed within ViVac, important information has been gathered on the mode of action for ViscoGel as adjuvant. This knowledge will promote further research and development on vaccine design. An exploitation plan has been set up for the use of ViscoGel, including a business model for the development of a commercial product(s). A summary of the exploitation plan is found below.

Lytix Biopharma AS (Partner 2)

Lytix Biopharma contributed to ViVac with their background technology, a novel type of cationic peptides with cell-penetrating capacity. It was hypothesised that the cationic peptides could enhance the mucoadhesiveness of mucosal vaccine formulations. Furthermore, the cell-penetrating activity would increase the epithelial penetrating capacity of vaccine formulations intended for mucosal administration. In ViVac the peptide LTX-315 was evaluated for the target application therapeutic allergy vaccination. LTX-315 has so far been applied in cancer treatment, since this type of peptides possesses strong anti tumour activity. In ViVac LTX-

315 was successfully formulated with ViscoGel and Viscosan (Viscogel's proprietary chitosan). Both LTX-315 alone and the ViscoGel/Viscosan formulated peptide were evaluated for potentially advantageous characteristics for application in sublingual allergy vaccination (SLIT). Although LTX-315 generated a slightly enhanced immune stimulation after sublingual delivery of a model allergen, this application of LTX-315 was not considered promising enough to pursue further development. Discussions were initiated between Lytix and Viscogel on other possible collaboration opportunities beyond ViVac (subject not disclosed) and a dose-finding experiment for ViscoGel and LTX-315 was performed during the ViVac project. Except for formulation development no new IP had been generated at the end of the project.

Inmunotek SL (Partner 3)

Inmunotek is focused on the development and commercialization of innovative approaches to allergy diagnosis and treatment using non-recombinant (purified) and recombinant allergens. The key goal is to eliminate the drawbacks of conventional allergy treatment with unpurified natural extracts. In ViVac Inmunotek's allergen technology was combined with Viscogel's adjuvant technology with the aim to develop new therapeutic allergy vaccines. Preclinical characterisation was performed on the immune response to both recombinant birch pollen allergen Bet v 1 and a purified birch pollen extract enriched for natural Bet v 1 in formulations with ViscoGel. It was found that ViscoGel might be a promising adjuvant candidate for use in subcutaneous allergy vaccination (SCIT). Further development is needed in order to obtain new IPRs in this area. ViVac has generated scientific collaboration between Inmunotek and Karolinska Institutet (Partner 7) on allergen characterisation and between Inmunotek and Viscogel on a cancer vaccine project.

DISSEMINATION OF PROJECT RESULTS

ViVac project results were managed with the following objectives:

- to generate and protect new IPRs
- to present results at scientific conferences and in peer-reviewed journals
- to promote collaboration between ViVac partners and to attract new collaborators and partners
- to support out-licensing of product candidates

Dissemination of results

All participants in ViVac are entitled to publish project results after review by the ViVac steering committee (SC, consisting of representatives for the three SMEs). The SC has the right to delay a publication for a reasonable time period in order to ensure appropriate action if the data to be presented could be the subject matter of intellectual property protection. The funding by the FP7 program "Research for SME:s" has been, and will be, acknowledged in all publications and presentations of ViVac results.

ViVac results have so far been presented at the following vaccine/adjuvant-, allergy- and chitosan conferences:

- IMV (Modern Vaccines Adjuvants & Delivery Systems) in Porto, Portugal, April 6-8, 2011
- EAACI (European Academy of Allergy and Clinical Immunology) meeting in Istanbul, Turkey, June 11-15, 2011
- 4th International Conference on Drug Discovery & Therapy, Dubai, United Arab Emirates, 12-15 February 2012
- IAMV (Modern Vaccines Adjuvants & Delivery Systems), Copenhagen, Denmark, July 4-6, 2012
- 6th IberoAmerican Chitin Symposium / 12th International Conference on Chitin and Chitosan, 2-5 September, Fortaleza, Brazil

One report on ViVac results has been published in a peer-reviewed journal:

- Neimert-Andersson T., *et al.* "Improved immune responses in mice using the novel chitosan adjuvant ViscoGel, with a *Haemophilus influenzae* type b glycoconjugate vaccine" *Vaccine*, (2011) 29:8965-73

Additional publications will result from ViVac. Publications of mechanism of action for ViscoGel as adjuvant and on clinical trial results are planned.

Viscogel AB has published three press releases directly related to the ViVac project.

<u>Scientific /business collaboration</u>

To support the collaboration between the ViVac project partners three ViVac project meetings have been organised:

- ViVac Kick-off meeting in Stockholm, September 2-3, 2010
- ViVac Half-time meeting, Istanbul, June 10-11, 2011
- ViVac Final project meeting, Stockholm, April 16-17, 2012

Collaborations have been initiated between the SME partners, as described above. In addition Viscogel has together with Karolinska Institutet received a four years grant from the Swedish Research Council to a PhD student who will be funded during four years to investigate immune mechanisms of action for ViscoGel. These partners (KI and Viscogel) have also started collaboration on the development of allergy vaccines with ViscoGel. The ViVac project consortium has provided a scientific/business network for potential future collaborations between project participants.

As a result of the presentations and publication of preclinical results on ViscoGel as an adjuvant, collaborative and business agreements have been established between Viscogel and vaccine companies, both within veterinary and human medicine.

IPR management

The IPR strategy to define pre-existing know-how, the establishment and protection of intellectual property (IP) and ensuring confidentiality of information shared in the ViVac project have been critical activities to ensure a successful commercialization of the results.

The process for handling IPR in ViVac was regulated by the grant agreement, stating that the ownership of the project results will remain with the SMEs and the RTD performers should be remunerated 100% for their work. During the ViVac project the results have been carefully evaluated to make sure that novel IPR opportunities will be protected. No applications for novel intellectual property rights (patent applications, trademark rights etc.) have been filed during the ViVac project. The results/foreground obtained in ViVac will though constitute a valuable foundation from which new IPRs and out-licensing of products/technologies will be managed according to the business model of each SME.

EXPLOITATION OF RESULTS

The target applications of the ViVac project i.e. i) prophylactic vaccination against common infectious diseases, and ii) therapeutic allergy vaccination, both represent billion €markets. A successful outcome of the ViVac project would significantly improve the competitive situation for the SME partners and create new commercialisation opportunities.

Market segments, vaccine market

Prophylactic vaccines Expansion of the vaccine market is primarily driven by new innovations and increased market growth, which is in part driven by the focus on infectious diseases and the threat of pandemics from new types of influenza. The market has also proved willing to pay higher prices for more effective products. The rapid growth, approximately 30 percent per year during the period 2004-2007, can be compared with the figure of 8 percent annual growth for the pharmaceutical industry as a whole. CAGR during the period 2007-2017 is expected to be 11.5 percent. Vaccine sales during 2010 are estimated to USD 28 billion according to GBI Research[1]. Following a number of structural deals, the vaccine market has come to be dominated by the major pharmaceutical companies (Big Pharma), which currently account for about 85 percent of the total vaccines market. Sales of vaccines in the veterinary market are estimated at USD 5.9 billion[2] with annual growth of about 1 percent.

A large part of successful development in the vaccine field has been through the development of more efficient adjuvants. ViscoGel's properties provide the potential for improved

[1] Adult and Adolescent Vaccines Market to 2017- GBI Research, 2011

[2] Veterinary Vaccines Market to 2017- GBI Research, 2012

adjuvants that can be utilized for improved and entirely new vaccines for both human and veterinary medicine.

Therapeutic vaccines The market for therapeutic vaccines has great potential to add new treatment concepts in many indication areas, including allergies, nicotine addiction, autoimmune and cardiovascular diseases. A substantial number (hundreds) of products are under development and a few of them have reached the market like Provenge, a prostate cancer vaccine approved in the US and Oncophage, a kidney cancer vaccine approved in Russia.

The high incidence of allergic diseases has highlighted the need for efficient treatments, and the allergy vaccine area has been assessed as an interesting market. It is estimated that 10-30% of the population in the US[3] and at least 20% in Europe suffers from allergic rhinitis[4]. According to the World Allergy Organization report in 2008, approximately €14 billion are spent globally each year on allergic rhinitis and 300 million persons worldwide are estimated to suffer from asthma. Birch pollen allergy affects approximately 20% of the population in exposed areas, e.g. in central and northern Europe[5].

Safety risks, long treatment time and complex dosages have prevented more widespread use of allergy vaccines. Most patients today exclusively receive symptomatic treatment. Medications in fact represent the largest cost component of direct medical expenditures for asthma and rhinitis[6]. Thus it is of importance to weigh the costs for a lifelong consumption of drugs against the cost for allergy vaccination[7]. Over longer time periods it has been shown that patients treated with SCIT or SLIT vaccination display a lower mean annual cost than patients who only receive symptomatic treatment[8],[9], [10]. The anti-allergy drug market is anticipated to exceed 14.7 BUSD by 2015 in the US alone according to GIA[11]. Worldwide allergy vaccine sales were 642 MUSD in 2010 and could be worth billions if new technologies succeed in achieving long-term relief of symptoms, significant cost advantages

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[11] Allergy drugs- A US market report, Global Industry Analysts, Inc. 2012

compared with medication and more convenient dosage as necessary for practical use. One can anticipate that allergy vaccines will prove cost-effective in the long run and could thus also improve the overall health economics.

Potential partners

The vaccine market is currently dominated by five large players, Merck&Co, Sanofi Pasteur, GlaxoSmithKline, Pfizer and Novartis[12]. MedTrack databases list over 500 vaccine companies with vaccine candidates in development[13].

While Big Pharma dominates the market for vaccines to end customers, much of innovative technical development is performed by smaller biotechnology companies with which the major companies are interested in creating alliances. Examples of such alliances include the close collaboration between Intercell AG with both Novartis and GSK with regard to the IC-31 adjuvant and the collaboration between Sanofi and Crucell on new production methods for influenza vaccines. Big Pharma has an obvious interest in new market best-sellers and these have been achieved through acquisitions and alliances for specialized vaccines such as Prevnar (Pfizer), Gardasil (Sanofi Merck) and Cervarix (GSK).

Competitors

Several competing adjuvants are in the process of development, but only four adjuvants are currently found in approved vaccines globally. These are:

- Aluminiumhydroxid (alum) has been a standard for adjuvants since the beginning of the 1900s. A consensus exists in the industry that a need exists for improved adjuvants capable to induce cellular responses
- AS03 (GSK), which is based on squalene and used in Pandemrix for H1N1 vaccination. Not approved for use in the US.
- AS04 (GSK), which is based on aluminum hydroxide and monophosphoryl lipid A (MPL) and used in hepatitis and HPV vaccines.
- MF59 (Novartis), which is based on squalene and used in influenza vaccines. Not approved in the US.

The main competition of a novel therapeutic allergy vaccine is conventionally used therapeutic drugs targeting the allergy symptoms. The anti-allergy drug market is currently dominated by antihistamins, which represent the most widely prescribed anti-allergy products, followed by corticosteroids.

ViVac business model

Exploitation strategy of results The strategy is to market novel technologies to leading global companies in vaccines, pharmaceuticals and drug delivery. The business model builds on initially demonstrating Proof-of-Concept (PoC) for the selected application areas, that is, clear

[12] Infectious Diseases Vaccine Market Overview: Key companies & Strategies, Datamonitor, 2010
[13] MedTrack search 2011-11-12

data to demonstrate that the technology works (Figure 3). Based on this data and the SME's intellectual property rights, potential partners are identified for further evaluation, development and commercialization. The partnerships are facilitated by license or collaboration agreements with a revenue model that includes upfront payments, milestones and royalties.

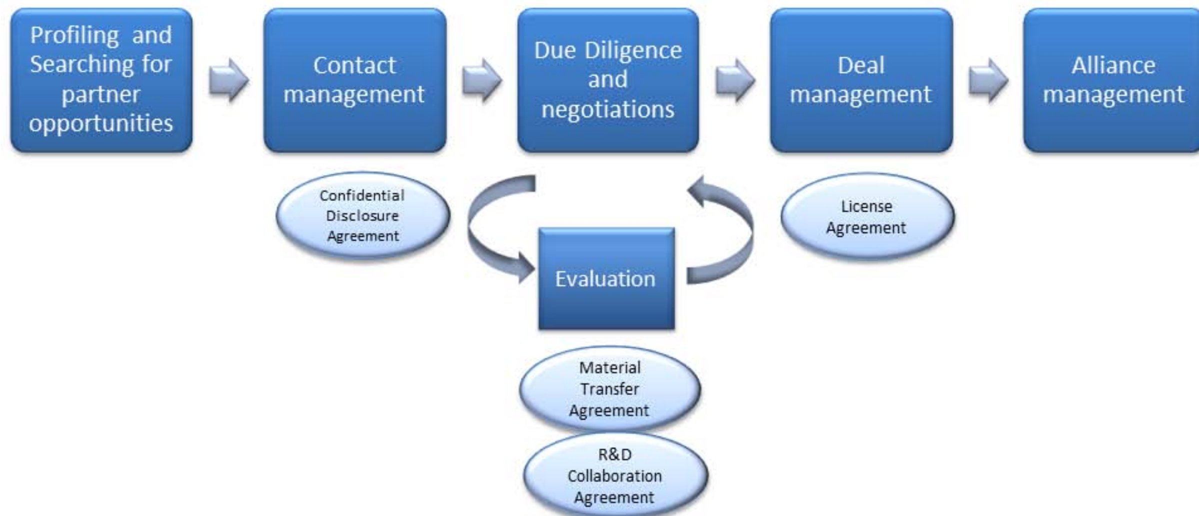

Figure 3. Business model

Profiling and searching for partner opportunities Potential partners are sought within the existing network as well as search databases (e.g. MedTrack, Thompson Pharma) to identify vaccine companies worldwide, with a potential need for novel technologies. Based on the ideal profile of a partner the search strategy is progressively refined. The search results are then segmented into indication areas, development phase, size etc. down to a limited target list of vaccine company contacts that will match the need of an ideal partner to SME.

Contacting management The preparation of contacting potential partners includes composition of first-stage non-confidential marketing material of the SME and its technologies. Business Partnering conferences (e.g. World Vaccine conferences, BIO, Biopartnering) with one-to-one partnering activities are great opportunities to get personal meetings with business development representatives from target companies and to discuss potential collaboration opportunities and identify novel needs. Apart from providing the chance to present or receive business opportunities, the conferences are also important for the SME to build strong networks with key opinion leaders within the vaccine community. Confidential Disclosure Agreements (CDA) are signed with companies that are interested in further evaluation and more extensive information exchange of confidential material.

Due diligence and initial negotiations Before it is possible to enter full negotiations on business terms with a potential partner it is necessary to make a more thorough valuation of the assets of the SME. The due diligence process is a deeper evaluation phase within several areas depending on the targeted deal (License agreement, acquisition, joint venture etc) such as scientific research, intellectual property, contractual relations and operational liabilities and assets. The main terms of the business deal are negotiated under a Term Sheet. The purpose of

the term sheet is to capture the core of the proposed deal and to serve as a framework for the following negotiation.

Evaluation phase Most likely the Due Diligence phase and business negotiations are preceded by an evaluation phase where the collaboration partner will test the adjuvant technology pre-clinically in combination with their own vaccine candidates, which are in development. These R&D collaborations may enhance the interest of the SME's technologies within the company and also enable identification of novel future product candidates and business opportunities. The evaluation phase will be implemented under Material Transfer Agreements (MTA) as well as R&D Collaboration Agreements. A successful outcome of the evaluation phase will add value to the SME and increase the chances of continued successful term sheet negotiations

Deal management The final deal or deals can be structured in many ways depending on how to address different complex situations. The main types of structure will be licenses, acquisitions and joint ventures.

Alliance management – making the deal work The most likely outcome during the commercialization is license agreements where the SME's license the IPR related to the technology to a larger vaccine development company with financial capacities to continue the development into a marked product. The license agreement will not only be a plain license but rather a strategic alliance between the companies where the continued collaboration and effective development is of great importance. Successful alliance management is the key to ensure the continued development and commercialization of the new product based on the SME's technologies.

Exploitable results from ViVac

NEW / EXPLOITABLE FOREGROUND	**DESCRIPTION, UTILIZATION**
Characterisation data on Viscosan and ViscoGel	Supporting the regulatory documentation on Viscosan/ViscoGel
ViscoGel manufacturing	Process development resulting in a GMP manufacturing process
Formulation development	New improved formulation processes and analyses. Data on ViscoGel formulation with Bet v 1, LTX-315, OVA and Act-Hib
Toxicity data obtained in mice and rabbits	Supporting regulatory documentation for clinical use
Preclinical POC for ViscoGel as adjuvant	Data obtained on the innate and adaptive immune response induced by ViscoGel will be of importance for further development of ViscoGel based vaccines
POC data in man for ViscoGel as adjuvant	Exploitation of ViscoGel as adjuvant for human vaccines

Concluding Remarks on Potential Impact, Dissemination and Exloitation of Results

There is a considerable global need for new vaccines that are able to induce efficient protection to infectious diseases. An adjuvant enhances the efficacy of the vaccine and lower the production cost through reduction in the quantity of antigen. In total only four adjuvants have been licensed in the EU and US. There is thus a significant opportunity for new adjuvants. Indeed, in later years the prospects for the global vaccine market have dramatically improved and significant growth potential is indicated. Vaccination strategies are also sought for therapeutic indications e.g. cancer and allergy, where the adjuvants' immunomodulatory capacity represents a significant exploitation possibility.

In ViVac, the ViscoGel technology was proven to constitute a simple, safe, versatile and clinically applicable adjuvant system matching the requirements for a novel promising adjuvant platform. Such a platform provides means to increase the efficacy and duration of a vaccine. In addition it will potentially reduce the costs for manufacturers and payers, and improve the supply in a pandemic, which is of interest to governments and global health organizations.

Several new foregrounds were obtained during the ViVac project, supporting the development of ViscoGel as an adjuvant for prophylactic and therapeutic vaccines. The positive results from the Phase I/II clinical trial for ViscoGel as adjuvant in man have paved the way for new partnering agreements with vaccine companies. Valuable scientific collaborations and potential new partner contacts have been generated for the participating SMEs. The novel results/foregrounds will constitute an important foundation from which new IPRs and out-licensing of products/technologies will be managed.

In conclusion, the ViVac project has significantly improved the competitive situation for the SME partners and created new commercialisation opportunities. On a societal level, the development of an adjuvant technology applicable in novel prophylactic and therapeutic vaccines will have a potential impact on health and quality of life, as well as a beneficial effect on health economics.