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Report against Grant Agreement – Amendment 2

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(a) Final Publishable Summary Report

Executive Summary

While vaccination is of major importance for maintaining public health, most current vaccines are inactivated. As such, only a limited amount of vaccine antigen – the immunogen – can be employed to activate the host immune defence, which in turn will provide only moderate protection. Current inactivated influenza virus vaccines require annual updating due to poor long-term immunity and antigenic drift of the virus. Moreover, inactivated vaccines often favour induction of humoral immunity, whereas efficacious immunity requires both humoral and cell-mediated immune defences. A major player in this efficacy is the replication of the vaccine, providing greater levels of immunogen over a more prolonged period compared with inactivated vaccines. The UniVax goal is to develop a vaccine capable of replicating, thus inducing both humoral and cell-mediated immunity, without the risks associated with a vaccine derived from a pathogen by attenuation. UniVax thus developed the first synthetic influenza vaccine based on self-replicating RNA replicons targeted to dendritic cells by synthetic delivery vehicles, adjuvanted with an efficacious synthetic adjuvant.

The UniVax replicons – termed RepRNA – are derived from a non-cytopathic porcine pestivirus, which is non-infectious for humans. It is defective due to the deletion of at least one structural protein-encoding gene, rendering it impossible to generate infectious progeny. This has the advantage over replicons derived from viruses with a cytopathic nature, which reduces the duration of immune response induction. The RepRNA is delivered into dendritic cells by synthetic, biodegradable nanoparticulate delivery vehicles – lipid/lipoplex-based, polyplex/lipopolyplex-based, polysaccharide-based and virus-like particle-based – for translation and replication to generate high levels of immunogen. Our RepRNA carries insertion sites that efficiently accommodate any vaccine or therapeutic gene of interest; for UniVax, influenza virus genes were inserted – encoding haemagglutinin (HA (H5, H1)), nucleoprotein (NP), neuraminidase (NA (N1)), M, and PB1. An essential further component is co-formulation with novel mucosal adjuvants, such as cyclic -di-AMP. This potentiates robust humoral and cellular immune responses, including cytotoxic and the multifunctional T lymphocytes related to robust protective immunity. The cyclic-di-AMP has now been shown as efficient for both mucosal and parenteral RepRNA vaccine delivery.

Several different biodegradable delivery systems, and modifications therein, were assessed. While many could deliver DNA or small RNA molecules, such as siRNA or mRNA, this could relate to RepRNA delivery, but was not indicative of delivery leading to efficient RepRNA translation. This is probably due to the larger size of RepRNA and therefore more complex interaction with the delivery vehicle components. Similarly, use of cell lines did not reflect delivery to dendritic cells. From the large number of each compound assessed, efficient formulations for RepRNA delivery leading to translation were identified, allowing selection of prototypes for testing *in vitro* and *in vivo*.

With the RepRNA being an RNA molecule, it has the potential for activating innate immune defences, as observed with RNA-based adjuvants. However, the RepRNA is derived from a pestivirus, which interferes with this recognition by innate defences. Accordingly, adjuvants for parenteral or mucosal immunisation were studied. The most efficient was cyclic-di-AMP, offering the advantage of being synthetic and applicable via parenteral or mucosal routes.

An additional assessment looked at targeting the delivery to dendritic cells. Surface hyaluronic acid targeted CD44. A large number of glycoconjugates were also assessed as ligands for dendritic cell receptors (again cell lines proved misleading). The relative capacity of different glycans for binding to dendritic cells was compared on mononuclear cells from porcine and human donors. A number of

the probes were selected as highly efficient at binding, and further defined microscopically for the endocytic route employed.

Following the selection of the above components for the RepRNA vaccine, evaluation *in vitro* and *in vivo* was pursued. The former allowed identification of efficient delivery leading to translation, as well as formulations biasing the translation to particular genes or segments of the RepRNA. From this, the prototypes were selected for *in vivo* evaluation.

The final formulation with the cyclic-di-AMP adjuvant was assessed by both mucosal and parenteral immunisations. This employed, murine, porcine and ferret models. Evaluation assessed antibody induction, T-lymphocyte subset induction, cytokine profiling and individual lymphocyte activities. The formulations modified following *in vitro* evaluation and initial *in vivo* assessment confirmed their capacity to induce immune responses against the influenza virus antigens encoded by the RepRNA. Moreover, the power of the cyclic-di-AMP was further evidenced.

Overall, the work to date demonstrates that particular formulations will facilitate delivery of the RepRNA for translation in the manner observed with RepRNA delivery by virus-like replicon particles (VRPs). Certain delivery vehicles showed some efficacy by either the mucosal or parenteral route. Selected virosome-like HA-nanoparticles based on the Coatsome® technology, a particular PTG-lipopolyplex formulations and a modified chitosan-hyaluronic acid nanoparticulate vehicle all showed efficacy for application with both mucosal and parenteral RepRNA vaccine delivery. In comparison, the VRP-based delivery remains the most efficacious for delivering RepRNA leading to translation and induction of humoral and cell-mediated immunity. Nonetheless, the cyclic-di-AMP proved a potent adjuvant both parenterally and mucosally, and selection of the delivery formulations showing the greatest promise with RepRNA vaccines have been identified. Together, these form a solid basis to progress with the creation of a generic vaccine platform – for vaccines against infectious diseases and cancer, as well as therapeutic applications – based on nanoparticulate delivery of replicon RNA vaccines adjuvanted with cyclic-di-AMP.

Summary Description of Project Context and Main Objectives

Background

Vaccination is the primary means of maintaining human health against many pathogenic infections, and remains the cornerstone of prophylactic measures to counter influenza virus. Nonetheless, most current vaccines are inactivated or subunit entities; due to the limited amount of virus antigen present and the absence of self-replication to increase the immunogenic load, the efficacy of these current vaccines is limited. Without repeated vaccinations, one observes only moderate protection. This can be seen with seasonal inactivated influenza vaccines. Annual updating of the vaccine antigenic components is required, due to poor long-term immunity – can be observed following single immunisations with inactivated vaccines in general – and the capacity of the influenza virus pool to modify viral antigenic determinants. When the latter occurs to a relatively low degree, it is termed antigenic drift; major antigenic changes create antigenic shift, leading to major epidemics and pandemics in both the human and susceptible animal populations. Antigenic shift is even more problematic for current inactivated vaccines. Consequently, the so-called seasonal vaccines against influenza virus have to be updated annually.

The reason for the aforementioned efficacy problems with inactivated vaccines comes from the immune system requirements for solid protection against the pathogen targeted by vaccination.

Efficacious, protective immunity requires the humoral and cell-mediated defences observed with convalescent immunity following virus infection. Vaccines mimicking the virus – in terms of antigenic makeup and the replicative characteristics of live virus – would induce immune defences more closely related to those in response to the virus infection itself.

Replicating vaccines, such as live attenuated influenza virus (LAIV) and vector-based vaccines, offer several rounds of antigen production due to their replicative nature. These rounds of antigen production increase the immunogenic load encountered by the immune system, thus enhancing the efficacy of immune defence induction (Wei et al 2010 Science. 329:1060-4; Price et al 2010 PLoS One 5: e13162; Poon et al 2009 J Immunol. 182:3063-71; Zhou et al 2010 Mol. Therapy 18: 2182). The replicative nature also facilitates entry of viral antigen into both MHC Class I and Class II processing pathways, as would occur during the virus infection. This is particularly important for the cell-mediated immune defences, including the cytotoxic T-lymphocyte [CTL] responses. The likelihood that robust immunity – including both humoral and cell-mediated defences – develops is increased with replicating vaccines, enhancing resistance to antigenic drift and reducing repeat annual vaccination.

While LAIV and vector-based vaccines show high potential, they are still based on live viruses, and are therefore not synthetic. Moreover, one has to consider the potential reaction of the host against the vector itself –potential sensitivity to pre-existing immunity – which would reduce or nullify the vaccine potential. Moreover, reversion or recombination with other influenza viruses leading to increased pathogenicity remain risk factors with attenuated vaccines. LAIV and vector vaccines also lack adaptability for targeting. Vaccine targeting to dendritic cell (DC) receptors is important considering the critical role of DCs in promoting and regulating immune responses (McCullough et al 2012; Lewis & Reizis 2012 Cold Spring Harb Persp. Biol. 4:a007401; Paluka et al 2010 Immunity 33:464-78). They are major sentinels detecting and collecting antigen in the periphery (including dermal layers and mucosal surfaces), transporting into lymphoid tissues and organs. DCs are termed "professional antigen-presenting cells": DC subsets are the most efficient for processing antigen through the MHC Class I pathway – activating CTLs – and the MHC Class II pathway – activating Th lymphocytes essential for efficient development of robust humoral and cell-mediated immunity (Heath et al2004 Immunol Rev. 199:9-26; Monu & Trombetta 2007 Curr Opin Immunol 19:66-72; Burgdorf & Kurts2008 Curr Opin Immunol20:89.95; Vyas JM et al 2008 Nature Rev Immunol 8:607-18). Their diversity of receptors is important for immunological targeting of vaccines (McCullough et al 2012 Ther Del 3:1077). DCs are also an important consideration when selecting adjuvants for vaccine formulation – they must promote/enhance the DC maturation essential for efficient immune response induction; immature cells can induce tolerance rather than activation.

The UniVax Goal

The UniVax goal considers the above parameters to develop synthetic platforms for efficacious self-replicating vaccines. Within the project, the main objective focuses on synthetic influenza vaccines. This is based on self-replicating RNA replicons; their production is synthetic, and they provide a replicative nature for the vaccine without any of the drawbacks associated with attenuated and vector-based vaccines. Delivery is offered by synthetic, nanoparticulate vehicles, providing potential for targeting to DC receptors. Formulation employs efficacious, synthetic adjuvants, to enhance promotion of both humoral and cell-mediated immunity.

The UniVax Innovative Vaccine Components

(i) RNA Replicons

Replicons are defective virus genomes, from which at least one structural protein-encoding gene was deleted. Replication and translation resemble the parent virus, but without infectious virus progeny or disease. This provides efficient induction of both humoral and cytotoxic cell-mediated immunity, leading to robust and durable immunity. They are biosafe products readily engineered to encode any vaccine antigen (McCullough et al 2012 Ther Del 3:1077).

The have limited applicability of unprotected ("naked") replicons due to RNA instability, led to development of virus-like particle vectors. These are termed virus or virus-like replicon particles (VRPs) (Rayner et al 2002 Rev Med Virol 12:279; Kofler et al 2004 PNAS 101:1951; Piggott 2009 Mol. Med. Rep. 2:753), in which replicon RNA is packaged by cell lines providing the missing gene product in trans. Delivery is dependent on the cell tropism of the VRP, which in turn is not readily modified for targeting DC receptors and may suffer from species restriction.

Most replicons are derived from human pathogens, such as the widely employed alphaviruses. Both the aforementioned safety considerations with LAIV and limitations due to anti-vector immunity in the host are again relevant. In contrast, the pestivirus replicons of UniVax are not derived from a human pathogen, rendering them (and their VRPs) safe for humans.

The cytopathic nature of many replicons such as alphavirus replicons would reduce the duration of replicon-dependent induction of immune responses. Moreover, the 5'-cap of alphavirus RNA is recognised by cellular innate defences, as are other structures formed by its RNA, leading to cellular attack. In contrast, pestivirus replicons are derived from a non-cytopathic porcine virus. They do not carry a 5'-cap, and any recognition of its RNA by the cell can be impeded by the leader autoprotease encoded by the genome (and replicon).

The pestivirus RepRNA to be employed in UniVax has been modified to carry insertion sites that efficiently facilitate the accommodation of any gene of interest. For UniVax, this will be different influenza virus genes, encoding HA, NA, NP, M or PB1.

(ii) Delivery of the Replicons

Although immune responses can be induced in small animals using non-replicating RNA such as mRNA, human clinical trials have highlighted the need for improved potency and delivery (Kutzler et al 2008, Ulmer et al 2006 Trends Mol Medicine 12:216). Self-amplifying RNA offers longer-lasting antigen expression than mRNA, and induces both humoral and cellular immune responses (Johansson et al 2012 Plos One 7:e29732; Geall et al 2012 PNAS 109:14604; McCullough et al 2012 Ther.Del 3:1077; McCullough et al 2014 MTNA 3:e173).

The pestivirus replicon (RepRNA) employed within UniVax can be delivered into DCs by VRP or the UniVax synthetic nanoparticles. Delivery must promote RepRNA translocation into the cytosol for translation and replication. Co-formulation with novel mucosal adjuvants, such as c-di-AMP, potentiates robust humoral and cellular immune responses, including cytotoxic and multifunctional T cells – the latter related to robust protective T-lymphocyte immunity. Such a potent adjuvant is important for the vaccine development, because the pestivirus RepRNA does not activate innate immune cells. This is in contrast to other replicons, including alphavirus replicons, relating to the absence of a 5'-cap with pestivirus RNA and the encoding of the pestivirus N^{pro} autoprotease with its interferon regulatory functions (see above). Accordingly, the efficiency of the RepRNA vaccine is dependent on the delivery vehicle interaction with cells, particularly DCs, and the presence of an efficient adjuvant.

Targeting delivery of RepRNA to DCs is important for focussing antigen translation within the main antigen-presenting cells. The most promising vaccine targeting employs ligation of C-type lectin receptors (CLRs) and SigLec receptors (SLRs) (Pichon & Midoux 2013 Meth Mol Biol 969:247). Certain CLRs can be exploited for glycan-conjugated vaccine targeting (Unger & van Kooyk 2011), while co-delivery of vaccine and adjuvant can promote co-ligation of receptors, enhancing immune responses (Dzopalic et al 2012 Immunol Res 52:20).

(iii) Synthetic Delivery Vehicles

Synthetic delivery vehicles have several benefits over viral delivery: (1) obviate safety and manufacturing concerns associated with viruses or cell culture-derived antigens; (2) not restricted by anti-vector immunity; (3) free of animal, plant or microbe impurities; (4) large-scale and cost-effectively manufacturing under GMP conditions; (5) engineered for cytosolic delivery – essential for translation of RepRNA-encoded antigens; (6) readily associate with adjuvants (Lai & Wang 2008 Exp Opin Drug Del 5:979; Tyagi et al 2008 Exp Rev Vacc 7:499).

Accordingly, UniVax partners employ nanoparticulate delivery of the innovative RepRNA vaccine covered by the following Patents.
Partner 1: US9249395, WO2009/146867, US9670466, EP08010222, EP2009003892, 4414/KOLNP/2010;
Partner 2: WO2009/106713;
Partner 7: WO2017212197, WO2009112402, WO2009050372
Partner 8: EP 05 022 771.9, EP 05 025 431, EP 05 02 4266, EP 09016050.8.

UniVax provides an integrated approach to overcome the drawbacks of individual technologies, employing the following core elements creating the delivery platforms:
(a) Virus-like replicon particles (VRPs), a core technology with Partner 1 (Suter et al 2011 Vaccine 29:1491), produced by transfecting complimenting cell lines (providing the gene product deleted from the RepRNA) to encapsidate the RepRNA in a virus-like particle having tropism for DCs; all delivery vehicle formulations will be compared with VRPs;
(b) Commercial liposome formulations custom made for Partner 1, resembling virosomes in structure and referred to as HA-nanoparticles (with or without addition of recombinant HA);
(c) Cationic lipid-based elements – biodegradable cationic lipids have been developed to promote efficient RNA delivery;
(d) Lipopolyplexes – Histidylated Lipid/Polymer/RNA particles (LPRs) combining advantages of lipidic and polymeric carriers (Partner 7; Perche et al. 2011 J.Drug Targ. 19:315) and controlled protonation (pKa) of histidines during endosomal acidification promoting early escape from the endocytic vesicles (cytosolic translocation);
(e) Polyol-based elements (Partner 8, now Partner 12) defined in terms of chitosan-based delivery (Partner 8 collaboration with Partner 1: FP6 project PANFLUVAC – Thomann-Harwood et al. 2012, J.Contr.Rel doi: 10.1016; FP7 PEOPLE project Replixcel – McCullough et al 2012 Ther.Del 3:1077; McCullough et al 2014 MTNA 3:e173); the core of these particles can promote endosomal escape (Nasti et al. 2009 Pharm. Res. 26:1918; Zaki et al 2011 Macromol. Biosci. 11:1747), while the outer shell accommodates targeting ligands (Malik et al., in preparation; Thomann-Harwood et al. 2012, J.Contr.Rel doi: 10.1016);
(f) Glycan groups identified for targeting DC receptors (Partner 3: initiated through Partner 3 collaboration with Partner 1 in ERA.Net RUS project HCRus), thus providing defined glycoconjugates (Partner 3) for targeting delivery vehicles to DCs.

Cationic elements known to promote the endosomal disruption required for RepRNA release into the cytosol are an important criterion. Simultaneously, acidification of the endocytic vesicle leading

to this disruption destabilises the delivery vehicle interaction with the RNA, permitting release into the cytosol. Such cytosolic release and decompaction (reversal of the ionic interactions between delivery vehicle and RNA) promotes translocation of the RepRNA to the site of the translation machinery. Hence, the rationale behind application of cationic lipids (Partner 2) and combination of pH- sensitive histidylated polymers and pH-sensitive liposomes (Partner 7), shown to facilitate cytosolic delivery of mRNA (Midoux et al 2009 Br J Pharmacol 157:166; Mockey et al 2007 Cancer Gene Ther 14:802; Perche et al. 2011 Nanomed 7:445).

(iv) Synthetic Adjuvants
Single-shot DNA and mRNA vaccines show poorer efficacy in large animals versus small. Required dosage of mg concentrations prohibits a "universal" approach. UniVax tackles this in a dual approach with self-replicating RepRNA delivery combined with innovative, new-generation adjuvant technology promoting immune activation in a controlled manner.
Different adjuvants with well-defined molecular targets and effector functions have been identified by Partner 9, including cyclic-di-nucleotides [DDX41/STING/TBK1-dependent induction of type I IFN] such as c-di-AMP, a pegylated derivative of the TLR2/6 agonist MALP-2 (BPPcysPEG), and pegylated α- galactosylceramide [CD1d agonist]). All are readily administered by mucosal or parenteral routes (Zygmunt et al 2012 PLoSONE 7:e30382; Ebensen et al 2011 Vaccine 29:5210; Prajeeth, et al 2011 Eur J Immunol 40:1272; Libanova, et al 2010 Vaccine 28:2249; Borsutzky et al., 2006, Vaccine 24:2049), with proven potential for conventional and prime- boost vaccination protocols. Both humoral and cellular anti-influenza responses have been induced at mucosal and systemic levels, characterized by mixed Th1/Th2/Th17 responses with both multifunctional and Tc-lymphocyte responses (Pedersen et al 2011 PLoS ONE 6:e26973; Madhun et al 2011 Vaccine 29:4973). Mucosal adjuvants also provide for stimulating strong local responses at the respiratory tract, reducing viral shedding in infected individuals and subsequent horizontal transmission. Partner 9 also identified adjuvants to overcome immune senescence in aging mice of different haplotypes.

The UniVax Main Objectives
(i) generate multimeric RepRNA vaccines encoding influenza virus antigens;
(ii) incorporate RepRNA in new generation, synthetic, biosafe nanoparticles – derived from liposome, lipid, polysaccharide and polymer technologies – to enhance DC delivery;
(iii) apply glycan technology for targeting DC receptors;
(iv) select safe new generation adjuvants for single-shot mucosal or parenteral delivery;
(v) identify vaccine/adjuvant prototypes by pre-clinical evaluation;
(vi) identify prototype RepRNA vaccines that may be assessed in a Phase I Clinical Trial.

Main S & T Results/Foregrounds.

Choice of the Pestivirus Replicon
The active component of the multimeric influenza vaccine being developed in this project consists of replicon RNA (RepRNA) based on a pestivirus (CSFV) genome. Replicons are genetically modified viral genomes; they are self-amplifying (self-replicating) vaccines, incapable of generating infectious progeny. This is achieved by deleting genes encoding for essential viral structural proteins. Their translation capacity to promote this self-replication also produces the vaccine antigens from inserted genes encoding for these antigens.

The pestivirus replicon has major advantages over other replicons:
(i) Pestivirus RepRNA is non-cytopathogenic, unlike the majority of replicons in use today – primarily alphavirus or flavivirus derived – which are cytopathogenic.
(ii) Pestivirus RepRNA efficiently translates and replicates in dendritic cells (DCs).
(iii) Importantly, pestivirus RepRNA will not kill DCs in which it is translating and replicating, rendering it more ideal as a vaccine than other replicons such as those derived from alphaviruses, which are cytopathogenic and thus reduce duration of immune induction.
(iv) The pestivirus genome and replicon do not carry 5'-triphosphates, in contrast with alphaviruses where the 5'-triphosphates can signal cell innate defence mechanisms to impede or even destroy the replicon, reducing their efficacy as vector vaccines.
(v) The pestivirus leader protein encoded by the genome (N^{pro}) interferes with cell-signalling pathways responding to the presence of the RNA that would otherwise lead to cell activation and interferon induction impairing replicon function.

These characteristics of the pestivirus replicon facilitate its retention in DCs for prolonged periods. As such, this fits well with the manner by which DCs slowly process antigen for presenting to the adaptive immune system over a prolonged period, thus promoting robust immune defences.

An important advantage for the UniVax consortium comes from the intellectual Property for the nanoparticulate delivery of the RepRNA. This is owned by Partner 1:

1. Tratschin JD, Ruggli N, McCullough KC. US9670466B2. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.
2. Tratschin JD, Ruggli N, McCullough KC. US9249395B2. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.
3. Tratschin JD, Ruggli N, McCullough KC. WO 2009/146867 AI. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.
4. Tratschin JD, Ruggli N, McCullough KC. EP 2130912A1. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.

Application of the Pestivirus Replicon (RepRNA)

Existing constructs from the Replixcel, HCVAX and HCRus projects provided the starting pestivirus replicon material for UniVax – namely plasmids carrying the RepRNA encoding reporter genes and influenza virus haemagglutinin (HA (H5)). These plasmids provided the starting material to produce the first RepRNA for testing with different biodegradable, nanoparticulate delivery technologies. For more details, see

1. McCullough et al (2014) Self-replicating Replicon-RNA Delivery to Dendritic Cells by Chitosan-nanoparticles for Translation In Vitro and In Vivo. Mol Ther Nucleic Acids. 3:e173
2. McCullough et al (2012) Functional RNA delivery targeted to dendritic cells by synthetic nanoparticles. Ther Deliv.3:1077-99

Production of the RepRNA from the above plasmids requires linearisation and transcription. Thereby, an essential step involves a unique endonuclease site which does not recognise any sequence within that for the replicon. In order to assure continued production of the RepRNA as required, a second endonuclease site was sought to obviate dependence on the availability of a single endonuclease. However, no other unique site was found. Therefore, a second unique site was engineered into the construct, providing a choice for the endonuclease to be used in the production process for the RepRNA. The "backbone" construct was thus prepared, in which the "foreign gene of interest" (GoA) encoded for luciferase.

From the luciferase-encoding construct, additional constructs have been generated. These encode for influenza virus HA (H1 Cal2009), neuraminidase (NA (N1)), M protein, PB1 protein and nucleoprotein (NP). A further construct encodes for the ovalbumin sequence detected by OTI and OTII cells. This allowed for assessment of delivery systems in the OTI/OTII model, but also has applicability in future products as a cancer vaccine.
For more details, see

1 Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York.

RepRNA Replicon Constructs

As described in the previous section, RepRNA constructs encoding a series of antigens have been created. In turn, these can translate to provide the means for inducing both humoral and cell-mediated arms of immune defence. The aim of UniVax was to formulate the RepRNA encoding for the influenza virus antigens for delivery, with particular emphasis on interaction with DCs.

In addition to new RepRNA prototypes encoding the aforementioned influenza virus antigens, replicons have been constructed to lack one, several or all the structural proteins of the original pestivirus sequence. The expression of the encoded influenza virus proteins was assessed alongside expression of certain pestivrus proteins still encoded by the replicon. By such means the relative efficiency of translating the GoA encoding the influenza virus antigen was compared with the endogenous genes – the former are translated via the ribosomal entry site naturally occurring in the 5'-NTR of the pestivirus RNA, while the latter are translated via an additional ribosomal entry site inserted after the GoA. This allows determination of the efficiency with which the GoA near the 5' end of the RepRNA can be recognised and translated compared with the genes downstream of this, including those required for replicon replication.

Considering that the majority of the partners in UniVax would be involved with either the delivery of the RepRNA or the in vivo assessment of the delivery (in certain cases, also in vitro assessment), training of these partners in the methodologies for producing RepRNA was pursued. Partner 1 was in charge of this training, due to their ownership of the intellectual property for nanoparticulate delivery of RepRNA and their long-term experience in producing this material from the plasmid constructs they had generated.
See the aforementioned patents and references, as well as

1 Suter et al (2011) Immunogenic and replicative properties of classical swine fever virus replicon particles modified to induce IFN-α/β and carry foreign genes. Vaccine. 29:1491-503

Accordingly, most of the partners in UniVax were trained by Partner 1 in replicon generation, as well as producing and quality assessing the RepRNA (Démoulins et al 2017 Methods in Molecular Biology vol 1499: Chapter 5). These partners went on to associate the RepRNA with particular delivery vehicle formulations, for assessment of the capacity to promote translation of the encoded influenza virus antigens. The partners having expertise with *in vivo* evaluations (Partner 9 and Partner 7) took the process to the next level for evaluating induction of humoral and cell-mediated immune responses (see below under the sections on *in vitro* and *in vivo* evaluations).

Formulation for delivery to dendritic cells

The UniVax Consortium has assessed several different biodegradable delivery systems, and modifications therein, namely

- virus-like replicon particles (VRPs) developed by Partner 1 for pestivirus RepRNA,
- cationic lipids (lipoplexes) developed by Partner 2 to promote efficient RNA delivery,
- lipopolyplexes developed by Partner 7 – Histidylated Lipid/Polymer/RNA particles (LPRs) combining advantages of lipidic and polymeric carriers, and controlled protonation (pKa) of histidines during endosomal acidification to promote early escape from endocytic vesicles (cytosolic translocation),
- polyplexes using PEI of different molecule weight, with or without cell penetrating peptides,
- chitosan-based complexes developed by Partner 8 (now Partner 12) whereby particle cores can promote endosomal escape, while the outer shell accommodates targeting ligands.
- new virosome-like delivery systems termed "HA-nanoparticle" designed for Partner 1 using commercial liposome formulations, introduced by the UniVax Consortium due to the success observed by consortium partners with virosome vaccines
  (see
  1. Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223)

All nanoparticulate delivery systems were physico-chemically characterized, with the exception of the VRPs. For each of the above delivery systems, a number of different components was analysed. The aim therein was to determine if modifications in the structure of the material or the formulations would impact on interaction with and delivery of the RepRNA. The exception was with the VRPs, which resemble the partner virus of the RepRNA and have been already well characterized; due to their high efficiency at delivery RepRNA for translation ad replication, they were also the reference point against which the other delivery systems were compared.

Assessment for applicability with the RepRNA initially characterized the capacity of different formulations, and different components, to package RepRNA and protect from RNase. This was elaborated with respect to delivery of the RepRNA to DCs and selected cell lines. The latter were employed for comparative purposes and to facilitate rapid identification of candidates for testing in DCs. Overall, the main criteria were that the delivery vehicles were

- efficient at complexing RepRNA,
- capable of interacting efficiently with DCs to promote cargo uptake,
- readily tolerated by cells and host,
- efficient at promoting RepRNA release within cells to facilitate translation of the encoded vaccine antigens.

Once efficient delivery was established, the final readout was translation of the delivered RepRNA. In the first instance this employed reporter genes; once translation following delivery by a particular formulation had been established, this was further assessed using RepRNA encoding influenza virus antigens.

Details of the assessment is shown below under sections on *in vitro* and *in vivo* evaluations.
For more details, see

1. Démoulins et al 2016 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;
3. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271;
4. Englezou et al 2018, Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids 12:118-134.

Targeting

The aim of the targeting approach was to identify ligands reactive with receptors on DCs with the potential for enhancing RepRNA delivery leading to translation. It was considered that this would involve either directly enhancing the delivery process, or influencing the endocytosis of the delivery vehicle in a manner favourable to RepRNA translocation for translation and replication. The UniVax consortium employed three approaches –

hyaluronic surface decoration to target the CD44 on DCs and indeed many cell types;

glycan probes targeting members of the C-type lectin, SigLec and galectin families of receptors on DCs;

RGD-containing peptides known for targeting lectins and related structures on DCs.

(i) Hyaluronic acid decoration

Chitosan nanoparticulate delivery vehicles were modified to carry surface hyaluronic acid. The approach proved particularly successful. There was no toxicity from these formulations, and delivery to cells proved most efficient. Translation of the delivered RepRNA was observed, also proving successful when assessed *in vivo*.

A new approach for hyaluronic acid (HyA) functionalisation based on the introduction of boronic acids on the polymer structure allowed for its decoration. Importantly, the boronic esters formed are hydrolysed at acidic pH, facilitating detachment of the cargo (such as RepRNA) in the endosomal environment developing following endocytosis of the delivery vehicle formulation with RepRNA. This effort focused on conditions with tyrosine-containing peptides and proteins optimised for enzymatic oxidation to react with boronate-HyA.

Work on a new class of synthetic and pH-reversible proteoglycans was also finalised and published. For more details see Bioconjugate Chem. 2018, 29, 8, 2550-2560.

More details are shown under the sections for *in vitro* and *in vivo* evaluation below.

(ii) Glycans

For the glycans, it was first necessary to select the probes to be employed. To this end, over 300 glycoconjugates were assessed for their relative capacity at binding to DCs compared with other mononuclear cells obtained from porcine and human donors. This permitted a clustering that facilitated definition of glycan binding capacity for the differenthuman and porcine mononuclear cells, with particular emphasis on the DCs and DC subsets. The analyses also permitted identification of species-common and species-specific glycan-binding receptors.

In addition, the binding of probes interactive with DCs was assessed on cell lines, in particular the DC2.4 cell line, which is supposed to resemble DCs, at least in their ability to present antigen to T-lymphocytes. However, the glycan binding profile for the DC2.4 cells was distinctive from that for DCs or even other mononuclear cells (see under the sections for *in vitro* evaluation below). This work demonstrated that DC2.4 cells could not be used for assessment of nanoparticulate vehicle delivery to DCs, whereby the risk a false impression on binding or a lack thereof would be forthcoming.

A selection of probes defined on the basis of interaction with DC subsets and monocytes were further analysed by microscopy to define binding capacity and internalisation efficiency, as well as the endocytic route employed by the cell. The aim was to ascertain that the glycans were indeed capable of promoting endocytosis by the DCs, and not just binding to the cell surface – some cell

surface receptors can bind ligands, but rapidly eject them either before or shortly after endocytosis. Only the glycans capable of promoting enduring endocytic processes would prove applicable for targeting RepRNA delivery. Such endocytosis is important due to the manner by which the cell will process the material, in turn impacting on the release of the RepRNA for translation.
For more details see also

1. Rapoport et al 2018. Glycan recognition by human blood mononuclear cells with an emphasis on dendritic cells. Glycoconjugate Journal, doi.org/10.1007/s10719-017-9811
2. Rapoport et al 2019. Glycan-binding profile of DC-like cells. Submitted for publication

(iii) RGD-containing peptides
The RGD peptides employed are known for their high immunogenicity *in vivo*, which presents a risk of inducing an immune response against the RGD-containing peptides. This precludes their applicability as generic targeting ligands for the RepRNA delivery vehicles. Accordingly, they were used primarily as a means for defining the endocytic processes employed by DCs, to facilitate identification of the processes into which the glycan probes and the delivery vehicles would target the RepRNA cargo. As such, particular processes were subsequently identified with the selected glycan probes. This allowed for a more informative conclusion on the applicability of the glycan probes for RepRNA delivery. Namely, as opposed to protein antigen delivery – requires a diffident late endocytic pathway for processing and presentation – the RepRNA requires cytosolic translocation to facilitate interaction with the ribosomal machinery for translation.

(iii) Evaluations
For more details of these targeting entities (hyaluronic acid, glycans, RGD peptides and hyaluronic acid with glycans), see under the section for *in vitro* evaluation, subsection (iv) Targeting RepRNA delivery, below. Modified delivery vehicles, selected from the *in vitro* evaluations, were employed to assess their influence on RepRNA cargo delivery and translation efficiency – for more details, see below under *in vitro* and *in vivo* evaluations.

Adjuvants
The characteristics of the pestivirus replicon – non-cytopathogenic and slow translation, together with a lack of 5'-triphosphates to activate cellular innate defences and the leader N^{pro} to regulate Type I interferon pathways – facilitate retention by and maintained translation/replication in DCs. This contrasts with other viruses and replicons, notably the commonly used alphavirus replicons. In turn, the pestivirus replicon characteristics increase the chances for developing robust immunity. Nonetheless, activation of innate immune mechanisms is required for maturation of the DCs, essential for efficient induction of adaptive immune responses, as opposed to tolerance or anergy. Accordingly, the delivery vehicles employed with the RepRNA require a potent adjuvant.

The adjuvants were formulated with a number of the delivery vehicles carrying RepRNA mentioned above. These delivery vehicles carrying the RepRNA were selected in the most part from the *in vitro* evaluations (see the section on *in vitro* evaluation below). Certain experiments compared several different adjuvants, for enhanced induction of humoral and cellular immune responses against the RepRNA-encoded vaccine antigen. Moreover, pulmonic or subcutaneous administration employed RepRNA alone or in delivery vehicle formulation, co-administered with the adjuvant. The final formulation with adjuvant was assessed by both mucosal and parenteral immunisations. Readouts compared adjuvanted with non-adjuvanted groups for induction of humoral immunity, T-lymphocyte profiling, T-lymphocyte activities, and cytokine profiling.

The most promising candidates were MALP-2 and cyclic-di-AMP (c-di-AMP), both having distinct modes of action. These offer additional advantages in being synthetical and applicable via both parenteral or mucosal routes. Importantly, the cyclic-di-AMP proved to be the most efficacious, by both parenteral and mucosal routes of immunisation. For more details, see below under the sections on *in vitro* and *in vivo* evaluations.
For more details, see also

1. Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223

In vitro Evaluation

(i) Efficiency of RepRNA delivery by different complexes.

Both primary DCs (murine, porcine, and human) and cell lines relevant to either replicon translation or as potential models for DCs were employed. For assessment of delivery efficiency, the RepRNA was fluorescently labelled to facilitate its tracking. As expected, VRPs proved to be particularly efficient at delivering RepRNA, measured by translation of the RNA rather than microscopic observation of labelled replicon. As for the other delivery systems, regardless of which was employed – cationic lipids (lipoplexes), lipopolyplexes (LPRs) PEI polyplexes (with or without cell penetrating peptides), chitosan-based complexes, or the new virosome-like HA-nanoparticle commercial liposome – delivery efficiency varied dependent on the components employed for each system. Nonetheless, all systems and components assessed demonstrated efficient delivery of labelled RepRNA to DCs and to certain cell lines, particularly the porcine kidney cell lines in which replication of the pestivirus replicon is well established.

For comparative purposes, DNA and small RNA molecules such as siRNA and mRNA were also employed, particularly when the partner was unable to use the RepRNA. While this proved a useful model for small RNA molecule delivery, the pattern obtained for delivery of labelled RNA did not relate to that seen with labelled RepRNA. While many of the delivery formulations could deliver DNA or small RNA molecules, this was not necessarily indicative of efficient RepRNA delivery. With some delivery formulations, RepRNA could be detected earlier than the small RNA molecules, whereas with other formulations it was the converse. It was considered by the consortium partners that the larger size of the RepRNA, and therefore its more complex interaction with the delivery vehicle components, likely had an impact on these observations. Nonetheless, if a formulation or particular component therein was found to be poor or unreliable for small RNA delivery, one could presume that a similar scenario would arise with the RepRNA. Similarly, delivery to cell lines did not guarantee delivery to DCs with the same efficiency. Nonetheless, the use of appropriate cell lines could prove of value for determining the potential for delivery to DCs.

(ii) Efficiency of RepRNA translation following delivery by different complexes.

From the above work, it became clear that certain formulations, or modifications to the components thereof, were more efficient than others at delivering the RepRNA. However, many of the formulations and components under test had been developed for delivery of small RNA molecules or DNA. As witnessed from the above, this could not guarantee success with the much larger RepRNA and its more complex interactions with the delivery vehicle components.

Accordingly, it was critical to ascertain if the observed efficiency of delivery would in turn provide efficiency of translation for the RepRNA. This was particularly important when the delivery components had proven effective with siRNA or DNA, because the ultimate site for delivery of these nucleic acids has no relationship to the site for delivery of the RepRNA, namely the ribosomal machinery for translation. In contrast, delivery components successful with mRNA could

identify candidates for delivery of RepRNA, due to their similar requirements for the ribosomal machinery. Nonetheless, one had to consider that major differences between mRNA delivery and RepRNA delivery could come from the greater size of the RepRNA and its more complex interaction with the delivery vehicle components. In other words, compaction of the RepRNA by the delivery vehicle might prove more problematic than with mRNA. Certainly, identification of efficient delivery would not distinguish this problem, although microscopic compartmentalisation of the delivered RNA within the cell could indicate the likelihood of compaction or decompaction, dependent on the image and site of intracellular localisation obtained. Ultimately, assessment of translation would bring to light any problems with release of efficiently delivered RNA.

Those delivery formulations and components found to provide for efficient delivery of RepRNA to cells, particularly when the assessment had employed DCs, were further investigated for efficiency of delivery leading to translation of the encoded genes. This could not be assessed in combination with the delivery, because it was considered that the labelling of the RepRNA may have modified the RNA to impeded with ribosomal entry and/or movement along the RNA for translation.

Irrespective of the delivery system employed, delivery leading to translation was seen to be dependent on both the formulation and the cells employed for assessment. Indeed, certain cell lines proved much less efficient for assessment of the translation. Moreover, the results demonstrated the importance for screening with primary DCs. Neither the efficiency of associating the RepRNA with the delivery vehicle, nor the efficiency of the RepRNA delivery into the cells could be related to this translation. Clear delivery of the RepRNA was essential, but only certain formulations facilitated the apparent release of the RepRNA for translation of the encoded antigens. By modifying the delivery vehicle component, one could modify this efficiency of delivery leading to translation, even when the delivery efficiency was apparently unaltered. Moreover, efficient delivery of small RNA molecules had no relevance to identifying formulations for efficient delivery of RepRNA that would ensure translation.
See also

1. Démoulins et al 2016 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;
3. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271;
4. Englezou et al 2018, Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids 12:118-134.

(iii) Influence of delivery vehicle complexing with the RepRNA on translation efficiency.

<u>A. RepRNA functionality and cell sensing</u>

It was considered that the RepRNA may have been non-functional in certain complexes or compacted to a degree that would not be reversed adequately for ribosomal entry and translation of RepRNA-encoded antigens. Thus, the functionality of the RepRNA was assessed using virus replicon particles (VRP). These are constructed in complementing cell lines to create the original virus-like particles, but carrying the replicon in place of the virus genome. VRPs were efficiently delivered to cells – DCs and cell lines – promoting efficient translation of the encoded antigens, including the influenza virus antigens. These results clearly demonstrated that the RepRNA was indeed translation- and replication-competent.

Another consideration was the capacity of the cell to respond against the presence of this foreign RNA. It has been well established that both pestiviruses and their replicons do not induce cellular

innate responses (see above under the Section "Choice of the Pestivirus Replicon"). When another replicon to that employed within UniVax – an alphavirus replicon obtained from a third party outside the consortium – was assessed, there was the expected cellular innate response. However, this is irrelevant to the pestivirus replicon employed within UniVax. As described above under the Section "Choice of the Pestivirus Replicon", the pestivirus replicon does not possess the 5'-triphosphate cap of alphaviruses – strong inducers of cellular innate defences – and also encodes a leader autoprotease N^{pro} that regulates cellular innate defence pathways. Moreover, attempted regulation of cellular innate activity would pose problems for vaccine application. Such cellular activity is required for the vaccine adjuvant to promote DC maturation and thus efficient induction of adaptive immune responses.

Nonetheless, the readouts obtained from particular delivery vehicles with the aforementioned third-party alphavirus replicon were similar to those obtained using the same delivery vehicles with the pestivirus replicon employed within UniVax. Accordingly, it appeared that related events were indeed occurring that impacted on both the pestivirus and other replicons in terms of their efficiency for translation following delivery by these vehicles.

B. Delivery vehicle interaction with RepRNA

Accordingly, it was considered that the discrepancy between delivery and translation of the RepRNA with the different delivery systems may have related to the characteristics of interaction between the RNA and the delivery vehicle components. Analyses with different PEI-based formulations added some clarity to the situation. While certain formulations were efficient at promoting translation of all encoded antigens, other formulations provided for an imbalanced translation, often favouring translation of the endogenous replicon genes with little or no read-out from the gene of interest. This would imply a differential degree of compaction along the RepRNA, either preventing ribosomal entry or inefficiently protecting from RNase.

For this work, translation of the RepRNA "gene of interest" (GoI) – influenza virus hemagglutinin or nucleoprotein antigens (or the luciferase of RepRNA encoding this enzyme used as an indicator in certain experiments) – was compared with the RepRNA structural gene (E2). The GoI was under control of the ribosomal entry site within the 5'-NTR of the replicon, whereas the E2 was situated after the GoI and therefore under the control of the second (inserted) internal ribosomal entry site. By such means, it was possible to determine if all the RepRNA sequence was being translated, or only one segment dependent on which ribosomal entry site was involved. Certain formulations were found to be efficient for delivery leading to translation, regardless of which gene was under scrutiny. In contrast, other formulations promoting apparently similarly efficient delivery, provided for translation favouring either the GoI or the E2. Indeed, it was noted that particular formulation favoured delivery leading to decreased translation of the GoI with concomitant increase of E2 translation (compared with other formulations providing for translation of both genes. This would impact on the efficiency of the formulation as a vaccine, allowing for translation of downstream genes, but poor for translation of the GoI, namely the Influenza virus antigens.

These studies with the PEI polyplexes demonstrated that three major parameters influenced the outcome of delivery in terms of RepRNA translation:
the delivery component molecular weight,
the ratio of the delivery component to the RepRNA,
and the presence of membrane perturbing entities (in the case of the PEI polyplexes, these were inclusion of cell penetrating peptides).

The above results demonstrated that different delivery formulations could differ in their interaction with the RepRNA such that only certain genes would be translated. That is, a major issue to be assessed with delivery formulations for RepRNA is the degree of compaction of the RepRNA. Even with efficient delivery, and apparent translocation to the cytosol for translation, it is necessary to define if all genes are being translated, or if there is interference with a portion of the replicon. Thus, delivery of these large self-replicating RNA molecules require definition with respect to translation of different genes, rather than just the GOI, for identifying optimal delivery for the desired immune activation *in vivo*.
See also

1. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271

Clearly, careful selection of the delivery vehicle components
permits efficient formulation with RepRNA to protect from RNase,
promotes delivery to DCs,
and facilitates translation of the vaccine antigens leading to induction of immune responses (see the section on *in vivo* evaluation below).

While different delivery vehicle formulations were shown capable of delivering to both DCs and cell lines, this was dependent on the components of the delivery formulations. Moreover, the efficiency of delivery did not relate to efficiency at promoting translation of the delivered RepRNA. Modification of these delivery vehicle components has allowed delivery leading to increased translation efficiency of the delivered RepRNA. This was particularly notable with certain PEI-based polyplexes, newly developed PTG-lipopolyplexes, and modified chitosan-based delivery vehicles. In addition, the new virosome-like HA-nanoparticle delivery vehicle also showed *in vitro* delivery promoting translation. Nonetheless, overall the most efficient means of RepRNA delivery leading to translation of the RNA is via the VRP.
These new formulations were evaluation by mucosal (intra-pulmonary) and parenteral vaccination *in vivo* (see below under "*in vivo* evaluation").

(iv) Targeting RepRNA delivery.

A. Selection of targeting ligands.

The main focus for identification of potential targeting ligands assessed over 300 glycoconjugates for their relative capacity at binding to DCs compared with other mononuclear cells obtained from porcine and human donors. This collaborative effort involving Partner 3 (SYN) with Partner 1 (IVI) allowed clustering of the probes into different groups dependent on their relative binding to the different cell populations present. This clustering facilitated a definition of glycan binding capacity of the different mononuclear cells, with particular emphasis on the DCs and DC subsets, particularly with the human mononuclear cells. The analyses also permitted identification of species-common and species-specific glycan-binding receptors.

B. Assessment of glycan binding

The binding of probes interactive with DCs was also assessed on cell lines, in particular the DC2.4 cell line – supposed to resemble DCs in their ability to present antigen to T-lymphocytes – and activated THP-1 cells – supposed to reflect human DCs and macrophages, dependent on the mode of activation. However, the glycan binding profile for the DC2.4 cells was distinctive from that for DCs or even other mononuclear cells. Indeed, many of the glycans showing strong binding with DCs were poorly reactive (if at all) with DC2.4 cells). This work demonstrated that while DC2.4 cells could be employed for antigen presentation studies, they could not be used for assessment of nanoparticulate vehicle delivery to DCs. Importantly, the risk was that false readouts could be

obtained, and one might be left with a false impression on lack of binding. As for the THP-1 cells, there was less discrepancy in the binding of glycan probes to these cells, compared with DCs. However, the profile tended to relate more to monocytes and monocyte-derived cells rather than the blood DC populations.

These results showing how different cell lines can vary considerably in their support of RepRNA delivery/translation can be related to the analyses on the glycan targeting ligands. Yet, it is now clear that the binding patterns obtained with cell lines, including cell lines supposedly related to DCs, were not the same as the binding observed with the primary DCs or even monocytes. This was particularly notable with the murine DC2.4 cell line. Accordingly, the selection of glycan probes for further analysis was on the basis of binding to human and porcine DCs, in particular efficient interaction with different human blood cell populations as well as differentiating the degree of binding to DC subsets and monocytes.

C. Assessment of endocytosis

The selected glycan probes were further analysed by microscopy to define binding capacity and internalisation efficiency, as well as the endocytic route employed by the cell. The aim was to ascertain that the glycans were indeed capable of promoting endocytosis by the DCs, and not just binding to the cell surface – some cell surface receptors can bind ligands, but rapidly eject them either before or shortly after endocytosis.

The RGD-containing peptides allowed refined definition of the DC endocytic pathways, combining with markers for different compartments and structures within these. This high level of definition was also applied to the glycan probes, which had been extensively pre-screened for their binding profiles for DCs. Only the glycans capable of promoting enduring endocytic processes would prove applicable for targeting RepRNA delivery. Such endocytosis is important due to the manner by which the cell will process the material, in turn impacting on the release of the RepRNA for translation.

Certain of the glycan probes were found to promote efficient forms of endocytosis, but not all probes tested gave clear indication of efficient endocytosis. When endocytosis was clearly detectable with a particular glycan, the process into which the probe would target its delivery was found to be dominated by slow endosomal involvement typical of macropinocytosis or caveolar endocytosis. Both processes are important for RepRNA delivery – being slowly maturing (acidifying) processes, they would create an environment more readily accommodating cytosolic translocation. Of course, for the latter this would further depend on the characteristics of the delivery vehicle and to what degree compaction of the RepRNA would impact on cytosolic release the RNA.

D. Glycan probes for evaluation

On the basis of the above analyses, selected probes were modified to interact with certain of the delivery vehicles. Partner 3 (SYN) synthesised probes in their biotinylated form and then coupled with the respective biotin-functionalized carriers through streptavidin-mediated conjugation. This allowed for conjugation with low molecular weight chitosan/hyaluronic acid nanoparticles – with a strategy for functionalisation of the surface hyaluronic acid on chitosan nanoparticles assessed by Partner 12 (IIT) – and PEI-based polyplexes – with functionalised PEI assessed by Partner 1 (IVI). The selected probes were based on GalNAcα1-3Galβ and Neu5Acα2-8Neu5Acα2-8Neu5Ac, either in monomeric or polymeric form. Negative controls were included as well. The different glycan probes were:

A_{di}-biot (GalNAcα1-3Galβ), "Adi Mono"

$(Sia)_3$-biot (Neu5Acα2-8Neu5Acα2-8Neu5Ac) “Sia Mono”
αLacNAc-biot (Gala1-4GlcNAcb) “Neg Mono”
Biot1-pHAA-Sug(20) cat #87 Sug = GalNAcα1-3Galβ (A_{di}) “Adi Poly”
Biot1-pHEAA-Sug(20) cat #73 Sug = Neu5Acα2-8Neu5Acα2-8Neu5Ac (Sia_3) “Sia Poly”
Biot1-pHAA, no Sug, as negative control (no Sug ligand) “Neg Poly”.

How the conjugation with streptavidin affected the carrier properties was studied. An increase both in size and dispersity was observed, but the characteristics of low molecular weight carriers were only slightly affected. The screening allowed for the provision of the conjugated delivery vehicles for assessment *in vitro* and *in vivo*, namely their influence on RepRNA cargo delivery and translation efficiency.

In vivo Evaluation

(i) In vivo evaluation models

Following the *in vitro* identification of the most potentially efficacious delivery formulations delivering RepRNA for translation in dendritic cells, the prototypes were assessed by immunogenicity studies in mice and pigs. Certain delivery vehicle formulations were identified as showing promise for efficacious immunogenicity. Interestingly, this was noted with examples of each type of delivery system initially under investigation – lipoplex, polyplex, lipopolyplex and chitosan-based nanoparticle formulations.

This extensive work employed a number of *in vivo* experiments. The evaluations employed conventional immunogenicity studies with RepRNA encoding influenza virus antigens in mice and pigs. In addition, pre-immune mice were also employed. In order to enhance the capacity of the in vivo assessment, the TCR Ova model (OTI and II) together with a RepRNA encoding the Ova antigen was also employed. Assessment using the TCR Ova model offers a means of crossing *in vitro* with *in vivo* assessment. $CD8^+$ and $CD4^+$ ovalbumin-specific T cells were isolated from OTI and OTII mice, respectively (this allows analysis of both the helper and cytotoxic cell-mediated immune responses). Naïve TCR transgenic CD8+ and CD4+ T cells were enriched, labelled with CFSE, and injected intravenously into the recipient animals. After 24h, the animals were vaccinated with the prototype vaccine formulations, carrying RepRNA encoding for ovalbumin, selected as described under the *in vitro* evaluation section 6 above. The proliferative capacity of the injected ovalbumin-specific T cells was analysed after 5 days.

With both the conventional vaccination models in mice and pigs, and the TCR Ova model, in-depth analysis assessed most aspects of immune responsiveness. This included antibody induction, T-lymphocyte subset induction, cytokine profiling and assessment of individual lymphocyte activities (see below under the different subheadings).

(ii) Evaluation of initial delivery vehicle formulations

The initial lipoplex, polyplex and lipopolyplex formulations did not promote the clear specific immune response anticipated from the *in vitro* evaluations. However, modifications of the polyplex and lipopolyplex formulations showed increased efficiency *in vitro* for delivery to DCs and cell lines leading to translation of the encoded gene of interest. This included assessment of different PEI molecular weights and formulation modifications. In addition, modifications to the chitosan-based nanoparticles in terms of molecular weight and formulation were brought into the evaluations, again based on initial *in vitro* assessment.

(iii) RepRNA requires particular delivery vehicle formulations

These approaches identified new polyplexes, lipopolyplexes and chitosan-based vehicles with high promise for increased translation of the delivered RepRNA. To this group were added the new virosome-like HA nanoparticles, again showing high promise from the *in vitro* evaluations in DCs and cell lines. New *in vivo* assessments employed both murine and porcine models; the murine experiments compared a mucosal route (intra pulmonary) and parenteral route of injection.

These experiments confirmed the capacity of the new modified formulations to induce immune responses against the influenza virus antigens (or Ova) encoded by the RepRNA. Particularly interesting was the observation that not all formulations of the particular type of delivery vehicle (lipoplex, polyplex, lipopolyplex, chitosan-based particles and lipid/HA-nanoparticles) would prove effective. While this related to the situation observed *in vitro* whereby only certain formulations of each type of vehicle would deliver RepRNA leading to translation, the selectivity and restraint of delivery vehicle efficiency was more acute *in vivo*.

Moreover, when forms of each type of delivery vehicle that were effective *in vitro* were employed *in vivo*, certain formulations were more efficient at delivering RepRNA inducing one compartment of immune responses – humoral or cell-mediated. Nonetheless, particular formulations were identified that delivered RepRNA leading to induction of both humoral and cell-mediated immune responses.

(iv) Importance of the adjuvant in RepRNA/delivery-vehicle formulations
Importantly, the power of the cyclic-di-AMP has been clearly demonstrated, and that it is more potent with the RepRNA than any of the other synthetic adjuvants investigated. Cyclic-di-AMP adjuvant plays an essential role with the RepRNA for ensuring efficient induction of immune defences. In particular, cyclic-di-AMP proved to be a powerful mucosal adjuvant. This was observed with all delivery vehicle types assessed, including the VRPs.

(v) Evaluation of the final selected RepRNA/delivery-vehicle formulations
Using the data obtained from all previous *in vitro* and *in vivo* evaluations, final experiments were performed in mice and ferrets using the RepRNA/delivery-vehicle formulations showing the greatest efficacy following both mucosal and parenteral administration. These were one selected lipopolyplex, one selected chitosan/hyaluronic acid nanoparticle and one selected virosome-like HA-nanoparticle (without the surface HA, due to the *in vitro* evaluations showing that this did not influence the efficiency of delivery leading to translation of the delivered RepRNA).

The vaccines were composed of nanoparticulate delivery vehicles carrying RepRNA encoding for influenza virus HA (H1), NA (N1), NP and M1. This was an advance on previous experiments in which the delivered RepRNA encoded for either HA or NP. It is important to note that the RepRNA molecules encoded for a single Influenza virus antigen. This was designed to avoid the problems associated with multiple ribosomal entry site insertions, which are required for multiple foreign genes, in turn causing dramatic reduction in translation efficiency of the foreign genes.

Following vaccination (prime or boosts), there were no observed indicators for toxicity or signs of other adverse reactions with any of the formulations under test. This related to the previous observations with RepRNA encoding for HA or NP. Specific immune responses were detected in the animals vaccinated with the different delivery formulations. Interestingly, antigen-specific lymphocyte proliferation and cytokine responses were also observed with the RepRNA plus cyclic-di-AMP adjuvant, without delivery vehicle, implying that the adjuvant could also offer delivery potential. This had been observed in previous experiments using mice, but was less clear with the immunization of pigs (although the RepRNA/cyclic-di-AMP vaccination was not without immunogenic effect); in this context, it should be stressed that there the immune response

induction was dependent on the amount of RepRNA delivered to the mice, but only a single dose was tested in the pigs. Importantly, the particular HA-nanoparticle formulation, particular lipopolyplex formulation, and particular chitosan/hyaluronic acid formulation delivery of RepRNA also provided evidence for levels of protection against H1N1 virus challenge in mice and ferrets.

For the successfully efficient delivery formulations for RepRNA, each formulation showed a more pronounced efficacy for particular immune response components. Certain formulations favoured more an IL-2 and/or IL-12 response, while other formulations favoured IFN-γ-producing CD8+ T lymphocytes. While the lipopolyplex and chitosan/hyaluronic acid nanoparticles favoured antigen-specific T-lymphocyte responses, the HA-nanoparticle delivery was particularly efficient – for both $CD4^+$ and $CD8^+$ lymphocytes. Importantly, all delivery systems required the presence of the cyclic-di-AMP adjuvant. Intriguingly, when the combination of RepRNAs (encoding HA, NA, NP and M1) were employed with just the cyclic-di-AMP (no other delivery vehicle), an efficient induction of both antigen-specific $CD4^+$ and $CD8^+$ lymphocytes was observed.

The quality of immune effector functions has been related to the induction of multifunctional T cells, as a correlate of protection against influenza virus (Darrah et al. 2007). The above delivery formulations – lipopolyplex, chitosan-based, HA-nanoparticle and the cyclic-di-AMP adjuvant without delivery vehicles – all delivered RepRNA leading to antigen-specific induction of at least one cytokine – IL-2, IL-4, IL-6, IL-17, IFN-γ or TNF-α. Dominance of a particular cytokine was dependent on which delivery system was employed. Nonetheless, antigen-specific induction of IFN-γ and IL-17 dominated the profile regardless of the delivery. HA-nanoparticle delivery of RepRNA proved to be particularly efficient in this respect, while the lipopolyplex delivery of RepRNA was particularly effective for $CD8^+$ lymphocyte IL-17 and TNF. The multifunctional T-lymphocytes were also induced by RepRNA delivery with the different formulations. This was most notable when employing HA-nanoparticles, the lipopolyplex, or the cyclic-di-AMP without any other vehicle.

Current status of RepRNA/delivery-vehicle formulations

These results clearly demonstrated that a particular delivery formulation could not guarantee success for delivery of RepRNA leading to translation and induction of immune responses *in vitro*. Even formulations shown to be successful for small RNA molecules, such as mRNA or siRNA, could not be relied upon to provide the conditions required for successful RepRNA delivery leading to immune response induction. The effort of the UniVax consortium demonstrated that the delivery vehicle must be created specifically for RepRNA, taking into consideration the size and complexity of this molecule, the very particular requirements for its delivery, and the peculiarities associated with RepRNA release and translation for inducing immune responses.

A number of *in vivo* immunisations, using either mucosal delivery or parenteral delivery (sub-cutaneous or intra-muscular), identified formulations with clear efficacy for delivery, resulting in clear induction of specific immune responses. For all, the inclusion of the cyclic-di-AMP adjuvant was essential. The efficacy was especially notable after intra-pulmonary injection. One particular HA-nanoparticle formulation with RepRNA proved effective for leading to induction of both humoral and cell-mediated responses, as did a particular lipopolyplex and a particular PEI polyplex. These formulations were effective by both mucosal and parenteral routes of administration. In contrast, the cationic lipid lipoplex formulation chosen for mucosal delivery was different from that chosen for parenteral administration. As mentioned above, the particular HA-nanoparticle formulation, particular lipopolyplex formulation, and particular chitosan/hyaluronic acid formulation delivery of

RepRNA also provided evidence for levels of protection against H1N1 virus challenge in mice and ferrets.

These *in vivo* experiments provided clear evidence of immunogenicity for nanoparticulate delivery of RepRNA. An important discovery was the particularly powerful immunomodulatory capacity of cyclic-di-AMP, both parenterally and mucosally. Moreover, these results have allowed the selection of the delivery formulations showing the greatest promise for efficacy at inducing influenza virus-specific immune responses. The most efficient delivery system remains that based on VRPs, with which the added value of including the cyclic-di-AMP adjuvant was observed.
For more details see

1. Démoulins et al 2016. Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017. Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;
3. Démoulins et al 2017. Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271;
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5. Englezou et al 2018. Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids, in press.
6. Démoulins et al 2018. Virus replicon particle vaccine encoding nucleoprotein and hemagglutinin of influenza A elicit a robust adaptive immune response in pigs. (submitted for publication)

Immunological Evaluation of Biobank material

(i) Biobank evaluation in relation to Clinical Trial

A clinical trial at Haukeland University Hospital in Bergen, Norway was used to evaluate local and systemic immune responses after LAIV in children and adults. Clinical trial samples have been stored in a biobank of blood and saliva samples collected at different time points.

Overall, the biobank samples provide material from influenza H1, H5 and H7 infections or vaccinations, including seasonal vaccine studies. These have been employed to dissect the immune responses, in terms of human B and T cell epitopes, particularly potential universal epitopes. The HA stalk is highly conserved allowing the influenza A viruses to be divided into two groups. HA head and stalk specific antibody binding has been assessed, together with the avidity of binding and the functionality of stalk-specific antibodies using virus neutralization and antibody-dependent cellular cytotoxicity (ADCC). HA stalk-specific antibodies may have an important role in protection through neutralization and ADCC in people who respond poorly to traditional inactivated vaccines.

The data obtained to date shows that particularly young children respond well to LAIV with serological responses to H3N2 and B strains, along with local and systemic antibody secreting and memory B cell responses. The H1N1 strain did not elicit antibody responses, although T cell responses were detected in blood and tonsils. LAIV induces systemic and local T cellular responses including protection associated cross reactive T cells which may provide heterologous protection in children. This information is especially valuable to the RepRNA vaccine of UniVax, which possesses at least similar characteristics of immunogenicity to an LAIV. In particular, this concerns the ability of RepRNA to self-replicate and more closely mimic the situation with an Influenza virus infection, and therefore the type of immune responses induced.

(ii) Biobank evaluation with respect to immune responses of the elderly

Immune responses following influenza vaccination in elderly were assessed employing biobank material from 234 volunteers aged ≥ 65 years. Vaccine recipients were stratified in responder and non-responder by their increase of the hemagglutination inhibition (HAI) titres. PBMCs drived from responders and non-responders were used for in-depth multi-parametric flow cytometry analysis. While responders showed enhanced functionality upon vaccination, non-responders displayed a more suppressive immune phenotype. The acquired data is still being processed, together with correlation studies to gain a broad picture of vaccine-induced processes relative to age and responder status.

For more details see

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Potential Impact

Overall Impact of UniVax vaccine development

UniVax employed a number of different delivery vehicle formulations for synthetic delivery of RNA vaccines based on the self-amplifying (self-replicating) replicon technology, using a non-cytopathogenic pestivirus replicon to ensure duration of antigen expression in the targeted cells (patents US9249395B2, US9670466B2, WO 2009/146867, EP2130912, EP08010222, 4414/KOLNP/2010).

The delivery employed nanoparticulate vehicles derived from modified chitosan, newly developed PTG-lipopolyplexes (patents WO2017212197, WO2009050372), PEI-based polyplexes (patent WO2009112402), cationic lipid-based lipoplexes (patent WO2009/106713), and a new virosome-like vehicle based on Coatsome® technology referred to as HA-nanoparticles. These were also assessed using targeting ligands based on glycan technology, as well as hyaluronic acid or recombinant baculovirus Influenza virus HA. Modification of the delivery vehicle components allowed selection for the most efficient delivery leading to translation of the delivered replicon. An essential component of the developed formulations was the presence of the mucosal adjuvant cyclic-di-AMP. Interestingly, the cyclic-di-AMP alone with the replicon also provided evidence of efficacy.

Assessment proved efficacity for both mucosal and parenteral vaccinations, for which certain delivery vehicles proved more notable, namely the new virosome-like HA-nanoparticle delivery vehicle. The newly developed PTG-lipopolyplexes, certain PEI-based polyplexes, and the modified chitosan-based delivery vehicles also showed efficacy for both mucosal and parenteral vaccinations. With the cationic lipid lipoplex formulation, that selected for mucosal delivery was different from that chosen for parenteral administration. In comparison, it was noted that the most efficacious delivery of replicon vaccines for induction of both humoral and cell-mediated immune defences employed the technology for generating virus-like replicon particles (see below).

Impact of Self-Amplifying RNA Vaccines

A major advance in the evolution of vaccines has been the development of nucleic acid-based (particularly RNA) vaccines, together with application of synthetic biology for both the vaccine and the delivery systems. While mRNA vaccines have shown their worth, they have the same failing as inactivated vaccines, namely they are non-replicating and therefore limited to the size of the vaccine payload for the amount of antigen delivered (or translated in the case of mRNA). Such mRNA molecules have limited half-life within the cell. In order to enhance the duration of RNA vaccine translation and the generation of antigen, self-replicating (also known as self-amplifying) RNA vaccines have been developed. These are vaccines based on replicon technology – replicons are defective virus genomes, rendered defective by removal of one or more genes encoding structural proteins essential for virus assembly and therefore incapable of generating infectious progeny. The latter characteristic of replicons provides an additional advantage over live, attenuated virus vaccines, which cannot guarantee safety from reversion or recombination to generate more pathogenic progeny.

Thus, replicon vaccines offer a high impact for the future development of safe and efficacious vaccines. Replicons derived from certain virus families – such as the so-called positive-strand viruses of pestiviruses, alphaviruses and flaviviruses – are readily modified to facilitate insertion of any gene of interest, such as genes encoding vaccine antigens or a therapeutic product. A further advantage is offered by such positive-strand replicons – as with the parent virus genome, they function as mRNA upon release within the cells, to encode all necessary proteins for both the

vaccine and initiating the replicative cycle of the replicon. For this reason, they are also referred to as self-replicating mRNA entities.

Of course, the duration of their effectiveness within the targeted cell relies on the nature of the replicon, which often mimics that of the parent virus in terms of cytopathogenicity. For a more durable induction of immune defences, it is desirable that the replicon vaccine does not kill the targeted cell, but can continue at a low rate to translate the vaccine antigen; this increases the fine-tuning of the developing immune response and thus the efficiency and robustness over the long term of the vaccination. Consequently, non-cytopathogenic replicons such as those derived from pestiviruses offer this advantage over those derived from cytopathogenic viruses such as alphaviruses and flaviviruses.

Application of such replicon-based vaccines has primarily employed the technology for generating virus-like replicon particles (VRPs). Whilst efficient, VRPs rely on complementing cell line to provide the gene product missing from the replicon, allowing for the generation of particles carrying the replicon. Whilst this is a most efficient means of delivering the replicon to target cells, an alternative is the use of biodegradable, synthetic nanoparticulate particles. By such means, both the replicon and the delivery vehicle can be generated synthetically without recourse to cells.

The generic nature of the UniVax creation

Most importantly, UniVax provides a generic platform for creating innovative, biodegradable, biosafe vaccines. As biosafe, replicative entities, they have potential to replace less efficacious inactivated (non-replicating) vaccines. When vaccinations have to be repeated on a regular or even annual basis, the advantages of a self-replicating vaccine offer a much-reduced vaccination programme, or even single shot vaccination. This is both more efficient and more agreeable to individuals being vaccinated. Importantly, the nature of the RepRNA with the UniVax innovative vaccine formulation provides particularly high generic potential. The plasmids carrying the RepRNA constructs can be easily modified to encode any antigen, therapeutic agent or gene of choice. The insertion sites within the RepRNA sequence allow for replacement of the so-called “gene of interest” in a rapid and efficient manner. This allows for rapid modifications of the vaccine in case of antigenic change, as occurs during pandemic Influenza, or replacement with a new sequence for whatever vaccine or therapeutic use is sought. Moreover, the generic nature of the UniVax creation is particularly pertinent considering the current problem with measles.

The only question now remaining is that surrounding the compaction of the rather large RepRNA molecule. UniVax partners are continuing research along these lines to enhance cytosolic release of the delivered RepRNA in a decompacted manner that will enhance the translation of both the gene of interest and the downstream genes facilitating replication of the RepRNA and therefore enhanced immune defence induction for robust and long-lasting immunity form a small number or even single shot administration.

The particular case for influenza

Influenza is a serious public health problem affecting more than 100,000,000 people per year. During seasonal epidemics, influenza infects up to 10% of adults and 20% to 30% of children. Whilst many recover from the symptoms within one to two weeks, there is a high risk for severe illness (3-5 million cases) or death (250,000 to 500,000) (WHO); most deaths occur among the elderly. The most effective way to prevent the disease is vaccination, although the vaccine among the elderly reduces severe illness and complication by only up to 60%. Annual vaccination is currently recommended for pregnant women, children 6 to 59 months of age, healthcare workers

with patient contact, the elderly and people with underlying chronic health conditions such as respiratory, cardiac, metabolic, neurological and immunosuppressive diseases.

An additional complication with influenza virus arises from the different forms of which the disease can be considered – seasonal, pandemic and zoonotic. It is important that a universal vaccine can protect against these different forms. This formed part of the UniVax approach for evaluation of prototype vaccines.

A major innovative approach of UniVax is the application of self-amplifying (self-replicating) replicon RNA (RepRNA) vaccines. Replicons have been generated encoding influenza HA (H1 and H5), NA (N1), NP, M1 and PB1 proteins. These are delivered by synthetic, biodegradable, nanoparticulate vehicles, formulated with defined adjuvants for parenteral or mucosal delivery. Early assessment focused on delivery of replicons encoding HA and NP, and compared with replicons delivered as VRPs. The vehicles selected as proving most efficient were tested with replicons encoding HA (H1), NA (N1), NP and M proteins.

The UniVax impact on human health

The Elderly

The European population is living increasingly longer due to improved health and treatment. This increases the number of persons in the high risk group for influenza infection of the elderly. It has been projected that by 2050 the ratio of workers per retiree will change from four to two (http://eurex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2012:396:0008:0011:EN:PDF).

The Young

WHO estimated that pneumonia is the number one killer of young children in third world countries, with 1.8 million deaths in their report of 2009. Influenza can weaken resistance of the patient, which related to its significant association with pneumonia probably by facilitating entry and complications by bacterial pathogens (Brooks et al 2009. Pediatr Infect Dis J 29:1-6). WHO also estimates that 20% of children are infected annually with seasonal influenza. Consequently, the development of a universal influenza vaccine that protects against seasonal and pandemic influenza would fulfil the millennium goals of improved maternal health and reduced child mortality. Moreover, such vaccines have increased value if protection can be assured against influenza virus strain variation. In terms of pandemics, any influenza vaccine would greatly benefit from a system with which modification of the vaccine to counter the newly emerged virus strain would be rapidly generated. This is a major advantage of the replicon technology employed in UniVax.

The UniVax Impact

The outcome of the UniVax project has been the first synthetic replicating RNA vaccine against influenza. UniVax has generated essential data on integrating innovative technologies for RepRNA, synthetic delivery to dendritic cells, glycoconjugate-based targeting of dendritic cells, and mucosal adjuvants. The first ever prototype synthetic RepRNA vaccines with innovative new generation mucosal adjuvants have been evaluated pre-clinically, providing data on enhancing efficacy of vaccine delivery for inducing both humoral and cell-mediated immune defences.

UniVax has a direct outcome in terms of a novel clinical technology, with considerable benefits for health of the human population. The project also provides tools in basic research (RNA delivery systems to DCs, new adjuvants, replicative RNA technologies). It can now enable the widespread use of nucleic acid delivery to dendritic cells and other “immune cells” such as monocytes, macrophages, natural killer cells or even lymphocytes with the purpose of investigating cellular functions and overexpressing or silencing genes in therapeutic strategies.

Innovative Impact
UniVax has employed patented (within the UniVax Consortium) technologies for synthetic, biodegradable nanoparticulate delivery of self-amplifying replicon RNA vaccines. Novel RNA delivery systems targeting dendritic cells have been generated, evaluated together with new synthetic mucosal adjuvants. Efficient, standardized RNA delivery technologies as a basis for vaccines as well as other gene therapy applications are now available. This represents tremendous progress for affected patients and their families. The economic impact of such technology comes from its immense applicability beyond the vaccine field (both infectious diseases and cancer).

UniVax integrated the technologies for the individual modules of the replicon RNA vaccines, adjuvants, delivery vehicles, and glycoconjugates for dendritic cell targeting. The resultant synergistic approach for improving delivery and induction of both humoral and cell-mediated immunity is particularly innovative. Association of these synergising technologies strengthened the efficiency of biodegradable particle-based nucleic acid delivery, greatly facilitating and standardising the formulation process.

The UniVax technologies are applicable to a broad spectrum of diseases and clinical scenarios, requiring vaccination or therapeutic applications. An important advance with the UniVax innovative approach is the controlled and targeted delivery of self-amplifying replicon RNA by nanoparticulate carriers to dendritic cells, without recourse to antibodies. Together with the power of the synthetic adjuvants developed within UniVax, this is expected to be of high value beyond the UniVax project. It is anticipated that other new high-efficacy vaccines will be developed in the future. The UniVax partners have realised a completely new synthetic vaccine/therapeutic process – based on self-amplifying replicon RNA, highly efficacious adjuvants and nanoparticulate delivery systems with glycan-based targeting ligands – which does not currently exist. Biodegradability, biocompatibility of a natural product, amphiphilicity, easy handling, and efficient preparation of the cargo have been brought together in one vaccine/therapeutic agent.

IPR and Exploitation Opportunities
Intellectual property protection for a future industrial partner is provided by the aforementioned patents
Partner 1 (IVI): US9249395B2, US9670466B2, WO 2009/146867, EP2130912, EP08010222, EP2009003892, 4414/KOLNP/2010;
Partner 2 (OZB): WO2009/106713;
Partner 7 (CNRS): WO2017212197, WO2009112402, WO2009050372
Partner 8 (HZI): EP 05 022 771.9, EP 05 025 431, EP 05 02 4266, EP 09016050.8.

Development, registration, global promotion, marketing, distribution and sales of the UniVax influenza vaccine is now open for partnership and further development to a pharmaceutical firm interested in applying this new innovative technology. The knowledge and capacities of the UniVax partners, together with their connections in the life science industry, allows for facilitated exploitation by industrial partners. By such means can commercialisation of several aspects of the project technologies be facilitated. This allows for straightforward exploitation, technology transfer and dissemination to additional and associated industrial fields, and increased competitiveness of European SMEs. Moreover, the UniVax developments and patent protected IPR facilitates extension of the self-amplifying replicon RNA formulated with nanoparticulate delivery systems and highly efficacious synthetic adjuvants to the treatment of other diseases beyond influenza, leading

to additional patent rights for vaccines and opening a gate to a novel class of therapeutics. This is particularly pertinent considering the current problems surrounding the ongoing measles epidemic.

Strategic Impact
The strategic impact of UniVax is applicable to a wide spectrum of vaccinations and therapeutic indications. UniVax has developed the first multimeric and synthetic universal influenza vaccines; this is based on replicon RNA and synthetic nanoparticulate technologies, together with synthetic adjuvant and glycan targeting ligand technologies. As such, the UniVax creation has as a direct outcome as a novel clinical technology with benefits for human health (see above). Moreover, the UniVax technologies provide tools in basic research (self-replicative RNA technologies, RNA delivery systems and targeting ligands for dendritic cells, new synthetic mucosal adjuvants). It will enable the widespread use of nucleic acid delivery to dendritic cells and other "immune cells" such as monocytes, macrophages, natural killer or even lymphocytes. Thereby, application will impact on investigations of cellular functions, as well as assessing the overexpression or silencing of genes through therapeutic strategies. The UniVax project deliverables now provide unique components of highly flexible modules that can be combined in an optimal manner for a selected application – vaccination; non-integrative gene delivery (for expression or silencing).

This strategic impact of Univax revolves around standardized synthetic self-replicating RNA-based vaccines, the replicon vaccines. Thereby, the innovative technologies for synthetic self-replicating RNA replicons, synthetic delivery vehicles, glycoconjugate-based targeting and mucosal adjuvants have been are integrated. The "vaccine" is readily formulated, and can be employed with standard operating procedures to cGMP grade. This development represents significant progress over current viral vector-based vaccine technologies, which present concerns in terms of manufacturing, reproduction and safety. Univax has enable the generation of novel synthetic self-amplifying replicon-based vaccines, for which SOPs and standardized protocols are available, allowing for generation of comparable results amongst different users. This provides a clear advantage as well as contributing to standardization efforts of authorizing agencies such as EMA and FDA.

Product Impact
UniVax has broadened the scope for nucleic acid delivery technologies and application, as well as developing new life sciences research reagents. In this context, UniVax outputs cover new replicative (self-amplifying) RNA vaccine technology, new synthetic adjuvants, new engineered self-amplifying RNA delivery systems, new dendritic cell-targeted RNA delivery.

Most techniques, protocols and reagents developed in specialized research laboratories emerging during basic and applied researches never become available to the broad scientific community. Licensing agreements with UniVax Partners allows the development of research tools and kits that can be commercialized for use in basic and applied research. The publications from the UniVax partners have also contributed to a broad dissemination of the project results and products within the scientific community, as well as providing essential information within the public domain for further development. Thus, marketable products developed within UniVax are available for dissemination to the scientific community and vaccine/pharmaceutical industries. Participation in the development of universal influenza vaccines is estimated with a turnover at 200-300 M€.

Market Impact
Taking influenza as an example, seasonal epidemics were reported by WHO to cost the lives of 250,000 to 500,000 people/year. In 2007, the annual damage to the U.S. economy was $71-167 billion (Molinari et al 2007. Vaccine 25:5086-96). In 1996, the cost due to influenza in Germany was €2.5 billion (Szucs, MedKlin, 2001); €4.52 billion were spent in 2010 on Vaccine R&D. The

pharmaceutical industry invested >€12 million for health interventions each day; 31 new drugs were licensed in Germany in 2010. Yet, influenza therapy is rather ineffective and expensive – influenza viruses are highly adaptable at resisting anti-viral drugs. Vaccination is the most effective method to counter influenza. Yet, current influenza vaccines are strain restricted, of limited efficacy, and require long periods of production, due to the reliance on eggs for the vaccine production (potentially of high risk due to the availability of adequate supplies of high quality embryonated eggs). Considering the growth of the vaccine market worldwide, it has been proposed that RNA-based therapies will be a key element in 2020.

UniVax focussed its self-amplifying replicon RNA vaccine – delivered by synthetic biodegradable, nanoparticulate vehicles, and formulated with synthetic adjuvants of particular applicability to mucosal vaccination – against influenza virus. This can be Extended to other diseases and clinical situations requiring therapeutic applications. As such, this yields a much higher benefit for the involved partners, and the European job market and industry. Besides the vaccination market, UniVax technologies can address specific needs in life sciences, especially research and application linked to nucleic acid delivery systems. The gene delivery systems market opportunities cover a wide number of scientific areas. Transfection technologies are widely used in cell research, recombinant protein production, drug discovery, vaccine and gene therapy. The growing markets of bioproduction, gene therapy, “nucleic acid” vaccine and drug discovery boost the development of transfection technologies. In terms of geographic location, North America forms about 40% of the market, Europe about 30% (principal actors Germany, France and the United Kingdom), Asia 25%, and others 5%. Life sciences research occurring in universities, research institutes, hospitals, and pharmaceutical and biotechnology companies is a major driving factor for the market.

Scientific progress in cellular and molecular biotechnology has also led to development of advanced therapies – such as gene therapy – offering new opportunities for treatment of diseases and dysfunctions of the human body. The innovative products developed in UniVax provide a means for regulating genes in cells participating in the immune response, such as dendritic cells. These products can also be used in other nanomedicine fields. Nanomedicine has already produced a number of significant products in which the nano-dimension has made a significant contribution to product effectiveness.

UniVax has responded by providing (1) the first influenza virus vaccine based on the non-cytopathogenic pestivirus replicon RNA; (2) formulation with unique synthetic adjuvants of particular applicability to mucosal vaccination without any side-effects; (3) delivery via synthetic, biodegradable nanoparticulate delivery vehicles; (4) formulation together to generated a unique selling product; (5) patent protection.

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(b) Use and dissemination of foreground

Section A

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4. Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223.
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9. Islam et al 2017 Influenza A haemagglutinin specific IgG responses in children and adults after seasonal trivalent live attenuated influenza vaccination. Vaccine. 35(42):5666-567
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13. Mohn 2018 Immune responses after live attenuated influenza vaccination. Hum Vaccin Immunother. 14(3):571-578.
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Section B

Measures taken to protect RepRNA vaccine delivery formulations

Prior to UniVax, the coordinator of the UniVax project was involved with preparing and submitting a patent application concerning a unique vaccine formulation composed of the pestivirus RepRNA carrying foreign genes of interest, delivery by synthetic means using biodegradable nanoparticulate delivery vehicles. Currently, this has resulted in the allowance of two patents granted. These are United States Patent US 9,249,395 B2 (PCT filed 30th May 2009),
United States Patent US 9,670,466 B2 (filed 22nd December 2015 as a continuation of patent US 9,249,395).
Both have a priority date of 4th June 2008.
The claims of the invention include amongst others
(i) a particulate delivery vehicle comprising the pestivirus replicon lacking codons for one or more structural proteins, and carrying a foreign gene;
(ii) encapsulation of the replicon in a particulate delivery vehicle to deliver the RNA into cells for replication and translation of the replicon;

(iii) a method of prophylaxis against a disease caused by an infectious agent;
(iv) a method of treatment of a disease caused by a lack of a gene.
A search of other patents, allowed or pending, has shown that the priority date of Patent US 9,249,395 B2 is prior to all others.

Status of development of RepRNA vaccine

The active component of the UniVax vaccine is the self-amplifying (self-replicating) RepRNA based on a pestivirus (classical swine fever virus) genome. This was developed almost two decades ago by deleting genes encoding for essential viral structural proteins. The characteristics of this pestivirus replicon facilitate its retention in dendritic cells for prolonged periods. Such prolonged translation fits well to the dendritic cell slow processing of antigen for presenting to the adaptive immune system; presentation over a prolonged period promotes more robust immune defences. Early assessment of the RepRNA employed the aforementioned virus replicon particle technology. This demonstrated the efficacy of the RepRNA vaccine as a single shot vaccine inducing humoral and cell-mediated immunity. The work was expanded under the FP6 project PANFLUVAC (044115) and FP7 project Replixcel (7270014) to replace the virus replicon particle technology with chitosan-based, synthetic, nanoparticulate delivery vehicles (*McCullough et al. 2012 Ther Deliv 3:1077-99; McCullough & Ruggli 2013 International Innovation June 2013: 90-92; McCullough et al. 2014 Mol Ther Nucleic Acids 3:e173; McCullough et al 2014 Vaccines 2:735-754; McCullough et al 2015 J Blood Lymph 5:e1000132*).
From these early successes, patent application was made for vaccines based on RepRNA delivered by synthetic, biodegradable nanoparticulate delivery vehicles. This has been granted (*US 9,249,395 B2 and US 9,670,466 B2*)) with a priority date of 4th June 2008.
Existing constructs encoding reporter genes (luciferase and eGFP) and the influenza virus genes for haemagglutinin (HA (H5)) were employed in UniVax to generate additional constructs. These encode for influenza virus HA (H1), nucleoprotein (NP), neuraminidase (NA (N1)), M protein, and PB1 protein. Moreover, replicons have been constructed to lack one, several or all the structural proteins of the original pestivirus sequence (*Démoulins et al, in preparation*).

Status of development of RepRNA vaccine delivery formulations

1. Characteristics of RepRNA translation

Expression of the RepRNA encoded influenza virus proteins has been assessed alongside expression of certain pestivrus proteins still encoded by the replicon. By such means the relative efficiency of translating the “gene of interest” – encoding the influenza virus antigen – was compared with the endogenous genes, which are translated via a different ribosomal entry site inserted after the gene of interest. This allowed determination of the efficiency with which the gene of interest near the 5’ end of the RepRNA was recognised and translated compared with the genes downstream of this, wherein one also finds the replicon genes encoding for the replication complex.

2. Characteristics of the delivery vehicles

This assessment of translation also determined how the mode of RepRNA delivery influenced the ultimate interaction with the cellular ribosomal translational machinery. Delivery formulations were derived from lipid/lipoplex-based, polyplex/lipopolyplex-based, polysaccharide-based and virus-like particle-based; these were assessed to identify the most efficient for delivery and translation of the RepRNA (*McCullough et al. 2014 Mol Ther Nucleic Acids 3:e173; Démoulins et al 2016 Nanomedicine 12:711-722; Démoulins et al 2017 in “RNA Vaccines: Methods and Protocols”, ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499; Démoulins et al 2017 J Control Release 266:256-271; Englezou et al 2018 Mol Ther Nucleic Acids, in press*). The UniVax partners

have assessed several different biodegradable delivery systems, and modifications therein. For more details on the individual delivery formulation technologies, see *Démoulins et al (2017 in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499).*

Assessment of delivery vehicle efficiency characterizes their capacity to package RepRNA and protect from RNase, together with delivery of the cargo to dendritic cells for promoting translation of the encoded antigens. The main criteria for the delivery vehicles were (i) biodegradability; (ii) readily tolerated by cells and host; (iii) capable of interacting efficiently with dendritic cells to promote cargo uptake; (iv) efficient at complexing RepRNA; (v) efficient also at promoting RepRNA release within cells to facilitate translation of the encoded vaccine antigens.

All biodegradable nanoparticulate delivery systems were physico-chemically characterized, and selected on their capacity to deliver functional nucleic acid. While many of the delivery formulations could deliver DNA or small RNA molecules, such as siRNA or mRNA, this was not necessarily indicative of efficient RepRNA delivery, probably due to the larger size of the RepRNA and therefore its more complex interaction with the delivery vehicle components. Nonetheless, in a number of cases, initial assessment with DNA or small RNA molecules could identify formulations which would not work, or formulations which might prove of value. Similarly, delivery to cell lines did not guarantee delivery to dendritic cells with the same efficiency. Nonetheless, the use of appropriate cell lines could prove of value for determining the potential for delivery to the dendritic cells.

A large number of each compound employed in the delivery vehicle formulation has been assessed, together with modification of formulations employing variations of each compound. Accordingly, the UniVax Consortium identified efficient formulations with the RepRNA encoding different genes of interest, including reporter genes and the genes encoding influenza virus antigens. These formulations promoted both delivery and translation/replication of the RepRNA, allowing selection of prototypes for testing *in vitro* and *in vivo* (see below).

3. Targeting approaches

Polysaccharide formulations of the nanoparticulate delivery vehicles were assessed with surface hyaluronic acid, known to target CD44 on dendritic cells as well as other cell types. This proved a valuable approach, with which particular modifications to the chemical components of the delivery vehicle had strong influences on the efficiency of the hyaluronic acid enhanced targeting. There was no toxicity from these formulations, and delivery to cells proved most efficient. Translation of the delivered RepRNA was also observed, and this also proved successful *in vivo* (see below).

It was also considered important to assess ligands for other cell receptors, including those on dendritic cells. The aim of this approach was to employ specific glycan structures that should bind to members of different families of receptors, such as C-type lectins, SigLecs and galectins. Unfortunately, there was little or no information on which ligands and receptors should or could be targeted. Accordingly, the relative capacity of different glycan structures (glycoconjugates) for binding to dendritic cells compared with other mononuclear cells was assessed. Over 300 glycoconjugates were studied on mononuclear cells obtained from porcine and human donors. This facilitated a definition of glycan binding capacity for the different mononuclear cells. A number of the probes were selected on the basis of highly efficient interaction with different human blood cell populations, in particular differentiating the degree of binding to dendritic cell subsets and monocytes. These efficiently-binding glycans have been further analysed by microscopy to compare binding capacity with internalisation efficiency and the endocytic route employed by the cell. For more details see *Rapoport et al, 2018 Glycoconjugate Journal doi.org/10.1007/s10719-017-9811.*

4. Adjuvants

With the RepRNA being an RNA molecule, it has the potential for activating innate immune defences, which in turn can influence development of adaptive immune responses, as observed with RNA-based adjuvants. However, the RepRNA is derived from a pestivirus, which possesses the means to interfere with this recognition by the innate immune system. While this is of value to the RepRNA vaccine in terms of its interaction with and retention by dendritic cells, it leaves a gap for the required immunogenicity of a vaccine. Accordingly, the delivery vehicles employed with the RepRNA require a potent adjuvant. In this context, adjuvants for parenteral or mucosal immunisation have been studied. The most promising candidates were MALP-2 and cyclic-di-AMP, both having a distinct mode of action. These offer the further advantage of being manufactured synthetically and applicable via parenteral or mucosal routes. A number of comparative experiments have shown that the cyclic-di-AMP offers a number of advantages, which is why our current *in vivo* evaluations are focused on delivery formulations containing this adjuvant (see below). For more details, see *Ebensen et al 2017 Front Immunol. 28, 8:1223*.

Status of validation of RepRNA vaccines

Both primary dendritic cells (murine, porcine, and human) and cell lines relevant for either replicon translation or as models for dendritic cells have been employed. Certain delivery formulations were more efficient than others at delivering the RepRNA leading to translation of the encoded genes. Neither the efficiency of associating the RepRNA with the delivery vehicle, nor the efficiency of the RepRNA delivery into the cells could be related to this translation. Certainly, clear delivery of the RepRNA was essential, but only certain formulations facilitated the apparent release of the RepRNA for translation of the encoded antigens. These were the formulations employed for validation *in vivo* (see below). For further details see *McCullough et al. 2014 Mol Ther Nucleic Acids 3:e173; Démoulins et al 2016 Nanomedicine 12:711-722; Démoulins et al 2017 in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499; Démoulins et al 2017 J Control Release 28;266:256-271; Englezou et al 2018 Mol Ther Nucleic Acids, in press*.

Status of validation of RepRNA vaccine delivery formulations

1. *In vitro* evaluation

As mentioned above, different delivery vehicle formulations capable of delivering RepRNA to dendritic cells and cell lines was dependent on the components of the delivery formulations, but efficiency of delivery did not guarantee translation of the delivered RepRNA (*Démoulins et al 2016 Nanomedicine 12:711-722; Démoulins et al 2017 J Control Release 266:256-271Englezou et al 2018 Mol Ther Nucleic Acids, in press*). Modification of these initially successful delivery vehicle components has further increased the efficiency of delivery in terms of leading to translation of the delivered RepRNA. These newer formulations were selected for further evaluation *in vivo*, by mucosal and parenteral vaccination (see below).

It was considered that the RepRNA may have been non-functional in certain complexes or compacted to a degree that would not be reversed adequately for ribosomal entry and translation of RepRNA-encoded antigens. Thus, the functionality of the RepRNA was assessed using virus replicon particles (VRP). These are constructed in complementing cell lines to create the original virus-like particles, but carrying the replicon in place of the virus genome. VRPs were efficiently delivered to cells, promoting efficient translation of the encoded antigens, including the influenza

virus antigens. These results clearly demonstrated that the RepRNA was indeed translation- and replication-competent.

Analyses with different PEI-based formulations showed that certain formulations were efficient at promoting translation of all encoded antigens, whereas other formulations provided for an imbalanced translation. This would imply a differential degree of compaction along the RepRNA, either preventing ribosomal entry or inefficiently protecting from RNase (see *Démoulins et al 2017 J Control Release 266:256-271*). It is now clear that careful selection of the delivery vehicle components permits efficient formulation with RepRNA to protect from RNase, promote delivery to dendritic cells, and facilitate translation of the vaccine antigens leading to induction of immune responses. (see also "In vivo evaluation" below).

2. *In vivo* evaluation

Following the *in vitro* identification of the most potentially efficacious delivery formulations delivering RepRNA for translation in dendritic cells, prototypes were assessed by immunogenicity studies in mice and pigs. Certain delivery vehicle formulations were identified as showing immunogenic promise. Interestingly, this was noted with examples of each type of delivery system initially under investigation – lipoplex, polyplex, lipopolyplex, chitosan-based and liposome nanoparticle formulations. While these RepRNA vaccine delivery formulations were initially selected primarily from *in vitro* evaluations, the final formulation with adjuvant was assessed by both mucosal and parenteral immunisations. Initial experiments compared several different adjuvants, for enhanced induction of humoral and cellular immune responses against the RepRNA-encoded vaccine antigen. Importantly, the cyclic-di-AMP proved to be the most efficacious, by both parenteral and mucosal routes of immunisation (*Ebensen et al 2017 Front Immunol. 8:1223*).

In-depth analysis assessed most aspects of immune responsiveness, with both conventional vaccination models and the TCR Ova model. On the one side, this employed both murine and porcine models, while on the other side comparison was made in mice of a mucosal route and parenteral route of injection. Evaluation of the immunological readout assessed antibody induction, T-lymphocyte subset induction, cytokine profiling and individual lymphocyte activities. The aforementioned experiments with the new modified formulations confirmed their capacity to induce immune responses against the influenza virus antigens encoded by the RepRNA. Moreover, the power of the cyclic-di-AMP has been further evidenced.

Overall, the work to date demonstrates that particular formulations will facilitate delivery of the RepRNA for translation in the manner observed with RepRNA delivery in a single shot by VRPs, although this may prove more efficacious dependent on the route of administration. The experiments were then extended to assess delivery vehicle formulations with RepRNA in terms of relating vehicle modifications to increased efficacy at inducing immune defences. A number of *in vivo* immunisations have been performed, using either mucosal delivery or parenteral delivery. These formulations displayed clear efficacy for delivery, especially after mucosal delivery, resulting in clear induction of specific immune responses.

Taken together, the *in vivo* experiments provided clear evidence of immunogenicity for the RepRNA vaccine formulations. An important discovery was the particularly powerful immunomodulatory capacity of cyclic-di-AMP, both parenterally and mucosally. Moreover, these results have allowed the selection of the delivery formulations showing the greatest promise for efficacy at inducing influenza virus-specific immune responses.
For more details see

1. Démoulins et al 2016 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;
3. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 266:256-271;
4. Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223
5. Englezou et al 2018, Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids, in press.

Activities with respect to potential commercialisation of the RepRNA vaccine delivery formulations

We would be interested in seeking a partner to commercialise the RepRNA vaccine delivery formulation described above. For this, we are able to offer the advantage of allowed patents (US 9,249,395; US 9,670,466) with a priority date preceding other patents of similar genre, together with additional patents with the same priority date (WO2009/146867, EP2130912, EP08010222, EP2009003892, 4414/KOLNP/2010). This is further enhanced by additional patents within the UniVax Consortium covering the adjuvant (EP 05 022 771.9, EP 05 025 431, EP 05 02 4266, EP 09016050.8), PEI polyplexes (WO2009112402), lipopolyplexes (WO2017212197, WO2009050372) and cationic lipids (WO2009/106713).

In the context of UniVax, the RepRNA vaccine delivery formulation relates to vaccination against influenza. Influenza is a serious public health problem affecting more than 100,000,000 people per year. Most recover from the symptoms within a week without requiring attention, but there is a high risk for severe illness (3-5 million cases) or death (250,000 to 500,000) (WHO); most deaths occur among the elderly. The most effective way to prevent the disease is vaccination, although the vaccine among the elderly reduces severe illness and complication by only up to 60%. Annual vaccination is currently recommended for pregnant women, children 6 to 59 months of age, healthcare workers with patient contact, the elderly and people with underlying chronic health conditions such as respiratory, cardiac, metabolic, neurological and immunosuppressive diseases.

UniVax has generated essential data on integrating innovative technologies for RepRNA, synthetic delivery to dendritic cells, glycoconjugate-based targeting of dendritic cells, and mucosal adjuvants. Prototype synthetic RepRNA vaccines with innovative new generation mucosal adjuvants are being evaluated pre-clinically, providing data on enhancing efficacy of vaccine delivery for breadth and duration of protection. UniVax has as a direct outcome a novel clinical technology with great benefits for population health. It will enable the widespread use of nucleic acid delivery to dendritic cells and other "immune cells" such as monocytes, macrophages, natural killer cells or even lymphocytes with the purpose of investigating cellular functions and overexpressing or silencing genes in therapeutic strategies.

This information has been provided to the UTILE programme.

UniVax IPR (intellectual property rights) situation and business strategy

	Own Patents/applications	Major claims and UniVax relationship
IVI	(i) US9249395B2 Priority Date 2008 06 04 Pestivirus replicons providing an RNA-based viral vector system (ii) US9670466B2 Priority Date 2008 06 04 Pestivirus replicons providing an RNA-based viral vector system (iii) EP2130912A1/EP20090757250 Priority Date 2008 06 04 (i) CA2723999A1 Priority Date 2008 06 04 (ii) WO2009146867A1 Priority Date 2008 06 04	A particulate delivery vehicle comprising a single-stranded RNA pestivirus replicon lacking essential codons or all codons for one or more structural proteins required for formation of infectious virus, and carrying a foreign gene. Priority Date 2008 06 04 A particulate delivery vehicle comprising (1) a single-stranded RNA replicon lacking essential codons or all codons for one or more structural proteins required for formation of infectious virus, and carrying a foreign gene, (2) wherein said replicon is encapsulated inside said particulate delivery vehicle (2.1) to protect said replicon against RNase degradation and (2.2) to deliver said replicon to cytoplasm of cells (2.3) for replication of said replicon in cytoplasm. *(Claim 1 US9249395: Priority Date Jun 4 2008; Claim 1 US9670466: Priority Date Jun 4 2008)* Wherein the particulate delivery vehicle is selected from the group consisting of: liposomes, microparticles, nanoparticles and nanocapsules. *(Claim8 US9249395B2:Priority Date Jun 4 2008; Claim 3 US9670466: Priority Date Jun 4 2008)* Wherein the particulate delivery vehicle is aVirus Replicon Particle (VRP). *(Claim 4 US9670466: Priority Date Jun 4 2008)* These IVI patents protect the development of replicons with the delivery platforms of UniVax for commercialisation (see also below).
CNRS	1-F. Lemoine, V Matéo, P. Midoux, C. Pichon, T. Benvegnu, L. Lemiègre. Synthetic lipid archaeum diethers. WO2017212197 - 2017-12-14 2-H. Cheradame, M. Sassatelli,C. Pichon, P. Midoux, P. Guégan. Polymer derived from linear polyethylenimine for gene transfer. WO2009112402 - 2009-09-17 3- Clément, J.C., Midoux, P., Mével, M., Yaouanc, J.J., Pichon, C. Novel lipophilic compositions and uses thereof. WO2009050372 - 2009-04-23	1-DC targeting: Tri-antennea mannosyl lipid comprised in liposomes of mannosylated LPR nanoparticles 2- Cationic polymer forming mRNA complexes for encapsulation of mRNA in mannosylated LPR nanoparticles 3- Imidazolyl lipophosphoramides used to prepare mannosylated liposomes in our mRNA LPR formulation

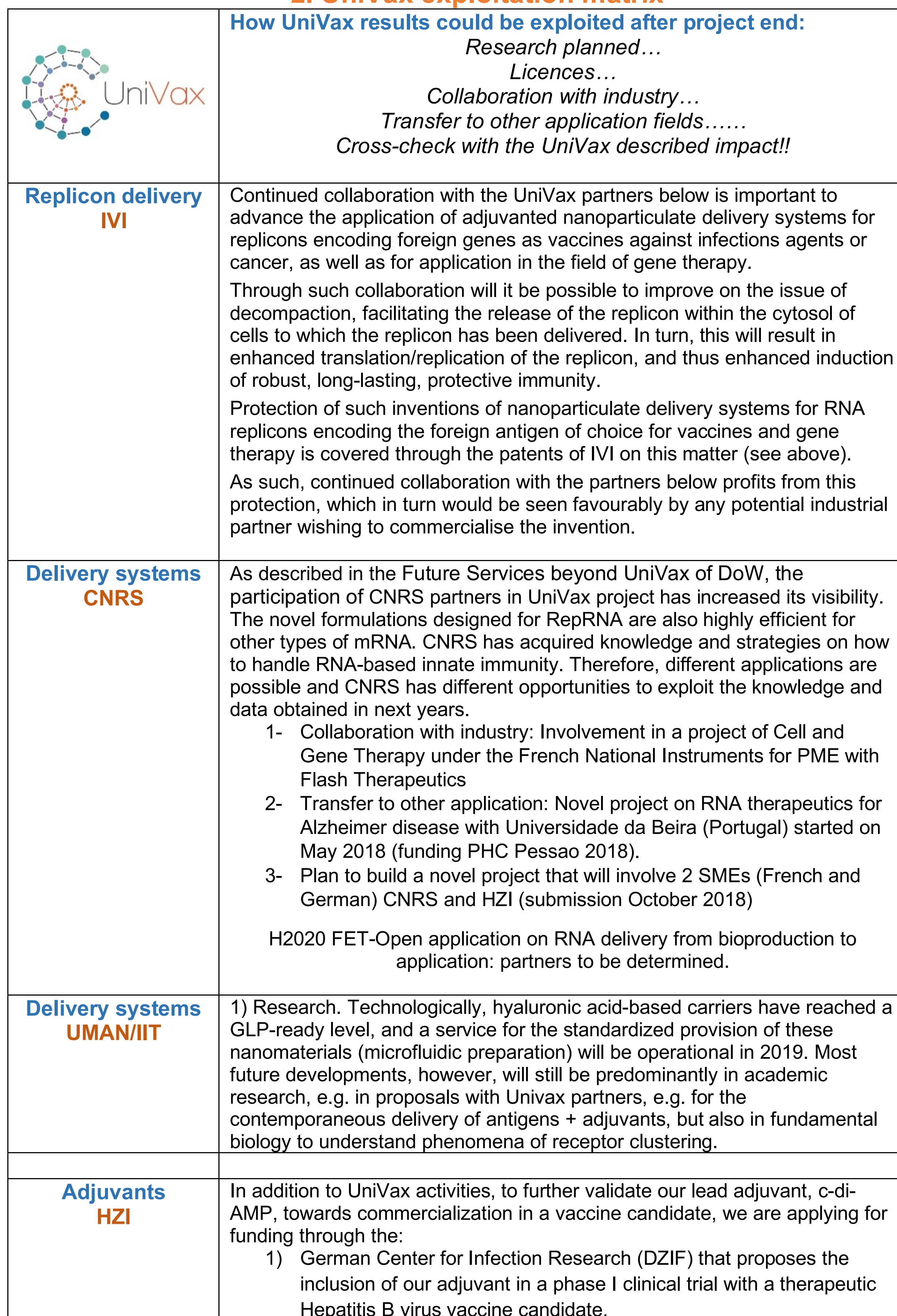
HZI	**EP 05 022 771.9:** GalCerMPEG als mukosales Adjuvanz, T. Ebensen, CA. Guzmán, M. Morr, 19.10.2005 **EP 05 025 431:** BPPcysGlyc4armPEG als Adjuvanz T. Ebensen, CA. Guzmán, M. Morr, 22.11.2005 **EP 05 02 4266:** c-di-AMP and derivatives as mucosal and/or parenteral adjuvant; T. Ebensen, CA. Guzmán, M. Morr 08.11.2005 / 01.12.2006 **EP 09016050.8:** BPPcysOvamPEG as adjuvant delivery system T. Ebensen, CA. Guzmán, W. Tegge und M. Morr, 28.12.2009	**Adjuvant** **Mucosal application** Intranasal Sublingual Pulmonary **Parenteral application** Intramuscular Subcutaneous

	External Patents/Applications	Major claims and UniVax relationship
IVI	***OTHERS:*** (i) US9770463B2 Priority Date 2010 07 06 (ii) US9192661B2 Priority Date 2010 07 06 (iii) US20130171241A1 (Application) Priority Date 2010 07 06 (iv) US9254265B2 Priority Date 2010 08 31 (v) US20130189351A1 (Application) Priority Date 2010 08 31 (vi) US20170239371A1 (Application) Priority Date 2011 06 08 (vii) US20170079916A1 (Application) Priority Date 2015 09 23 (viii) WO2018010815A1 (Application) Priority Date 2016 07 15	***OTHERS:*** Do not cover different delivery vehicles; Priority Date post-dates the **IVI OWN** patents, both granted and pending (Application). Therefore, the IVI patents hold Prior Art over these "Others" patents.
HZI	Despite the fact that adjuvants have been used to increase the immunogenicity of vaccines for more than 70 years, only a handful have been licensed for human use (e.g., aluminum salts, the micro-fluidized squalene-in-water emulsion MF59, monophosphoryl lipid A (MPL A), AS03, AF03, LT and virosomes). Thus, the development of new delivery systems, combinations of already known adjuvants and novel adjuvants, which are able to promote broad and sustained immune responses at both systemic and mucosal level, still remains a major challenge in vaccinology.	Despite the fact that adjuvants have been used to increase the immunogenicity of vaccines for more than 70 years, only a handful have been licensed for human use (e.g., aluminum salts, the micro-fluidized squalene-in-water emulsion MF59, monophosphoryl lipid A (MPL A), AS03, AF03, LT and virosomes). Thus, the development of new delivery systems, combinations of already known adjuvants and novel adjuvants, which are able to promote broad and sustained immune responses at both systemic and mucosal level, still remains a major challenge in vaccinology. Many vaccine formulations need to be improved and customized for application in specific groups of individuals (e.g., children or elderly). On the other

	Many vaccine formulations need to be improved and customized for application in specific groups of individuals (e.g., children or elderly). On the other hand, the advent of subunit vaccines has resulted in formulations with improved safety profiles, but purified antigens are considerably poorly immunogenic, making critical their co-administration with novel adjuvants. There are several candidate adjuvants in the pipeline, which are based on well-characterized moieties able to exert their biological activity through stimulation of defined cellular targets and/or signalling cascades. An in-depth understanding of the underlying mechanisms of action of these compounds, together with the availability of defined synthetic derivatives will certainly facilitate rational vaccine design and improve the safety profiles of the resulting candidates. It is important to highlight that in contrast to the compounds available for UNIVAX, most of the available adjuvant are not amenable for mucosal vaccination. Furthermore, by and large, the existing adjuvants promote either humoral or cellular responses, whereas one of the UNIVAX compounds is able to stimulate both effector mechanisms. o AS02 patent family directed to vaccine compositions comprising 3D-MLA and QS21 is owned by GlaxoSmithKline (EP 0 671 948 B1; EP 0 761 231 B1; US 5,750,110 o MPL™, patented by Corixa Corp. The patents are drawn to an attenuated form of the lipid A component of bacterial lipopolysaccharide (LPS). LPS, and lipid A, are potent immunostimulators, but have deleterious side effects, such as pyrogenicity (fever) (EP 0 971 739 B; EP 1 194 166 B1; US 6,491,919) o AS04 an adjuvant composition comprising an immunostimulant which is 3-de-O-acylated monophosphoryl lipid A, adsorbed onto an aluminium salt particle (EP 1 126 876 B1; US 7,357,936) o MF59 is the subject of a large patent family titled "Adjuvant formulation comprising a submicron oil droplet emulsion"(EP 0 399 843 B)	hand, the advent of subunit vaccines has resulted in formulations with improved safety profiles, but purified antigens are considerably poorly immunogenic, making critical their co-administration with novel adjuvants. There are several candidate adjuvants in the pipeline, which are based on well-characterized moieties able to exert their biological activity through stimulation of defined cellular targets and/or signalling cascades. An in-depth understanding of the underlying mechanisms of action of these compounds, together with the availability of defined synthetic derivatives will certainly facilitate rational vaccine design and improve the safety profiles of the resulting candidates. It is important to highlight that in contrast to the compounds available for UNIVAX, most of the available adjuvant are not amenable for mucosal vaccination. Furthermore, by and large, the existing adjuvants promote either humoral or cellular responses, whereas one of the UNIVAX compounds is able to stimulate both effector mechanisms. o AS02 patent family directed to vaccine compositions comprising 3D-MLA and QS21 is owned by GlaxoSmithKline (EP 0 671 948 B1; EP 0 761 231 B1; US 5,750,110 o MPL™, patented by Corixa Corp. The patents are drawn to an attenuated form of the lipid A component of bacterial lipopolysaccharide (LPS). LPS, and lipid A, are potent immunostimulators, but have deleterious side effects, such as pyrogenicity (fever) (EP 0 971 739 B; EP 1 194 166 B1; US 6,491,919) o AS04 an adjuvant composition comprising an immunostimulant which is 3-de-O-acylated monophosphoryl lipid A, adsorbed onto an aluminium salt particle (EP 1 126 876 B1; US 7,357,936) o MF59 is the subject of a large patent family titled "Adjuvant formulation comprising a submicron oil droplet emulsion"(EP 0 399 843 B) o Virosomes patent application "Phospholipid Virosome" of Mymetics corp. (# 10/544,939, based on WO 04/071492)

	○ Virosomes patent application "Phospholipid Virosome" of Mymetics corp. (# 10/544,939, based on WO 04/071492)	
UMAN/ IIT	Immunotherapy Compositions, Method of Making and Method of Use Thereof (US 2005/0244505 A1) Particulate Delivery Systems and Methods of Use (US 6,630,169 B1)	This patent US 2005/0244505 A1 refers to delivery employing only chitosan or PLGA/PEI **microparticles** and modulation of the immune response. Patent claims cover microparticulates and microstructures **NOT** nanoparticulate delivery systems. Microparticulates interact with different cells compared to those interacting with nanoparticles. Also, microparticles tend to be phagocytosed by macrophages rather than endocytosed by dendritic cells, which would impair their applicability as vaccines. This patent does **NOT** define replicons to be employed, particularly replicons carrying a foreign gene. **Was only an application; abandoned 14.04.2019.** This patent US 6,630,169 B1 refers also only to microstructures, **NOT** nanoparticulate delivery systems; importantly, it also refers to delivery using inhalers, atomizers or nebulizers, none of which is relevant to the **IVI OWN** patents. The same criticism of microstructures applies to this patent as to that above. This patent does **NOT** define replicons to be employed, particularly replicons carrying a foreign gene. **Moreover, this patent expired on 14.04.2019.** The absence of nanoparticulate delivery or replicon definition in these patents renders them irrelevant to **IVI OWN** patents. Thus, these patents **DO NOT** influence the dominance of the **IVI OWN** patents, as witnessed by the allowance of the US patents.

2. UniVax exploitation matrix



UniVax	**How UniVax results could be exploited after project end:** *Research planned…* *Licences…* *Collaboration with industry…* *Transfer to other application fields……* *Cross-check with the UniVax described impact!!*
Replicon delivery **IVI**	Continued collaboration with the UniVax partners below is important to advance the application of adjuvanted nanoparticulate delivery systems for replicons encoding foreign genes as vaccines against infections agents or cancer, as well as for application in the field of gene therapy. Through such collaboration will it be possible to improve on the issue of decompaction, facilitating the release of the replicon within the cytosol of cells to which the replicon has been delivered. In turn, this will result in enhanced translation/replication of the replicon, and thus enhanced induction of robust, long-lasting, protective immunity. Protection of such inventions of nanoparticulate delivery systems for RNA replicons encoding the foreign antigen of choice for vaccines and gene therapy is covered through the patents of IVI on this matter (see above). As such, continued collaboration with the partners below profits from this protection, which in turn would be seen favourably by any potential industrial partner wishing to commercialise the invention.
Delivery systems **CNRS**	As described in the Future Services beyond UniVax of DoW, the participation of CNRS partners in UniVax project has increased its visibility. The novel formulations designed for RepRNA are also highly efficient for other types of mRNA. CNRS has acquired knowledge and strategies on how to handle RNA-based innate immunity. Therefore, different applications are possible and CNRS has different opportunities to exploit the knowledge and data obtained in next years. 1- Collaboration with industry: Involvement in a project of Cell and Gene Therapy under the French National Instruments for PME with Flash Therapeutics 2- Transfer to other application: Novel project on RNA therapeutics for Alzheimer disease with Universidade da Beira (Portugal) started on May 2018 (funding PHC Pessao 2018). 3- Plan to build a novel project that will involve 2 SMEs (French and German) CNRS and HZI (submission October 2018) H2020 FET-Open application on RNA delivery from bioproduction to application: partners to be determined.
Delivery systems **UMAN/IIT**	1) Research. Technologically, hyaluronic acid-based carriers have reached a GLP-ready level, and a service for the standardized provision of these nanomaterials (microfluidic preparation) will be operational in 2019. Most future developments, however, will still be predominantly in academic research, e.g. in proposals with Univax partners, e.g. for the contemporaneous delivery of antigens + adjuvants, but also in fundamental biology to understand phenomena of receptor clustering.
Adjuvants **HZI**	In addition to UniVax activities, to further validate our lead adjuvant, c-di-AMP, towards commercialization in a vaccine candidate, we are applying for funding through the: 1) German Center for Infection Research (DZIF) that proposes the inclusion of our adjuvant in a phase I clinical trial with a therapeutic Hepatitis B virus vaccine candidate,

	2) Helmholtz Association validation fund that will support studies including our adjuvant ultimately leading to a phase I clinical trial with a prophylactic Hepatitis C virus vaccine candidate, and 3) European Union Horizon 2020 (stage 2) to test our adjuvant in a phase I clinical trial with a vaccine candidate against Chagas. It is also planned to continue the pursuit of producing a universal influenza vaccine adjuvanted with c-di-AMP.
Innovation in vaccination **UiB**	

(c) Report on the wider societal implications

See online questionnaire.