**Influenza Vaccine Research Funded by the European Commission FP7-Health-2013-
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Abstract

Due to influenza viruses continuously displaying antigenic variation, current seasonal influenza vaccines must be updated annually to include the latest predicted strains. Despite all the efforts put into vaccine strain selection, vaccine production, testing, and administration, the protective efficacy of seasonal influenza vaccines is greatly reduced when predicted vaccine strains antigenically mismatch with the actual circulating strains. Moreover, preparing for a pandemic outbreak is a challenge, because it is unpredictable which strain will cause the next pandemic. The European Commission has funded five consortia on influenza vaccine development under the Seventh Framework Programme for Research and Technological Development (FP7) in 2013. The call of the EU aimed at developing broadly protective influenza vaccines. Here we review the scientific strategies used by the different consortia with respect to antigen selection, vaccine delivery system, and formulation. The issues related to the development of novel influenza vaccines are discussed.

Key words: influenza; vaccines; broadly protective; EU consortia; correlates of protection

Effective influenza vaccination, are we there yet?

Each year during seasonal epidemics, influenza infects up to 10% of adults and 20% to 30% of children, resulting in three to five million cases of severe illness and 250,000 to 500,000 deaths worldwide.¹ To prevent influenza disease and related social-economical loss, vaccination has been the primary intervention for more than 70 years.² Currently marketed influenza vaccines include live attenuated influenza vaccine (LAIV), recombinant influenza vaccine and inactivated influenza vaccine (IIV), with IIV being the most widely used vaccine type worldwide. All vaccine types contain minimally the viral membrane glycoprotein hemagglutinin (HA), with or without the presence of neuraminidase (NA). Although currently available influenza vaccine formulations such as recombinant proteins, subunit and split virion preparations are relatively pure and show very little side effects, the vaccine concept – generating antibodies targeting against influenza virus surface glycoproteins – is not much different from the one first introduced in the 1940's. Influenza viral antigens from two influenza A virus strains (H1 and H3) and one or two influenza B virus strains (Yamagata and/or Victoria lineages) are included in the vaccine. These trivalent or quadrivalent influenza vaccines (TIVs or QIVs) are effective at eliciting neutralizing antibodies against the HA globular head, which prevent virus attachment and infection of host cells. However, immunodominant epitopes of the HA head are subject to antigenic variation due to random point mutations followed by selective pressure mediated by antibodies (antigenic drift) or genetic re-assortment, resulting in the emergence of viruses of novel subtypes (antigenic shift).² Thus, antibodies induced by previous influenza virus infections and/or vaccination will fail to recognize and neutralize new variants.

To afford protection against the most recent influenza viruses, annual vaccination with TIV or QIV containing the latest circulating strains is recommended. However, due to the long

vaccine production time, influenza vaccine strains need to be selected six to eight months prior to the start of the next influenza season so that the vaccines can be available on time.³ The selection is based on epidemiological data from 112 countries participating in the World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS). When the selected strains do not match antigenically with the actual circulating strains, the protective efficacy of influenza vaccines offered by strain-specific antibodies against the viral HA can be reduced substantially. The poor vaccine effectiveness during the 2014-2015 influenza season is an example of this. After the vaccine strain selection for the northern hemisphere in February, drifted H3 strains were observed that accounted for 17% of the circulating H3 viruses in the United States in May and became more dominant during the summer of 2014.³ The mismatched H3 strains, containing up to 7 amino acid differences in HA, caused more than half of the influenza cases in the northern hemisphere during the 2014-2015 season with exceptional excess morbidity and mortality reported, notably among the elderly 65 years and older.³⁻⁶ Aside from the mismatch between vaccine strains and the actual circulating strains, mutation at the antigenic sites of the H3 vaccine strain due to egg adaptation was for example also reported to be the cause of the low vaccine effectiveness during the 2012-2013 influenza season.⁷

The limitations of strain-specific protection and time-consuming vaccine production resulted in the delayed availability of effective vaccines against the 2009 pandemic virus. Human cases of infection with 2009 A(H1N1) virus (H1N1pdm09) were first reported in April and reached pandemic levels rapidly in June.⁸ Despite expedited processes, pandemic vaccines only became available in October, when the pandemic activity had already peaked in many countries. Although the disease burden of the 2009 pandemic was relatively mild compared to previous pandemics (1918, 1957 and 1968), it was estimated that thousands of lives could

have been saved if the vaccine would have been available one month earlier.⁹ This has been a “wake-up” call for public health officials and stakeholders from industry to face the shortcomings of current influenza vaccines. It is unpredictable which subtype or virus will cause the next pandemic and the population at large will be immunologically naïve and susceptible to the new virus. H5N1, H7N9 and other subtypes of influenza A viruses may have the potential to cause a pandemic if these viruses acquire human-to-human transmissibility. Due to the risk of vaccine mismatch and the danger of newly emerging influenza virus strains there is an urgent need for vaccines that offer broad protection against a variety of influenza virus strains.

European Consortia for Influenza Vaccine Research

In 2013, the European Commission has funded five consortia under the seventh Framework Programme for Research and Technological Development (FP7) to work on the next generation influenza vaccines (Table 1). The call of the EU aimed at the development of influenza vaccines which could provide longer-lasting and broader protection against a wider range of influenza virus strains than what current influenza vaccines on the market can offer.¹⁰ The final goal of the call is to develop influenza vaccines which can protect the general population from both seasonal and pandemic influenza. The influenza vaccines defined in the EU call is recognized as universal influenza vaccines and the same definition has been used by the WHO.^{11,12} Here we present a brief overview of scientific strategies used by these consortia with respect to antigen selection, vaccine delivery system, and formulation (Table 2). We order the projects according to their vaccine development stage from preclinical to clinical (phase I to Phase II).

Combinatorial Immunization Strategy to Educate the Immune System Towards Cross Recognition and Coverage Against Antigenic Drift in Seasonal Influenza Virus Exposure (EDUFLUVAC)

The EDUFLUVAC consortium coordinated by European Vaccine Initiative (DE) consists of seven partners. The proposed vaccine concept is to contain multiple variants of viral surface glycoproteins within a single influenza (sub)type.^{13,14} Since, by this approach, the relative concentration of common influenza epitopes is increased whilst that of strain-specific epitopes is decreased, the immune system is expected to learn what “the common characters” of influenza viruses are. This vaccination strategy earlier has proven to be functional in a malaria vaccine study.¹⁵ Studies in rabbits showed that antibody responses against malaria epitopes shared by different vaccine alleles/strains are readily amplified by both combined and sequential immunization (that mimics natural malaria infection) with the vaccine variants. The consortium will express the selected components (HA and/or NA) representing antigenic drift variants within the H1, H3, and B influenza viruses on baculovirus-based virus-like particles (VLPs). The VLP antigen expression system helps cross-linking B cell receptors and boosting humoral immune responses. EDUFLUVAC will evaluate the immunogenicity and efficacy of the final product in relevant animal models including mice, ferrets, and non-human primates. A vaccine candidate ready for clinical testing is to be expected at the end of the project in October 2017. Similar to the vaccine concept used by EDUFLUVAC, a recent study from Schwartzman *et al* shows that a mixture of *baculovirus*-based VLPs expressing influenza H1, H3, H5, and H7 HAs conferred the immunized mice protection against homologous (i.e. H1N1), intrasubtypic (drifted H7N9), and heterosubtypic (i.e. H2N1, H6N1, H10N1, and H11N1) viral challenge.¹⁶ The same study also shows that while the vaccine-elicited antibodies substantially contributed to the observed homologous and intrasubtypic protection, the observed heterologous protection was mediated by immune mechanisms other

than antibodies. The EDUFLUVAC consortium is investigating polyvalent VLPs (i.e. up to five HA variants of a (sub)type on a single VLP). Preliminary mouse immunogenicity studies show that for the H3 and B strains the response was broadened beyond the vaccine composition, i.e. strains isolated ~10 years after the last strain in the vaccine composition are neutralized by pentavalent VLP immunized mouse sera. The H3N2 and B strain data show that at least five components on a single VLP induce broadened responses.

Influenza Virus Universal Vaccine Development Program (FLUNIVAC)

The FLUNIVAC consortium coordinated by Erasmus University Rotterdam (NL) consists of eight partners. The FLUNIVAC vaccine will include nine conserved proteins identified from influenza viral surface proteins (HA, NA, and matrix 2 extracellular domain (M2e)), internal proteins (matrix 1 (M1), nucleoprotein (NP), and non-structural protein 1(NS1)) and the polymerase proteins (polymerase basic proteins 1 and 2 (PB1 and PB2) and polymerase acidic protein (PA)) to induce broadly reactive influenza-specific B and T cell responses.^{17,18} Vaccine antigen selection will be performed based on the breadth of protective immune responses elicited by individual proteins and various combinations of them. Altenburg *et al* from the consortium have pointed out that influenza viral NP and other conserved influenza proteins in vaccine preparations promotes cross-reactive CD4+ and CD8+ T cell responses and heterosubtypic protection.¹⁹ The FLUNIVAC vaccine will be viral vector-based. There are several advantages of this type of vaccine. The over-expression of viral proteins could potentially increase vaccine immunogenicity. In addition, the *de novo* synthesis of viral proteins in the cytoplasm of antigen presenting cells not only enhances antigen processing and presentation to CD8+ T cells, but also helps inducing antibodies of the proper specificity due to the preserved native conformation of antigens. The Modified Vaccinia Virus Ankara (MVA) vector, which has the capacity to express multivalent antigens and which possesses intrinsic

adjuvant activity to boost both humoral and cellular immune responses, is chosen by the consortium. MVA-based vaccine can be handled at biosafety level 1 and is able to induce antigen-specific immune responses even in the presence of pre-existing vector immunity.²⁰ A phase I study on a MVA-based influenza vaccine containing NP and M1 epitopes showed that the vaccine is also immunogenic in the older age group (≥ 50 years) and that the vaccine-elicited T cell responses were comparable between the younger (18-45 years) and older volunteers (≥ 50).²¹ Another Phase I/IIa study performed in young adults (18-28 years) showed that antibody responses elicited by the tested MVA-based H5 vaccine could be further enhanced by booster vaccinations.²² Based on these observations, pre-existing host immunity against MVA is not a concern for MVA-based vaccines. Aside from evaluating the durability and boost ability of immune responses elicited by the MVA-based vaccine candidates, FLUNIVAC will compare them with those elicited by adjuvanted vaccines. Moreover, the MVA-based vaccine platform will be optimized in terms of the expression rate and extent of the antigens and the unity and scalability of the production process. The consortium has constructed various recombinant MVA expressing wildtype and modified influenza virus proteins, aiming at the induction of improved cross-reactive antibody and T cell responses. The vaccine candidates will be evaluated in mouse models. The consortium will evaluate vaccine candidates in mouse models and have the most optimal candidate vaccine ready for phase I study at the end of the project in September 2017. Although there is no MVA-based vaccine available on the market yet, the recent licensure of a MVA-based smallpox vaccine (Imvamun®) in Canada and in the EU has paved a way for the future development of other MVA-based vaccines.²³

A Universal Influenza Vaccine through Synthetic, Dendritic Cell-Targeted, Self-Replicating RNA Vaccines (UNIVAX)

199 The UNIVAX consortium involving 11 partners is coordinated by the Institute of Virology
200 and Immunology (IVI), within the Swiss Federal Department of Home Affairs (CH). A major
201 innovative approach of UNIVAX is the application of self-amplifying (self-replicating)
202 replicon RNA (RepRNA) vaccines encoding influenza HA, NA, NP, M1 and PB1 proteins;
203 these are delivered by synthetic, biodegradable, nanoparticulate vehicles, formulated with
204 defined adjuvants for parenteral or mucosal delivery.^{24,25} Replicons are defective virus
205 genomes which translate and replicate without producing virus particles due to the lack of one
206 or more essential gene(s) encoding for structural protein(s).²⁶⁻²⁸ The RepRNAs of UNIVAX
207 are derived from positive-strand, non-cytopathogenic classical swine fever virus (CSFV).²⁹
208 Their positive-strand character allows the delivered (input) RNA to function as a mRNA,
209 without the need to co-deliver polymerase proteins, as is the case with negative-strand
210 viruses.²⁸ Being non-cytopathogenic – in contrast to most replicons employed today – the
211 levels of self-replicating RNA templates remain available within the targeted cells for
212 continued translation.^{26-28,30} When targeted to dendritic cells (DCs), these translation and
213 replicative characteristics result in high levels of vaccine antigen production within the cells
214 responsible for processing and presentation to the adaptive immune system; their self-
215 replicating nature provides antigen under the necessary conditions for inducing both humoral
216 and cell-mediated cytotoxic immunity.²⁸ This DC-targeting, achieved by the delivery vehicles,
217 is enhanced through application of glyco-conjugate ligands.³¹ These ligands have been
218 selected for efficient binding to DCs leading to internalisation into particular endocytic
219 pathways. Well-defined adjuvants are selected for their influence on DCs and functionality as
220 mucosal adjuvants, particularly when pertinent to influenza vaccines.³²⁻³⁶ The adjuvants are
221 also considered for their influence on the delivery of the RepRNA for translation in vitro and
222 in vivo. The UNIVAX consortium has recently reported the success of their approach,
223 demonstrated by successful translation of RepRNA encoding influenza virus HA (H5, H1)

and NP *in vitro* (human, porcine and murine DCs) and *in vivo* (mouse and rabbits).^{37,38} UNIVAX is currently exploring how the design of the nanoparticulate delivery, together with the added values of mucosal adjuvants and glyco-conjugate targeting ligands, enhances RepRNA vaccine efficacy for both parenteral and mucosal delivery.^{32-36,39} In addition to the main aim of inducing strong and long-lasting immune responses, the UNIVAX consortium is evaluating the safety, tolerability, and protective potential of the candidate vaccine.⁴⁰

Development of an Universal Influenza Vaccine Based on Tandem Core Technology (FLUTCORE)

The FLUTCORE consortium consists of seven partners and is coordinated by University College London (UK) and led by the SME iQur Ltd (UK). FLUTCORE aims at inducing broadly-reactive B and T cell responses against influenza A viruses by using conserved parts from the influenza M2e and HA stem as vaccine antigens.^{41,42} Sequence variability analyses have shown that influenza A viruses can be categorized into one of three distinct sequence patterns for M2e or one of two distinct sequence patterns for HA stem.^{43,44} Multiple conserved antigens are co-expressed on Hepatitis B virus (HBV)-based Virus-Like Particles (VLPs), with the help of iQur's Tandem CoreTM technology. Naturally, HBV core protein (HBc) spontaneously forms dimers, which are the building blocks of the resulting VLPs. Due to the repetitive structure of the VLPs and the intrinsic presence of Toll-like receptor (TLR) 2 and 7 stimulatory sequences in HBc, strong immunogenicity of HBc has been observed *in vivo*. In addition, immune responses against non-immunogenic antigens are observed when inserting those antigens into HBc. However, such constructs are inherently unstable but this was overcome by using Tandem Core which fuses two HBc with a flexible linker. This allows even large or hydrophobic residues to be inserted which was hitherto impossible.⁴⁵ Moreover, the Tandem Core VLP contains two major insertion regions that allow the incorporation of

several antigens simultaneously. The consortium has chosen a lead candidate vaccine which contains five conserved influenza antigens. This vaccine has shown strong immune responses and protection against lethal challenges in mice by both H1 and H3 HA subtypes simultaneously. The consortium is currently beginning GMP manufacture of the candidate vaccine and will carry out a Phase I clinical trial in the second quarter of 2017.

Universal Influenza Vaccine Secured (UNISEC)

The UNISEC consortium consists of 11 partners and is coordinated by the University of Groningen (NL). The consortium aims to bring forth a number of novel vaccine concepts that can afford broad protection against influenza virus infection and can be manufactured and used in a timely and cost-effective manner.^{46,47} A variety of vaccine candidates with different vaccine antigens (i.e. HA stem, M1, M2, and NP) and delivery systems (i.e. AAV vector, DNA, subunit, polypeptides, and fusion proteins) are available within the consortium and are being tested in comparative pre-clinical studies using different animal models (mouse, cotton rat, ferret and pig, etc.). UNISEC also develops various adjuvants and application devices up to phase I, IIa clinical trial studies.

Two of the UNISEC vaccine candidates at the most advanced stage of development, FLU-v (SEEK, UK) and M-001 (BiondVax, IL), are currently tested in human phase IIb studies. 222 healthy young adults aged 18-60 are recruited for each of the randomized, double blind and controlled study. FLU-v vaccine contains an equimolar mixture of four different synthetic polypeptides representing conserved epitopes identified from M1, M2, and NP. Earlier trials on FLU-v showed that the vaccine is safe and able to elicit influenza-specific T cell responses.⁴⁸ The vaccine-elicited cellular immunity was further shown to be correlated with reductions in both viral shedding and morbidity scores in a human challenge experiment.⁴⁹

The M-001 vaccine contains a single recombinant protein consisting of triplicates of nine conserved epitopes identified from the HA stem, M1, and NP. Earlier trials on M-001 show that the vaccine is safe and able to elicit influenza-specific non-neutralizing antibodies and T cell responses in both young and older (65+) adults.⁵⁰ Moreover, M-001 was shown to prime antibody responses elicited by conventional TIV and increase the sero-conversion rate induced by TIV.⁵¹ Based on these observations, the capacity of M-001 to be a pandemic primer is further tested in the current phase IIb study. Aside from safety, the breadth of immune responses, particularly cell mediated immunity, elicited by both FLU-v and M-001 are evaluated in the UNISEC clinical studies. Trial reports from both studies are expected in 2017.

One of the work packages of the UNISEC consortium is dedicated to standardizing and validating immune assays for the evaluation of humoral and cellular immune responses to vaccination in both pre-clinical and clinical studies. With this approach, the consortium aims at providing robust and reliable technology suitable for assessment and identification of new immune correlates of protection. Assays that are being validated so far include multiparametric intracellular cytokine FACS analysis, intracellular cytokine expression at the mRNA level, ELISpot, haemagglutination inhibition and (micro)neutralisation.

Major challenges faced by the five Consortia

For all five EU consortia, three general issues essential for the development of universal influenza vaccine need to be addressed, namely the breadth of protection, immune correlates of protection and clinical efficacy. Although each of the consortia works independently for its own project, a joint effort of experts from among the consortia was made to clarify these

general issues. These issues were also discussed in a recent WHO meeting on development of influenza vaccines that induce broadly protective and long-lasting immune responses.¹²

Breadth of protection

The first and foremost issue is the breadth of protection that a “universal” influenza vaccine should achieve. Theoretically, an influenza vaccine candidate could claim to be “universal” only, if it is shown that the candidate provides protection against all known circulating strains *and* future emerging strains which are obviously not yet known. To prove the claim for “universality” is thus extremely challenging and is perhaps a major leap from the seasonal TIV. It may be more realistic to improve the breadth of influenza vaccine protection to a reasonable and desired extent, such as pan-seasonal influenza or pan-influenza A protective. The breadth of vaccine coverage is restricted by the antigenic similarities and differences among group 1 (a.o. H1, H2, H5, and H9) and 2 (a.o. H3, H7) influenza A virus strains and influenza B virus strains. When deciding which vaccine antigens should be included, the public health impact of the epidemic strains should also be taken into consideration. During a recent informal scientific discussion between the European Medicines Agency (EMA) and both the FLUTCORE and the UNISEC consortium, it was suggested that in claims for the registration of a novel influenza vaccine it should be specifically mentioned in the registration dossier against which strains the protection was evaluated and is supported by the obtained results. Thus, it is not what a vaccine should achieve, but what it actually does achieve. In light of this, it may be better to call the influenza vaccine candidates to be brought forth by the five EU consortia “broadly protective” instead of “universal”.

Immune correlates of protection (COPs)

The second issue is how to evaluate the immunogenicity or efficacy of a broadly protective influenza vaccine. The 1:40 protective antibody titre and the standard seroconversion and seroprotection rates based on the conventional hemagglutination inhibition (HI) assay have long been accepted internationally as the main criteria to evaluate influenza vaccine efficacy. Such evaluation criteria, however, will not be applicable to novel influenza vaccines targeting conserved epitopes other than those on the HA globular head. Due to the awareness of the importance of antibodies without HI activity and cellular immunity in protection from influenza disease (Fig. 1), particularly in the elderly population, the “gold standard” of using HI assays for influenza vaccine evaluation has been removed from the EMA guidelines since 2014.^{52,53} Two recent studies on HA-stem vaccines published in *Science* and *Nature Medicine* further highlight the fact that influenza cross-protection can be achieved by vaccine-elicited antibodies with undetectable or negligible HI or neutralization activity.^{54,55} Intrastem-specific protection mediated by stem-specific antibodies was confirmed by passive antibody transfer and challenge models in both studies. Although scientists and regulatory authorities are both open to new assays measuring non-HI and/or non-neutralizing antibodies and cellular immune responses, protective levels of such additional immune responses still need to be established and confirmed in clinical settings.

The issue of COPs was extensively discussed during a recent workshop on immunoassay standardisation for universal flu vaccines organized by the EDUFLUVAC consortium (18-19 June 2015, National Institute for Biological Standards and Control (NIBSC), UK). It was agreed that there is no single assay or criterion for the evaluation of broadly protective influenza vaccine immunogenicity since such a vaccine could work through various mechanisms and concepts. Despite the need of case by case evaluation, it was also proposed that perhaps a panel of assays should always be performed to present the overall picture of

immune responses elicited by a given vaccine. Although the workshop concluded with no agreement on which assays should be included in the so-called basic evaluation panel, a “package” of vaccine immunogenicity data assessed by HI, virus neutralization (VN), and cell-mediated assays will be requested according to the new EMA influenza vaccine guideline that are to be released soon.⁵³ Since different assays differ in the immune effectors they measure (Fig. 1) and in their detection sensitivities, correlations of data obtained from different assays are not always observed. Nevertheless, global harmonization on immunological assays was stressed during the workshop. This is to be started with protocol and reference reagent sharing and is expected to facilitate the COPs finding for influenza. The assays being discussed during the workshop are summarized in Table 3, they include established serological assays, exploratory serological methods, cellular assays, and assays combining antibodies and effector cells. With the same concern in mind, the FLUCOP consortium funded by the Innovative Medicines Initiative (IMI) aims to standardize and validate assays for assessment of influenza vaccine COPs.⁵⁶ The consortium consists of 22 partners from vaccine manufacturers, academic institutes and regulatory and public health agencies coordinated by the European Federation of Pharmaceutical Industries and Associations (EFPIA). FLUCOP works on four research packages focusing on the standardization of HI and VN assays, the advancement of cellular assays, the development of NA-specific assays, and the exploration of other new technologies. It should also be stressed that due to the complexity of T cell assays, structured reporting of these assays based on Minimal Information about T cell Assays (MIATA) guidelines has been recommended to allow a comprehensive and objective review of the data.⁵⁷ Last but not the least, a head-to-head comparison of different influenza vaccines using the same protocol for trial study and laboratory readout is needed in order to gain a better insight of the immune protection against influenza.

372

373 *Clinical efficacy*

374 Except for the EDUFLUVAC vaccine, which is expected to elicit broadly neutralizing
375 antibodies able to prevent infection of cells by influenza virus, vaccines developed by the
376 other consortia are expected to induce immune effectors which impair replication of virus
377 within the host cells and/or to impair release of virus progeny from the infected cells. Such
378 vaccines are not expected to induce sterilizing immunity, but to reduce virus replication and
379 transmission, infection duration, and disease symptomatology. How to measure clinical
380 efficacy of an influenza vaccine that mitigates disease symptoms (but allows infection) is thus
381 an essential issue. Data on a combination of outcomes including laboratory-confirmed
382 influenza cases, viral loads detected from swab samples, subject-reported symptom severity
383 scores, and preferably pneumonia and influenza (P&I) hospitalization rates will need to be
384 collected and validated to find out the most appropriate clinical endpoint(s) for vaccine trial
385 studies. Aside from choosing relevant clinical endpoints for evaluation, it is important to set
386 the objective right when comparing the broadly-protective influenza vaccine candidates
387 developed by the consortia with current HA-based influenza vaccines. While the broadly-
388 protective vaccines are expected to be non-inferior to the HA-based vaccines during match
389 influenza seasons, the broadly-protective vaccines are expected to be superior during
390 mismatch influenza seasons.

391

392 In general, it is quite challenging to establish clinical efficacy and particularly broad
393 protection in the field. If the virus is not out there yet it is impossible to determine protection.
394 Even for circulating viruses the use of clinical endpoints drastically increases the number of
395 study subjects to include, when relying on natural infection, which has an attack rate of 3.5%
396 (95% confidence interval (CI) 2.30% to 4.60%) and 15.2 % (95% CI 11.40% to 18.90%) in

adults and children respectively.⁵⁸ Challenge studies in humans, though possible with small numbers of study subjects, are expensive and might not represent what is going on in a real scenario.

Other aspects related to novel influenza vaccine development

Enhancing vaccine immunogenicity

Like for most infectious pathogens, the highly conserved regions of the influenza virus proteins are poorly immunogenic.⁵⁹ One way to enhance immune responses and to achieve antigen dose-sparing is through the use of adjuvant. The FLUTCORE vaccine candidate and FLU-v from the UNISEC consortium both will be clinically tested in two formulations: without or with adjuvantation. Alum and Montanide ISA-51 (a water-in-oil emulsion adjuvant known to boost both humoral and cellular immune responses) are the selected adjuvants for the FLUTCORE vaccine and FLU-v, respectively. Immune responses elicited by one vaccination of the adjuvanted FLU-v and two vaccinations of the plain FLU-v will be evaluated. The UNIVAX vaccine will make use of a chitosan-based delivery vehicle which has already shown efficacy and is recognised for the mucoadhesive and potentially adjuvant properties associated with the chitosan component. Nonetheless, it is likely that the manner by which the RepRNA vaccine is delivered will not be sufficient to stimulate DCs for their migration into lymph nodes and further induction of adaptive immune responses. Accordingly, the UNIVAX consortium is analysing two synthetic mucosal adjuvants (macrophage-activating lipopeptide (MALP-2, a TLR2/6 agonist) and cyclic-di-nucleotides (c-di-AMP)) to promote efficacious immune induction by mucosal delivery. The adjuvants are formulated together with the candidate replicon vaccines encoding influenza virus antigens and delivered by biodegradable nanoparticles targeting DCs.

All the other vaccine candidates developed by the five consortia that are to be clinically tested, however, are largely free from adjuvants. Instead, vaccine immunogenicity will be enhanced through alternative strategies, such as increased antigen presentation through expression of repetitive antigens and delivery platforms with intrinsic adjuvant activity (e.g. the MVA vector or the HBV core). In addition to increasing immune responses, adjuvants are also known to facilitate the onset of immune responses, direct the phenotype and quality of immune responses, broadening of immune responses, and helping the formation of memory B and T cells which results in long-lasting immunity.⁶⁰ In light of this, EDUFLUVAC, UNIVAX and UNISEC plan to evaluate the added value of novel adjuvants on vaccine candidates available from their consortium. It should be stressed that the decision for the use of adjuvants needs to be justified by balancing the benefits (i.e. increased vaccine immunogenicity) and risks (i.e. increased adverse events following vaccination). It is important to properly design vaccine trials such that the effect of adjuvants on vaccine immunogenicity, safety, and tolerability can be reliably evaluated.⁶¹ For a thorough evaluation of the effects of adjuvants, a vaccine control arm (using vaccines without adjuvants or vaccines adjuvanted with licensed adjuvants) and an adjuvant control arm (using adjuvants alone without vaccine antigens) should always be included next to the testing vaccine arm (using vaccines containing the novel adjuvants).

Vaccine administration

Most of the influenza vaccines on the market today or in the development pipeline of the consortia are administered via the parenteral route (i.e. intramuscularly, intradermally, or subcutaneously). Parenteral immunization elicits robust systemic immune responses, but little or no response at the respiratory (mucosal) site, which is the portal of entry for influenza viruses. For influenza vaccines that aim at mitigating virus infection and replication, it is

especially crucial that the immune responses are elicited in the respiratory tract so that local lymphocytes can be quickly (re-)activated upon virus infection for immediate viral clearance. In order to elicit mucosal immunity, mucosal immunization is required. Intranasal vaccine delivery in humans so far only works well for live attenuated vaccines. Pulmonary immunization has been shown to be more potent than nasal immunization and is able to induce robust immune responses at both systemic and mucosal sites.⁶² Moreover, several older studies in humans prove the viability of a pulmonary influenza vaccination strategy.⁶³⁻⁶⁵

In addition, pulmonary immunization has the advantage that it can be performed with powder vaccine. When compared to liquid vaccines, powder vaccines have additional advantages including (i) increased stability and longer shelf-life; (ii) reduced costs for vaccine logistics; (iii) the prevention of spreading blood-borne diseases from contaminated needles; (iv) the elimination of vaccination barrier caused by needle-phobia, and (v) the facilitation of immunization procedure, especially during mass immunization.^{66,67} Through recent advances in spray (freeze) drying techniques and powder inhalation devices inhaled powder vaccines have become a viable alternative to current injected vaccines. State-of-the-art technology is available to the UNISEC consortium from the University of Groningen. With technology in the *Pharmaceutical Technology and Biopharmacy* group, customized powder formulation can be made for various novel vaccine concepts suitable for targeting different mucosal sites (i.e. intranasal, pulmonary, or sublingual).

Vaccine development and evaluation pathway

For any novel vaccine to reach the market, it has to complete a complicated and time-consuming process outlined in Fig. 2. This extensive evaluation process is to ensure the safety and efficacy of vaccine products. The safety demand for preventive vaccines is high, since

they are to be widely used in healthy populations and especially in children. Ball *et al* have summarized several statistical approaches used by the US Food and Drug Administration (FDA) for vaccine safety evaluation in various clinical and epidemiological settings.⁶⁸ In addition, international public health, and scientific organizations have established working groups on vaccine pharmacovigilance (i.e. Counsel for International Organizations of Medical Sciences (CIOMS) from World Health Organization (WHO) and the Vaccine Special Interest Group (VAXSIG) from the International Society for PharmacoEpidemiology (ISPE)), aiming for a better assessment of the benefits and risks (BR) of a vaccine throughout its developmental stages. Due to the limited sample size used for clinical trial studies, signals of rare adverse event following immunization (AEFI) including immunological disorders (i.e. Bell's Palsy, Guillain-Barré syndrome and narcolepsy) and vaccine-associated enhanced respiratory disease (VAERD) may only become apparent during the post-licensure stage. The new EMA influenza vaccine guideline will also demand for more effort on post-licensure BR monitoring of vaccine products, requesting continuous efficacy assessment and enhanced safety surveillance.⁵³ Nevertheless, dealing with vaccine safety should not start with a passive post-licensure surveillance plan, but rather with a proactive precaution analysis at the preclinical stage. Khurana *et al* have pointed out the need for a careful evaluation of VAERD during influenza vaccine development, especially for vaccines targeting to the influenza HA stem domain.^{69,70} The group has shown in pigs that in the case of a mismatched vaccination and virus infection, VAERD can be caused by HA stem-specific antibodies which are cross-reactive but not neutralizing. Ahmed *et al* have also pointed out a potential safety concern of having a high amount of influenza virus NP in vaccines.^{69,70} Influenza virus NP contains a peptide sequence which resembles human neuropeptide hypocretin (HCRT) receptor 2. Due to molecular mimicry, antibodies targeting the specific influenza NP peptide can cross-react

with the human HCRT receptor 2, which may affect the neurotransmission system and eventually lead to narcolepsy (sleep disorder).

Summary and Future Perspective

Covering a spectrum of the different concepts and approaches, influenza vaccines developed by the five EU consortia are expected to possess the following attributes: enhanced immunogenicity of non-immunodominant but conserved influenza virus antigens, broad protection against drifted and possibly shifted influenza strains, and timely production without the need of embryonated chicken eggs. The success of broadly-reactive influenza vaccines will bring the annual hassle of vaccine strain prediction, selection, and production from chicken eggs to an end. This, however, will need the joined forces of scientists, manufactures, and regulatory and public health authorities to resolve the general issues of establishing the possible breadth of vaccine protection and internationally harmonized and validated methods for the evaluation of vaccine immunogenicity and clinical efficacy. Future head-to-head comparative studies of different influenza vaccine concepts are especially valued for a better understanding of influenza protection mechanisms.

Authors Contributions

HL and EH contributed to the conception and design of the work. HL, ES, and EVD collected information for the work. All information collected is from the public portal. All authors interpreted information for the work. HL drafted the work. All authors revised it critically for important intellectual content. All authors approved the final version to be submitted. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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525

526 **Table 1 Project details of five European consortia working on influenza vaccine¹**

	EDUFLUVAC	FLUNIVAC	UNIVAX	FLUTCORE	UNISEC
EU contribution (EUR)	4,647,179	5,066,881	5,999,457	3,872,919	5,972,180
Funded period	2013.11.01-2017.10.31	2013.10.01- 2017.09.30	2013.10.01-2018.09.30	2013.09.01- 2017.03.31	2013.10.01- 2017.09.30
Coordinated in	Germany	Netherlands	Switzerland	United Kingdom	Netherlands
Coordinator	European Vaccine Initiative-EEIG (DE)	Erasmus University Rotterdam (NL)	Federal Department of Home Affairs: Institute of Virology and Immunology (CH)	University College London (UK)	University of Groningen (NL)
Other Participants	Redbiotec AG (CH); Instituto De Biologia Experimental E Tecnologica (PT); ETNA Biotech SRL (IT); Stichting Biomedical Primate Research Center (NL); Stichting Dienst Landbouwkund-ig Onderzoek (NL); Department of Health “Quarry House, Quarry Hill” (UK)	Artemis One Health Research BV (NL); Ludwig-Maximilians-Universitaet Muenchen (DE); Aimm Therapeutics BV (NL); Probiogen AG (DE); VIB (BE); Isconova (Novavax) AB (SE); Q-Biologicals NV (BE)	OZ Biosciences SAS (FR); Synthaur OOO (RU); Vitamfero SA (FR); Amvac Research GMBH (DE); Meditox S.R.O (CZ); Centre National De La Recherche Scientifique (FR); The University of Manchester (UK); Helmholtz-Zentrum Fuer Infektionsforsch-ung GMBH (DE); Universitetet I Bergen (NO); TP21 GMBH (DE)	University of Leeds (UK); Latvijas Biomedicinas Petijumu Un Studiju Centrs (LV); IQUR Limited (UK); Luxembourg Institute of Health (LU); 3P Biopharmaceuticals SL (ES); Institut Catala De La Salut (ES)	Academisch Ziekenhuis Groningen (NL); Peptcell LTD (UK); BiondVax Pharmaceuticals LTD (IL); Retroscreen Virology LTD (UK); Department of Health “Quarry House, Quarry Hill” (UK); Statens Serum, Institute (DK); Országos Epidemiológia Központ (HU); Nasjonalt Folkehelseinstitut-t (NO); Robert Koch-Institut (DE); Goeteborgs Universitet (SE)

527 Country Abbreviations: BE, Belgium; CH, Switzerland; CZ, Czech Republic; DE, Germany; DK, Denmark; ES, Spain; FR, France; HU,
528 Hungary; IL, Israel; IT, Italy; LU, Luxembourg; LV, Latvia; NL, Netherlands; NO, Norway; PT, Portugal; RU, Russia; SE, Sweden; UK, United
529 Kingdom

530 ¹ FP7 program addresses the importance of cooperation between academia and industry. The participation of SMEs is encouraged by the
531 industry-relevant project calls and EU funding.

532

533 **Table 2 Influenza vaccine concepts developed by the five European consortia supposed to enter clinical evaluation**

Consortium	EDUFLUVAC	FLUNIVAC	UNIVAX	FLUTCORE	UNISEC	
Immunization strategy	Multiple components from each (sub)type	Pre-selected conserved Ags	RepRNA encoding influenza virus Ags	Pre-selected conserved Ags	Pre-selected conserved Ags	
Antigen(s)	HA or NA	Including NP	Including HA, with selection from NA, NP, M1 and PB1	M2e and HA stem	M1, M2 and NP (FLU-v)	HA stem, M1 and NP (M-001)
Delivery system	Baculovirus-based VLP	Recombinant MVA vector	DC-targeting, biodegradable nanoparticles, delivering RepRNA, with defined mucosal adjuvants	HBV-based VLP	Synthetic polypeptides	Single recombinant protein
Immunity	Mainly humoral	Both humoral and cellular			Mainly cellular	
Developmental stage	Pre-clinical		Pre-clinical/Phase I		Phase II	

534 Abbreviations: Ags, antigens; DC, dendritic cell; HA, hemagglutinin; HBV, Hepatitis B virus; M1, matrix 1 protein; M2, matrix 2 protein; MVA,
535 Modified Vaccinia Virus Ankara; NA, neuraminidase; NP, nucleoprotein; PB1, polymerase basic protein 1; RepRNA, self-amplifying replicon
536 RNA; VLP, virus like particle.

537 **Table 3 Assays discussed during a recent workshop on immunoassay standardisation for**
538 **universal flu vaccines**

Serological assays	<p><i>Established:</i> Hemagglutination inhibition (HI) Single radial hemolysis (SRH) Virus neutralization (VN)</p> <p><i>Exploratory:</i> Enzyme-linked ImmunoSorbent Assay (ELISA) HA stem domain assays</p> <ul style="list-style-type: none"> • ELISA for anti-stem antibodies • Neutralization assay for anti-stem antibodies • In vivo challenge experiment for anti-stem antibodies <p>Neuraminidase inhibition assays</p> <ul style="list-style-type: none"> • Enzyme-linked lectin assay (ELLA) • Traditional Thiobarbituric acid (TBA) method <p>Other neuraminidase (NA)-specific assays</p> <ul style="list-style-type: none"> • ELISA for NA-specific antibodies • Westernblot for NA-specific antibodies • Plaque size reduction <p>Pseudotype-based assays⁷³ Recombinant virus-based assays⁵⁶</p>
Cellular assays	<p>ELISpot Cell Proliferation assays</p> <ul style="list-style-type: none"> • CFSE dilution assay <p>Cytotoxicity assays</p> <ul style="list-style-type: none"> • Chromium (⁵¹Cr) release assay • Cytotoxic T lymphocyte (CTL) assay with fluorescent-antigen-transfected- target (FATT) cells • Viral clearance assay <p>Intracellular cytokine staining (ICS)</p>
Assays combining antibodies and effector cells ¹	<p>Antibody-dependent cell-mediated cytotoxicity (ADCC)</p> <ul style="list-style-type: none"> • ADCC-NK cell activation assay <p>Antibody-dependent cell-associated viral elimination (ADCVE)⁷⁴</p> <ul style="list-style-type: none"> • ADCVE with peripheral blood mononuclear cell (PBMC) • ADCVE with respiratory cell line <p>Antibody-dependent respiratory burst (ADBR)</p>

539

540 ¹ Aside from the assays discussed during the workshop, FcγR dimer-binding assay and

541 antibody-dependent phagocytosis (ADP) assay are two recently available assays measuring

542 Fc-mediated antibody functions.^{75,76}

References

1. World Health Organization [Internet]. Influenza (seasonal) fact sheet [updated 2016; cited 2016 Mar 22]. Available from: <http://www.who.int/mediacentre/factsheets/fs211/en/>.
2. Centers for Disease Control and Prevention [Internet]. Pink book-influenza [updated 2015 Sep 21; cited 2016 Mar 22]. Available from: <http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/flu.pdf>.
3. Marill MC. After flu vaccine mismatch, calls for delayed selection intensify. Nat Med. 2015;21(4):297-8.
4. European Center for Disease Prevention and Control [Internet]. Summarizing the 2014-2015 influenza season in Europe [updated 2016; cited 2016 Mar 22]. Available from: http://ecdc.europa.eu/en/press/news/_layouts/forms/News_DispForm.aspx?ID=1231&List=8db7286c-fe2d-476c-9133-18ff4cb1b568.
5. Chambers BS, Parkhouse K, Ross TM, Alby K, Hensley SE. Identification of Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014-2015 Influenza Season. Cell Rep. 2015;12(1): 1-6.
6. Molbak K, Espenhain L, Nielsen J, Tersago K, Bossuyt N, Denissov G, et al. Excess mortality among the elderly in European countries, December 2014 to February 2015. Euro Surveill 2015;20(11). pii:21065. PubMed PMID: 25811643
7. Skowronski DM, Janjua NZ, Serres GD, Sabaiduc S, Eshaghi A, Dickinson JA, et al. Low 2012-13 influenza vaccine effectiveness associated with mutation in the egg-adapted H3N2 vaccine strain not entigenic frift in circulating viruses. PLoS One. 2014;9(3):e92153.
8. Centers for Disease Control and Prevention [Internet]. The 2009 H1N1 pandemic: summary highlights, April 2009-April 2010 [updated 2010 Aug 3; cited 2016 Mar 22]. Available from: <http://www.cdc.gov/h1n1flu/cdcresponse.htm>.

- 568 9. President's Council of Advisors on Science and Technology[Internet]. Report to the
569 president on reengineering the influenza vaccine production enterprise to meet the challenges
570 of pandemic influenza. 2010 [cited 2016 Mar 22]. Available from :
571 [https://www.whitehouse.gov/sites/default/files/microsites/ostp/PCAST-Influenza-](https://www.whitehouse.gov/sites/default/files/microsites/ostp/PCAST-Influenza-Vaccinology-Report.pdf)
572 [Vaccinology-Report.pdf](https://www.whitehouse.gov/sites/default/files/microsites/ostp/PCAST-Influenza-Vaccinology-Report.pdf).
- 573 10. Work Programme 2013 [Internet]. 2013 [cited 2016 Aug 31]. Available from:
574 http://ec.europa.eu/research/participants/data/ref/fp7/192012/a-wp-201303_en.pdf.
- 575 11. Cox NJ, Hickling J, Jones R, Rimmelzwaan GF, Lambert LC, Boslego J, et al. Report on
576 the second WHO integrated meeting on development and clinical trials of influenza vaccines
577 that induce broadly protective and long-lasting immune responses: Geneva, Switzerland, 5-7
578 May 2014. Vaccine. 2015;33(48):6503-10.
- 579 12. World Health Organization [Internet]. Eighth WHO meeting on development of influenza
580 vaccines that induce broadly protective and long-lasting immune responses [updated 2016;
581 cited 2016 Aug 31]. Available from:
582 [http://www.who.int/immunization/research/meetings_workshops/8th_influenza_vaccine_chic](http://www.who.int/immunization/research/meetings_workshops/8th_influenza_vaccine_chicago2016/en/)
583 [ago2016/en/](http://www.who.int/immunization/research/meetings_workshops/8th_influenza_vaccine_chicago2016/en/).
- 584 13. European Commission [Internet]. Combinatorial immunization strategy to educate the
585 immune system towards cross recognition and coverage against antigenic drift in seasonal
586 influenza virus exposure [updated 2015 Mar 11; cited 2016 Mar 22]. Available from:
587 http://cordis.europa.eu/project/rcn/110279_en.html.
- 588 14. EDUFLUVAC consortium [Internet]. Educate the immune system to recognise
589 common influenza epitope [updated 2016; cited 2016 Mar 22]. Available from:
590 <http://www.edufluvac.eu/>.

591 15. Kusi KA, Faber BW, Thomas AW, Remarque EJ. Humoral immune response to mixed
592 PfAMA1 alleles; multivalent PfAMA1 vaccines induce broad specificity. PLoS One. 2009;
593 4(12): e8110.

594 16. Schwartzman LM, Cathcart AL, Pujanauski LM, Qi L, Kash JC, Taubenberger JK. An
595 intranasal virus-like particle vaccine broadly protects mice from multiple subtypes of
596 influenza A virus. MBio. 2015;6(4):e01044-15.

597 17. European Commission [Internet]. InFLUenza virus UNIVersal VACcine development
598 program [updated 2015 Oct 12; cited 2016 Mar 22]. Available from:
599 http://cordis.europa.eu/project/rcn/109606_en.html.

600 18. FLUNIVAC consortium [Internet]. FLUNIVAC [updated 2016; cited 2016 Mar 22].
601 Available from: <http://flunivac.eu/>.

602 19. Altenburg AF, Kreijtz JH, de Vries RD, Song F, Fux R, Rimmelzwaan GF, et al.
603 Modified vaccinia virus ankara (MVA) as production platform for vaccines against influenza
604 and other viral respiratory diseases. Viruses. 2014;6(7):2735-61.

605 20. Brewoo JN, Powell TD, Jones JC, Gundlach NA, Young GR, Chu H, et al. Cross-
606 protective immunity against multiple influenza virus subtypes by a novel modified vaccinia
607 ankara (MVA) vectored vaccine in mice. Vaccine. 2013;31(14):1848-55.

608 21. Antrobus RD, Lillie PJ, Berthoud TK, Spencer AJ, McLaren JE, Ladell K, et al. A T
609 cell-inducing influenza vaccine for the elderly: Safety and immunogenicity of MVA-NP+M1
610 in adults aged over 50 years. PLoS One. 2012;7(10):e48322.

611 22. Kreijtz JH, Goeijenbier M, Moesker FM, van den Dries L, Goeijenbier S, De Gruyter
612 HL, et al. Safety and immunogenicity of a modified-vaccinia-virus-ankara-based influenza A
613 H5N1 vaccine: A randomised, double-blind phase 1/2a clinical trial. Lancet Infect Dis.
614 2014;14(12):1196-207.

- 615 23. Bavarian Nordic [Internet]. IMVAMUNE. Available from: [http://www.bavarian-](http://www.bavarian-nordic.com/about/company-overview.aspx)
616 [nordic.com/about/company-overview.aspx](http://www.bavarian-nordic.com/about/company-overview.aspx).
- 617 24. European Commission [Internet]. A “Universal” influenza vaccine through synthetic,
618 dendritic cell-targeted, self-replicating RNA vaccine [updated 2015 Jun 29; cited 2016 Mar
619 22]. Available from: http://cordis.europa.eu/project/rcn/109454_en.html.
- 620 25. UNIVAX consortium [Internet]. UNIVAX [cited 2016 Mar 22]. Available from:
621 <http://www.univax-fp7.eu/index.php?id=1292>.
- 622 26. Atkins GJ, Fleeton MN, Sheahan BJ. Therapeutic and prophylactic applications of
623 alphavirus vectors. *Expert Rev Mol Med*. 2008; 10: e33.
- 624 27. Khromykh AA. Replicon-based vectors of positive strand RNA viruses. *Curr Opin Mol*
625 *Ther*. 2000; 2(5): 555-69.
- 626 28. McCullough KC, Bassi I, Demoulins T, Thomann-Harwood LJ, Ruggli N. Functional
627 RNA delivery targeted to dendritic cells by synthetic nanoparticles. *Ther Deliv*. 2012; 3(9):
628 1077-99.
- 629 29. Tratschin JD, Ruggli N, McCullough KC. Pestivirus replicons providing an RNA-based
630 viral vector system. United States Patent US 20110189224 A1. 2008 Jun 4.
- 631 30. McCullough KC, Milona P, Thomann-Harwood L, Démoulins T, Englezou P, Suter R,
632 et al. Self-Amplifying Replicon RNA Vaccine Delivery to Dendritic Cells by Synthetic
633 Nanoparticles. *Vaccines (Basel)*. 2014; 2(4): 735-54.
- 634 31. Milona P, Bovin N, McCullough KC. Enhanced translational DC targeting: Wide
635 carbohydrate ligand screening on human and porcine dendritic cells. (submitted)
- 636 32. Ebensen T, Libanova R, Schulze K, Yevsa T, Morr M, Guzman CA. Bis-(3',5')-cyclic
637 dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant.
638 *Vaccine*. 2011; 29(32): 5210-20.

- 639 33. Libanova R, Ebensen T, Schulze K, Bruhn D, Nörder M, Yevsa T, et al. The member of
640 the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent
641 activity as mucosal adjuvant. *Vaccine*. 2010; 28(10): 2249-58.
- 642 34. Ebensen T, Guzman CA. Immune modulators with defined molecular targets:
643 cornerstone to optimize rational vaccine design. *Adv Exp Med Biol*. 2009; 655: 171-88.
- 644 35. Madhun AS, Haaheim LR, Nøstbakken JK, Ebensen T, Chichester J, Yusibov V, et al.
645 Intranasal c-di-GMP-adjuvanted plant-derived H5 influenza vaccine induces multifunctional
646 Th1 CD4⁺ cells and strong mucosal and systemic antibody responses in mice. *Vaccine*. 2011;
647 29(31): 4973-82.
- 648 36. Svindland SC, Pedersen GK, Pathirana RD, Bredholt G, Nøstbakken JK, Jul-Larsen Å,
649 et al. A study of Chitosan and c-di-GMP as mucosal adjuvants for intranasal influenza H5N1
650 vaccine. *Influenza Other Respir Viruses*. 2013; 7(6): 1181-93.
- 651 37. Demoulins T, Milona P, Englezou PC, Ebensen T, Schulze K, Suter R, et al.
652 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines. *Nanomedicine*
653 2015. pii: S1549-9634. PubMed PMID: 26592962.
- 654 38. McCullough KC, Bassi I, Milona P, Suter R, Thomann-Harwood L, Englezou P, et al.
655 Self-replicating Replicon-RNA Delivery to Dendritic Cells by Chitosan-nanoparticles for
656 Translation In Vitro and In Vivo. *Mol Ther Nucleic Acids*. 2014; 3: e173.
- 657 39. Svindland SC, Jul-Larsen A, Pathirana R, Andersen S, Madhun A, Montomoli E, et al.
658 The mucosal and systemic immune responses elicited by a chitosan-adjuvanted intranasal
659 influenza H5N1 vaccine. *Influenza Other Respir Viruses*. 2012; 6(2): 90-100.
- 660 40. Cox RJ, Pedersen G, Madhun AS, Svindland S, Sævik M, Breakwell L, et al.
661 Evaluation of a virosomal H5N1 vaccine formulated with Matrix MTM adjuvant in a phase I
662 clinical trial. *Vaccine*. 2011; 29(45): 8049-59.

- 663 41. European Commission [Internet]. Development of a universal influenza vaccine based
664 on tandem core technology [updated 2015 Mar 11; cited 2016 Mar 22]. Available from:
665 http://cordis.europa.eu/project/rcn/109344_en.html.
- 666 42. FLUTCORE consortium [Internet]. FLUTCORE [updated 2016; cited 2016 Mar 22].
667 Available from: <http://www.flutcore.eu/>.
- 668 43. Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal
669 influenza A vaccine based on the extracellular domain of the M2 protein. *Nat Med*. 1999;
670 5(10): 1157-63.
- 671 44. Nabel GJ, Fauci AS. Induction of unnatural immunity: prospects for a broadly
672 protective universal influenza vaccine. *Nat Med*. 2010; 16(12): 1389-91.
- 673 45. Peyret H, Gehin A, Thuenemann EC, Blond D, El Turabi A, Beales L, et al. Tandem
674 fusion of hepatitis B core antigen allows assembly of virus-like particles in bacteria and plants
675 with enhanced capacity to accommodate foreign proteins. *PLoS One*. 2015;10(4):e0120751.
- 676 46. European Commission [Internet]. Universal influenza vaccines secured [updated 2015
677 Mar 11; cited 2016 Mar 22]. Available from:
678 http://cordis.europa.eu/project/rcn/110139_en.html.
- 679 47. UNISEC consortium [Internet]. Universal influenza vaccines secured [updated 2016 Oct
680 16; cited 2016 Mar 22]. Available from: <http://www.unisecconsortium.eu/>.
- 681 48. Pleguezuelos O, Robinson S, Stoloff GA, Caparros-Wanderley W. Synthetic influenza
682 vaccine (FLU-v) stimulates cell mediated immunity in a double-blind, randomised, placebo-
683 controlled phase I trial. *Vaccine*. 2012;30(31):4655-60.
- 684 49. Pleguezuelos O, Robinson S, Fernandez A, Stoloff GA, Mann A, Gilbert A, et al. A
685 synthetic influenza virus vaccine induces a cellular immune response that correlates with
686 reduction in symptomatology and virus shedding in a randomized phase Ib live-virus
687 challenge in humans. *Clin Vaccine Immunol*. 2015;22(7):828-35.

688 50. Atsmon J, Kate-Ilovitz E, Shaikevich D, Singer Y, Volokhov I, Haim KY, et al. Safety
689 and immunogenicity of multimeric-001--a novel universal influenza vaccine. *J Clin Immunol.*
690 2012;32(3):595-603.

691 51. Atsmon J, Caraco Y, Ziv-Sefer S, Shaikevich D, Abramov E, Volokhov I, et al.
692 Priming by a novel universal influenza vaccine (multimeric-001)-a gateway for improving
693 immune response in the elderly population. *Vaccine.* 2014;32(44):5816-5823.

694 52. European Medicines Agency. Explanatory note on the withdrawal of the note for
695 guidance on harmonisation of requirements for influenza vaccines and of the core SmPC/PL
696 for inactivated seasonal influenza vaccines, EMA/CHMP/VWP/40560/2014 (2014).

697 53. Wijnans L, Voordouw B. A review of the changes to the licensing of influenza vaccines
698 in Europe. *Influenza Other Respir Viruses.* 2016;10(1):2-8.

699 54. Impagliazzo A, Milder F, Kuipers H, Wagner MV, Zhu X, Hoffman RM, et al. A stable
700 trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science.* 2015;
701 349(6254): 1301-6.

702 55. Yassine HM, Boyington JC, McTamney PM, Wei CJ, Kanekiyo M, Kong WP, et al.
703 Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat Med.*
704 2015;21(9):1065-70.

705 56. FLUCOP consortium [Internet]. Standardisation and development of assays for
706 assessment of influenza vaccine correlates of protection [updated 2016; cited 2016 Mar 22].
707 Available from: <http://www.flucop.eu/>.

708 57. Janetzki S, Hoos A, Melief CJ, Odunsi K, Romero P, Britten CM. Structured reporting
709 of T cell assay results. *Cancer Immun.* 2013;13:13.

710 58. Jayasundara K, Soobiah C, Thommes E, Tricco AC, Chit A. Natural attack rate of
711 influenza in unvaccinated children and adults: A meta-regression analysis. *BMC Infect Dis.*
712 2014;14:670.

713 59. Rappuoli R. The challenge of developing universal vaccines. *F1000 Med Rep.* 2011;3:
714 16.

715 60. Alving CR, Peachman KK, Rao M, Reed SG. Adjuvants for human vaccines. *Curr Opin*
716 *Immunol.* 2012;24(3):310-5.

717 61. van Doorn E, Liu H, Huckriede A, Hak E. Safety and tolerability evaluation of the use
718 of Montanide ISATM 51 as vaccine adjuvant: A systematic review. *Hum Vaccin Immunother.*
719 2016;12(1):159-69.

720 62. Liu H, Patil HP, de Vries-Idema J, Wilschut J, Huckriede A. Enhancement of the
721 immunogenicity and protective efficacy of a mucosal influenza subunit vaccine by the
722 saponin adjuvant GPI-0100. *PLoS One.* 2012;7(12):e52135.

723 63. Waldman RH, Mann JJ, Kasel JA. Influenza virus neutralizing antibody in human
724 respiratory secretions. *J Immunol.* 1968;100(1):80-5.

725 64. Waldman RH, Wood SH, Torres EJ, Small PA Jr. Influenza antibody response
726 following aerosol administration of inactivated virus. *Am J Epidemiol.* 1970;91(6):574-85.

727 65. Waldman RH, Coggins WJ. Influenza immunization: Field trial on a university campus.
728 *J Infect Dis.* 1972;**126**(3):242-8.

729 66. Tonnis WF, Lexmond AJ, Frijlink HW, de Boer AH, Hinrichs WL. Devices and
730 formulations for pulmonary vaccination. *Expert Opin Drug Deliv.* 2013;10(10):1383-97.

731 67. Amorij JP, Hinrichs WL, Frijlink HW, Wilschut JC, Huckriede A. Needle-free
732 influenza vaccination. *Lancet Infect Dis.* 2010;10(10):699-711.

733 68. Ball R, Horne D, Izurieta H, Sutherland A, Walderhaug M, Hsu H. Statistical,
734 epidemiological, and risk-assessment approaches to evaluating safety of vaccines throughout
735 the life cycle at the food and drug administration. *Pediatrics.* 2011;127 Suppl 1:S31-8.

69. Khurana S, Loving CL, Manischewitz J, King LR, Gauger PC, Henningson J, et al. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. *Sci Transl Med*. 2013;5(200):200ra114.
70. Ahmed SS, Volkmuth W, Duca J, Corti L, Pallaoro M, Pezzicoli A, et al. Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2. *Sci Transl Med*. 2015;7(294): 294ra105.
71. Krammer F, Palese P. Advances in the development of influenza virus vaccines. *Nat Rev Drug Discov*. 2015;14(3):167-82.
72. Wiersma LC, Rimmelzwaan GF, de Vries RD. Developing universal influenza vaccines: Hitting the nail, not just on the head. *Vaccines (Basel)*. 2015;3(2):239-62.
73. Trombetta CM, Perini D, Mather S, Temperton N, Montomoli E. Overview of serological techniques for influenza vaccine evaluation: Past, present and future. *Vaccines (Basel)*. 2014;2(4):707-34.
74. Jegaskanda S, Job ER, Kramski M, Laurie K, Isitman G, de Rose R, et al. Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies. *J Immunol*. 2013;190(4):1837-48.
75. Kristensen AB, Lay WN, Ana-Sosa-Batiz F, Vanderven HA, Madhavi V, Laurie KL, et al. Antibody responses with Fc-mediated functions after vaccination of HIV-infected subjects with trivalent influenza vaccine. *J Virol*. 2016;90(12):5724-34.
76. Ana-Sosa-Batiz F, Vanderven H, Jegaskanda S, Johnston A, Rockman S, Laurie K, et al. Influenza-specific antibody-dependent phagocytosis. *PLoS One*. 2016;11(4):e0154461.

Y HI active antibody

Y Y Y Non-HI active antibody