EDUFLUVAC FINAL REPORT

Grant Agreement number: 602640

Project acronym: EDUFLUVAC

Project title: Combinatorial immunisation strategy to educate the immune system towards cross recognition and coverage against antigenic drift in seasonal influenza virus exposure

Funding Scheme: Collaborative project

Period covered: 1 November 2013 – 31 October 2017

Name of the scientific representative of the project's co-ordinator:

Dr Odile Leroy, European Vaccine Initiative.

Tel: +49 6221565890

Fax: +49 6221565727

E-mail: odile.leroy@euvaccine.eu

Project website address: www.edufluvac.eu

Final publishable summary report

Executive summary

Current influenza vaccines afford only limited protection against seasonal as well as pandemic influenza. As influenza viruses can accumulate three-four amino acids substitutions per year and frequently change antigenically to escape population immunity, vaccine composition has to be updated and vaccines need to be administered annually. As a consequence, persistent monitoring and selection of viruses as well as production and formulation of vaccines are necessary every year. A major shortcoming is therefore that the relatively long production time results in a vaccine that does not always have a sufficient antigenic match with the epidemic strain. A significant advance in human infectious disease research would be the development of a broadly reactive influenza vaccine that can provide broad coverage against different strains within a subtype or even across subtypes.

In order to address the problem of antigenic drift and annual vaccine reformulation, the EDUFLUVAC consortium proposed the development of a novel influenza vaccine encompassing a combination of multiple influenza haemagglutinin (HA) or neuraminidase (NA) antigenic variants within a single subtype. The innovation of this approach lies in the selection of antigenic variants with maximal sequence diversity (each (sub)type is addressed separately), resulting in diluted strain-specific epitopes and enhanced presentation of common epitopes to the immune system, thereby increasing the breadth of the antibody response. Since, by this approach, the relative concentration of common influenza epitopes is increased while that of strain-specific epitopes is decreased, the immune system is expected to learn what "the common characters" of influenza viruses are. This approach builds on the concept of the Epitope Dilution Phenomenon as a practical strategy for the induction of broad, cross-variant antibody responses against polymorphic antigens. The consortium expressed the selected HA components representing antigenic drift variants within the H1, H3, and B influenza viruses as virus-like particles (VLPs) using the insect cells-baculovirus expression system. The VLP antigen expression system helps cross-linking B cell receptors and boosting humoral immune responses.

The initial assessment of the influenza VLPs was performed in the mouse model. 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 neutralised by pentavalent VLP immunised mouse sera. The H3 and B strain data suggest that at least five components on a single VLP are needed to induce broadened responses. Based on the immunogenicity results obtained in mice, the pentavalent VLPs were selected as lead candidates to conduct ferret and Non-Human Primates (NHPs) challenge studies to provide proof of concept of the EDUFLUVAC project. Challenge studies with the pentavalent H1 VLPs in both ferrets and NHPs did not induce a broadening of the humoral and/or cellular immune responses to the heterologous challenge virus A/California/04/2009 (H1N1)pdm09 and also did not lead to protection after challenge. In contrast, vaccination of ferrets with either a mixture of monovalent H3 VLPs or the pentavalent H3 VLPs induced a serological and cellular immune response against heterologous H3N2 strain and led to reduction in clinical severity and a lower virus load after challenge.

Besides the scientific scope of the project, the EDUFLUVAC partners were engaged in sound networking activities and organised three workshops with the aim to bring together the relevant and current efforts on universal influenza vaccine development and address critical topics in the field.

Context and objectives

Seasonal influenza occurs all over the world with an annual global attack rate estimated at 5 – 10% in adults and 20 – 30% in children, and causes about three to five million cases of severe illness and 250,000 to 500,000 deaths. Due to a high mutation rate (antigenic drift) as well as genetic re-assortment among viruses in animal reservoirs (antigenic shift), influenza viruses circulating in humans change continuously. However, current influenza vaccines are strain specific and offer very limited protection against new variants. As a consequence, persistent monitoring of viruses, annual selection, production of new vaccines and repeated annual immunisation are necessary. To overcome these weaknesses of the current vaccine, new strategies that can offer broadened recognition and cross protection are urgently needed.

Epitope Dilution Phenomenon. Evidence that broad protection within a single influenza subtype can be obtained by vaccination was provided in a study where sequential infection with two different combinations of three H1N1 viruses (viz. Historical: 1934, 1947 and 1957, Modern: 1991, 1999, 2007) conferred protection against challenge with a novel 2009 virus, while only limited protection was observed in ferrets infected with any single H1N1 virus (1). It was shown that sequential infections elicit and boost cross-reactive antibodies to novel H1N1 isolates. In other studies, an H1 subtype homologous DNA prime followed by Adenovirus 5 boost (2) and a centralised (i.e. consensus like sequence) Adenovirus-expressed H1 haemagglutinin (HA) (3) have also been shown to yield broadened influenza-specific immune responses. For the H3 subtype, evidence for broad protection was provided in a study where DNA prime containing a mixture of three HAs followed by a live attenuated-virus boost induced broad neutralising responses and protection to H3 influenza viruses (4). A further study demonstrated that sequential DNA prime with three divergent H3 HAs, followed by a boost with a divergent whole virus, enabled the generation of broadly protective H3 monoclonal antibodies (5). Similar observations were made for the H5 subtype, where immunisation with a mixture of three carefully selected variants expressed on the baculovirus surface yielded broad neutralising antibody responses to H5 – viruses from six different clades (6). The identification of monoclonal antibodies capable of heterosubtypic neutralisation of various HA subtypes (7,8) suggests that heterosubtypic immunity can be induced given the correct antigen. Recently highly conserved protective epitopes have also been identified for influenza B virus haemagglutinin (9). These data suggest that immune responses to influenza virus can indeed be broadened, but limited information is available on the maximum breadth that can be obtained and the number of vaccine components required for maximum breadth.

Recently it was shown that the specificity of antibody responses to the polymorphic malaria antigen apical membrane antigen 1 (AMA1) can be broadened by immunisation with a limited number of antigenic variants (10,11,12,13). The specificity of the antibody response was significantly broadened whilst retaining absolute antibody levels to each of the vaccine components and antibody function as shown by in vitro parasite growth inhibition. The mechanism underlying the broadening of antibody responses is the relative concentration of common epitopes diluting out strain specific ones. This concept represents the foundation of the Epitope Dilution Phenomenon (EDiP) aiming at covering polymorphism in variable vaccine antigens to ensure the efficient generation of broadly neutralising immune responses.

Phylogenetic analysis of AMA1 variation suggested that six vaccine components would be required to cover diversity (14). It was shown, however, that a mixture consisting of only three variants yielded antibody responses of similar breadth as a mixture of seven variants (15) and that simultaneous or sequential administration did not appreciably influence the breadth of the antibody response (16). A recent study defining the antigenic diversity of AMA1 shows that the number of growth inhibiting epitopes is limited, despite a high degree of phylogenetic variation (17). These

fundamental studies opened prospects for designing a combinatorial strategy for seasonal influenza vaccines that can induce broad coverage of circulating epidemic influenza strains.

Virus-like Particles as delivery system. The influenza vaccines produced by the consortium are virus-like particles (VLPs) in which HA or NA antigens are displayed on the surface of a protein scaffold – the influenza matrix (M1) protein – in a packed, orderly fashion. These VLPs present antigens in a highly repetitive form to the immune system in a similar way as an influenza virion and it is assumed that the antigen presented in this format can more efficiently trigger B cells (18). This also implies that the specificity of the B-cells activated by VLPs is highly dependent on the antigens on the VLP. A VLP expressing multiple variants of an antigen, in which the strain-specific epitopes will be diluted, is therefore more likely to activate cross-reactive B-cells capable of producing broadly neutralising antibodies (bnAbs). Moreover, these VLPs contain the M1 protein which does not confer direct protection, but can enhance responses to surface proteins by recruiting cross-reactive helper T cells (19).

VLPs are described as more immunogenic than subunit or recombinant protein immunogens and can stimulate strong humoral and cellular immune responses (20). They mimic virus structures, displaying antigens in their native conformations in a highly repetitive fashion, thereby enhancing the production of neutralising antibodies. On the other hand they lack the genetic information of their infectious counterparts, making VLPs safe and effective vaccine candidates. Enveloped VLPs like the ones in this project allow the vaccine proteins to be presented in their native state as membrane-bound proteins, rather than as the soluble ectodomains alone. In addition, VLPs produced using RBT's rePAX® system have the capacity to present multiple vaccine proteins to the immune system, making them an ideal platform for the induction of broadly neutralising antibodies.

Besides the repetitive antigen display provided by the three-dimensional surface, VLPs possess other features that makes them ideal as vaccine candidates. In addition to B-cell epitopes provided by the native surface antigens, both these antigens and the capsid or carrier proteins contain various T-cell epitopes that can be recognised by T-cells (21,22). VLPs tend to be suitable in size for efficient uptake by dendritic cells (DCs) for processing and presentation by major histocompatibility complex (MHC) class II and for directly promoting DC maturation and migration. Exogenous VLPs can also be taken up and processed via the MHC class I pathway (cross-presentation) for activation of CD8+ T-cells (23). Since VLPs will be generated using the insect cells-baculovirus expression system and knowing that insect cells do not add sialic acids to the N-glycans during posttranslational modifications, VLPs with HA can be effectively released from the insect cell surfaces (24), circumventing the need for the presence of neuraminidase (NA).

Major objectives of the project. In order to address the problem of antigenic drift and annual vaccine reformulation, the EDUFLUVAC consortium proposed to develop a combinatorial immunisation strategy to educate the immune system towards cross recognition and coverage against antigenic drift in seasonal influenza virus exposure. To achieve this objective, EDUFLUVAC aims at developing a novel influenza vaccine candidate encompassing a combination of multiple influenza haemagglutinin (HA) or neuraminidase (NA) antigenic variants within a single (sub)type displayed on the surface of a protein scaffold (influenza M1 protein) in a packed, orderly fashion using the proven, modern technology of VLPs. RBT's rePAX® technology applied in this project allows to specifically increase the surface expression of target proteins on VLPs, enabling the high densities of HA or NA needed to induce strong immune responses. In addition, because all genes are combined in a single baculovirus construct, the insect cells-baculovirus expression system is optimally suited for the generation of polyvalent VLPs.

The main objectives of the EDUFLUVAC project were:

- To develop a VLP-based influenza vaccine, produced using the insect cells-baculovirus expression system, that induces bnAb responses within influenza (sub)types.
- To develop methodology and Intellectual Property (IP) on polyvalent VLPs in preparation of potential marketing of the product.

The innovation brought by this consortium lay in the development of a vaccine that expresses several variant HAs or NAs on a VLP specifically designed to target B cells capable of mounting a bnAb response. Polyvalent VLPs are expected to elicit bnAb responses and therefore confer protection against epidemic influenza. To achieve this aim, the overall strategy of the EDUFLUVAC project was to:

1. Select HA and NA antigens representing antigenic drift within the H1, H3 and B (sub)types.
2. Generate baculovirus vectors for expression of one or more HAs or NAs.
3. Produce VLPs for immunological studies.
4. Develop in process and final product development assays for product characterisation.
5. Evaluate the immunogenicity of VLPs in mice and select the lead candidates
6. Provide proof-of-concept in relevant challenge animal models (ferret and NHPs) that multivalent VLPs can confer broad protection against influenza challenge.
7. Develop a complete Investigational Medicinal Product Dossier (IMPD) ready for transfer into cGMP VLP clinical manufacturing for early phase clinical testing.

Main Results and Achievements

Antigen selection

To define which strains would represent an appropriate coverage within one (sub) type, a methodology similar to Derek Smith's (25) was applied. Briefly, genetic maps of each (sub) type were prepared by using the differences in amino acids (only variable positions included) to calculate similarities instead of antiserum reactivities as in Smith et al. 2004, and then used in multi-dimensional scaling for strain selection, where 1-similarity is used to calculate distances between strains. The Smith methodology has been used previously in a combinatorial influenza vaccine approach (26), where representatives of three alternating major groups were selected.

To develop the multivalent seasonal influenza VLP vaccine, five HA antigens were selected for each of the H1, H3 and B (sub) types based on sequence diversity and serological cross-reactivity; the selected strains should represent major antigenic drift groups in each (sub) type. Concomitantly, three neuraminidase (NA) antigens from the N1 subtype were selected by similar criteria as outlined for the HA antigens. To address whether heterosubtypic neutralising antibodies can be induced by vaccination with VLPs, one HA variant from each of the six Group 2 HAs (H3, H4, H7, H10, H14 and H15) were selected to generate monovalent VLPs as well as a hexavalent VLP containing six Group 2 HAs.

In the combinatorial vaccination strategy undertaken by the consortium the following terminology is used: monovalent: VLPs with only a single HA or NA; polyvalent: VLPs expressing multiple HAs or NAs on a single VLP; multivalent: mixtures of mono- or polyvalent VLPs.

Generation of expression vectors

After the selection of all HA and NA antigens, baculovirus vectors encoding one or multiple surface antigens from each (sub)type were generated and used to produce influenza VLPs composed of (i) one single HA or NA – monovalent VLPs, (ii) three HAs or NAs – trivalent VLPs, (iii) five HAs – pentavalent VLPs, and (iv) six HAs – hexavalent VLPs.

The baculovirus constructs were generated using RBT's rePAX® technology. Briefly, it consists of a highly efficient sequence assembly platform, and multiprotein expression from a single baculovirus vector. Successful assembly is confirmed by restriction digest analysis of the plasmid vector, sequencing of clones showing the expected pattern, and subsequent transfer into the baculovirus expression vector system. After transfection of insect *Spodoptera frugiperda* (Sf)-9 cells with bacmids and generation of so-called V0 virus, expression tests in insect High Five cells were performed for identification/quantification of surface and capsid antigens by Western blot, ELISA and/or hemagglutination assay. Amplification of baculovirus stocks (V1) was performed in Sf-9 cells using the V0 virus stock initially generated. Baculovirus titres were determined using a virus counter (Virocyt, LLC, USA) and suitable quantities of V1 viruses were delivered to iBET for production of VLPs.

A total of 31 baculovirus constructs were initially planned to cover all target VLP configurations. However, due to the poor expression of two strains and the difficulty in generating the H3 pentavalent and the Group 2 hexavalent baculovirus constructs, additional vectors (four in total) had to be produced in order to generate the required VLPs for mouse immunogenicity studies, thus avoiding significant deviations from project objectives and timelines. For example, two initially selected strains representative of H3 and B subtypes had to be replaced by other strains with improved expression levels, and thus two new baculovirus vectors had to be generated. In addition, an alternative strategy was designed and executed by iBET to ensure the successful generation of

the pentavalent H3 VLPs (27). It consisted of generating a stable insect High Five cell line constitutively expressing two HA proteins and then infecting these cells with the trivalent H3 baculovirus construct already generated by RBT. A similar strategy was also put in place to produce the hexavalent Group 2 VLPs.

Rational optimisation of baculovirus vectors for expression in insect cells was also performed at Redbiotec AG. By manipulating the cloning conditions and/or the design of the expression cassettes (promoters, polyadenylation signals and order of the HA sequences), the stability and expression levels of the recombinant baculovirus vectors could be improved.

Production of mono and multivalent HA and NA VLPs

All monovalent, trivalent, pentavalent and hexavalent VLPs for mouse immunogenicity studies and challenge studies in ferrets and NHPs were successfully produced at iBET. In order to achieved this, rational process optimisation had to be carried out at iBET (in collaboration with EDUFLUVAC internal and external partners) and included (i) the implementation of a modular strategy for pentavalent H3 VLP production, (ii) the design of improved VLP production and purification platforms, and (iii) the development of supporting analytical techniques for VLPs characterisation.

1. Implementation of a modular strategy for pentavalent H3 VLP production

The pentavalent H3 baculovirus vector could not be generated possibly due to genetic instability and/or homology between closely related HA sequences. To overcome this issue and thus ensure production of all VLPs for studies in animal models, a robust insect High Five cell-based platform was successfully developed for production of pentavalent H3 VLPs by combining stable and baculovirus-mediated expression (**Figure 1**, Modular Strategy). The feasibility of this modular approach was demonstrated by infecting insect High Five cells constitutively expressing two different HA proteins from subtype H3 (HA1 and HA2) with a baculovirus encoding M1 and three other HA proteins from subtype H3 (HA3, HA4 and HA5) to produce the pentavalent H3 VLP. Rational optimization of infection conditions was conducted by (i) replenishing culture medium with key nutrients before infection and (ii) manipulating the cell concentration at infection. This combined strategy allowed an improvement of up to 4-fold in HA protein concentration. Importantly, the HA production levels attained with this modular strategy were similar to those of a traditional co-infection scenario in which parental insect High Five cells are co-infected with two different baculoviruses (**Figure 1**, Co-infection Strategy). Results demonstrate the potential of the modular approach herein developed for efficient production of pentavalent H3 VLPs, and were published in Vaccine Journal (27).

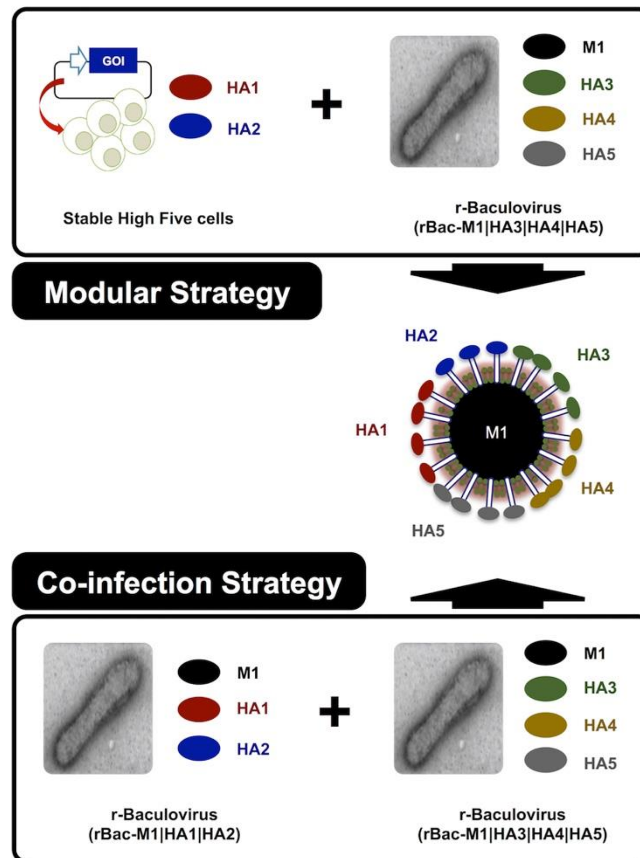

Figure 1. Combining stable and baculovirus-mediated expression for production of pentavalent H3 VLPs.

2. Design of improved VLP production and purification platforms

Upstream process development of influenza VLPs

A full factorial Design of Experiments (DoE) approach was used to evaluate the impact of three factors on VLPs production, namely cell concentration at infection (CCI), multiplicity of infection (MOI, number of virus particles per cell) and time of harvest (TOH) (**Figure 2**). Results obtained demonstrate that protein expression levels can be increased by 4-fold (compared to standard infection strategy) when insect High Five cells are infected at 2×10^6 cell/ml with 10 total virus particles/cell, and VLPs harvested at 48-72 hours post-infection (hpi), when cells viability is 50-80 %. This optimal infection strategy was used to produce all monovalent and multivalent VLPs for studies in animal models.

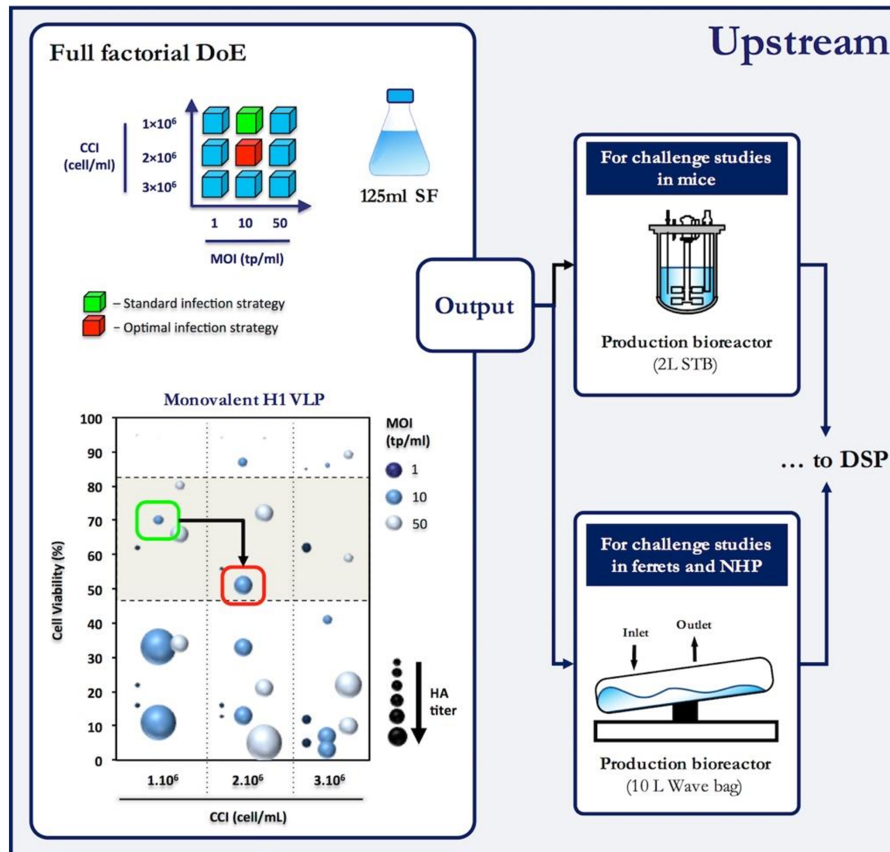

Figure 2. Full factorial DoE to identify best infection strategy for production of influenza VLPs.

Downstream process development of influenza VLPs

In order to increase product recovery yields during the purification process, iBET worked together with Merck Millipore (which joined the consortium in 2014 as an interested party) on the design of a purification platform for influenza VLPs which is scalable, "All-Filtration" and compliant with Good Manufacturing Practice (GMP) regulations. The resulting optimised scheme consists of a four-stage process: (i) a clarification step for cells and cell debris removal, (ii) two ultrafiltration/diafiltration steps for product concentration and impurities removal, and (iii) a final sterile filtration step before final formulation (**Figure 3**). Overall, this integrated "All-Filtration" purification platform improves efficiency, i.e. higher HA recovery as well as higher DNA, total protein and baculovirus removal, but also increases cost. Results were published in Journal of Chemical Technology and Biotechnology (28). Purification of monovalent and multivalent VLPs for challenge studies in ferrets and NHP was done using the aforementioned Downstream (DSP) scheme.

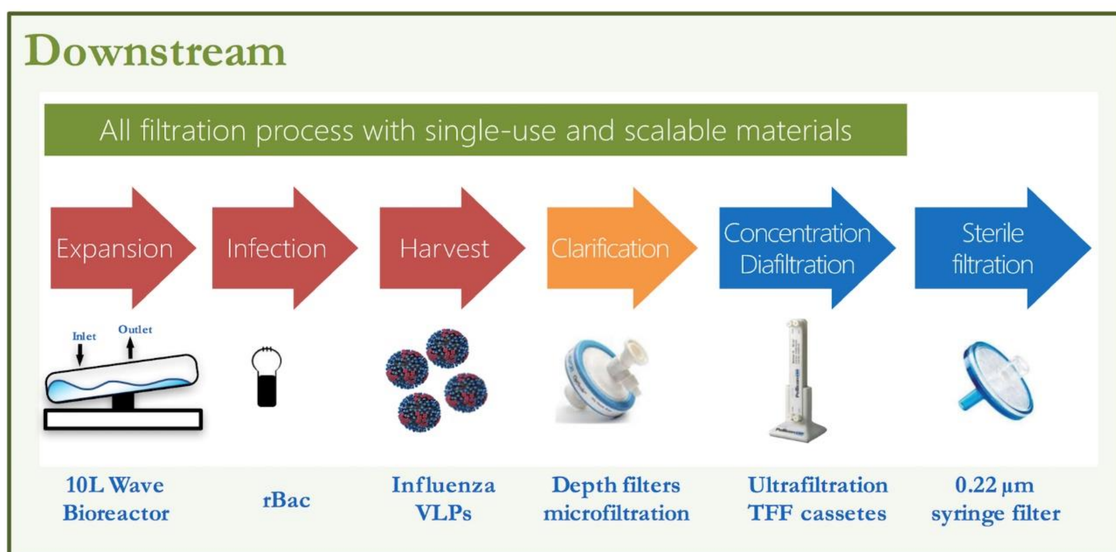

Figure 3. Integrated "All-Filtration" purification platform of influenza VLPs.

3. Development of supporting analytical techniques for VLPs characterization

The majority of the batch release assays defined in the analytical development plan of EDUFLUVAC project were successfully implemented at iBET (**Table 1**). These include (i) transmission electron microscopy to assess conformation and size of VLPs, (ii) Western blot to confirm the presence of HA, NA and M1 proteins in monovalent and multivalent VLPs, and (iii) single radial immunodiffusion to determine the concentration of immunologically active HAs, amongst others.

Table 1: List of analytical methods initially defined for the EDUFLUVAC project.

Method/Equipment	Description
Transmission electron microscopy *	Identity
Western blot	Identity
Single radial immunodiffusion *	Identity & Quantification/Potency
Capillary electrophoresis *,#	Quantification/Potency
SEC (as alternative to UPLC) *	Quantification/Potency
Nanoparticle tracking analysis	Particle size distribution
BCA assay *	Total Protein
AccuBlue assay *	Residual DNA
n.s. *,#	Residual Host Cell Proteins
MTT assay (as alternative to plaque assay)	Infectious baculovirus
ELISA	Antigen detection & quantification
Endosafe®-PTS™ quantitative LAL method *	Endotoxins
Sterility test using TSB and FTM *	Sterility
pH meter *	pH
PCR *	Mycoplasm (for viral seed lots)

** Methods identified in project proposal as critical/required; # not implemented; n.s. – not specified*

Additional methods to those initially defined in project proposal were used at iBET for at-line monitoring of VLPs during production and purification, and rational process optimisation in order to increase VLPs yield and purity (**Table 2**). These include (i) hemagglutination assay for quantitating the relative concentration of HA in VLPs, (ii) neuraminidase activity assay to determine the activity of immunologically active NAs, and (iii) mass spectrometry for identification of HA, NA and M1 proteins in bulk or purified samples, amongst others.

Table 2: List of additional methods implemented for the EDUFLUVAC project.

Method/Equipment	Description
Haemagglutination assay	HA quantification
^{1}H-NMR spectroscopy	Quantification of key nutrients
Protein deglycosylation	Assess the HA glycosylation pattern in VLPs
Neuraminidase activity assay	NA activity
Mass spectrometry	HA, NA and M1 protein identification
Real-time quantitative PCR	Total baculovirus quantification
Sucrose cushion	Concentration of VLPs
Sucrose gradient	Increase purity of final VLP samples
Bio-layer Interferometry	Influenza VLPs quantification
Click chemistry	On-line/at-line monitoring of Influenza VLPs
Isotope dilution mass spectrometry [#]	Quantification of HAs in multivalent influenza VLPs
Immunogold staining [##]	Co-localization of different HA (sub)types in a VLP

[#] *Under development (collaboration with NIBSC, UK);* [##] *under development (collaboration with IGC, Portugal)*

Noteworthy, two analytical techniques were developed within EDUFLUVAC's consortium: (i) a click chemistry approach for on-line/at-line monitoring of influenza VLPs (**Figure 4**), and (ii) a universal label-free in-process analytical tool for influenza VLPs quantification based on Biolayer interferometry technology applied on an octet platform (**Figure 5**). These methods have been published in Bioconjugate Chemistry (29) and in Biotechnology Journal (30), respectively.

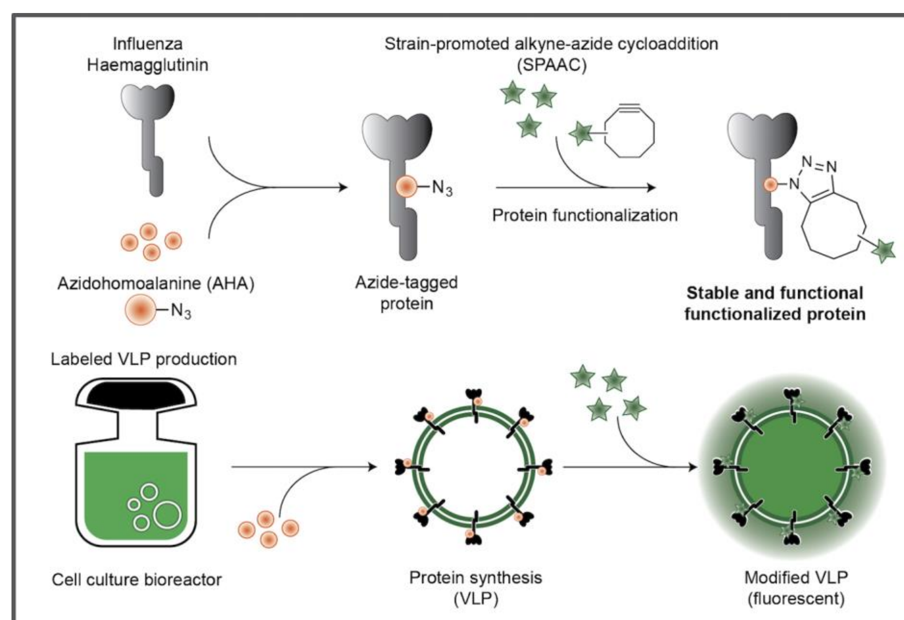

Figure 4. Click chemistry approach for on-line/at-line monitoring of influenza VLPs.

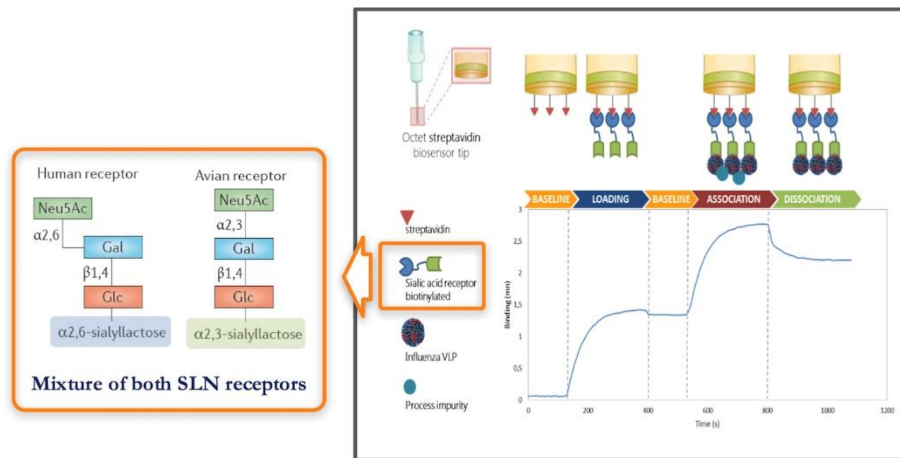

Figure 5. Universal label-free in-process analytical tool for influenza VLPs quantification based on Biolayer interferometry technology applied on an octet platform.

Mouse immunogenicity studies

The initial assessment of the EDUFLUVAC approach was performed in the mouse model. Monovalent and multivalent VLPs were evaluated in outbred (Swiss) mice following a vaccination schedule of three doses at four-weekly intervals. As the mice are influenza naïve, in contrast to the human target population, this vaccination schedule was chosen to allow the humoral immune response to mature. The total HA dose per vaccination was kept at 1.5 µg (1/10th of a human dose per (sub) type), and 0.5 µg for the NA, both irrespective of the VLPs' valence.

The main objective of these studies was to demonstrate that polyvalent VLPs induce an increased antibody breadth in the mouse model, yielding neutralising titres to later variants, not included in the vaccine composition. The main criterion for down selection of vaccine candidates was the breadth of VN assay titres. The VN assay is preferred over the HI assay because it is capable of detecting both anti head and stalk antibodies. Furthermore, competitive ELISA (cELISA) was performed to provide insight into the mechanisms and demonstrate the broadening of the antibody response.

Generation of reference mouse sera

Preliminary mouse studies focused on the generation of high titre reference sera from monovalent H1, H3, NA and B strain and from six Group 2 HAs VLPs to be used as reference standards for the enzyme-linked immunosorbent assays (ELISA) and for further characterisation using the virus Microneutralisation (MN) and the Haemagglutination Inhibition (HI) assays. In order to obtain sufficient quantities of mouse sera for all planned ELISA, high titre sera (i.e. >1:10000) had to be produced for each of the 20 selected monovalent HA VLPs (H1, H3, B and six Group 2 HAs) and three selected monovalent NA VLPs. For this purpose, groups of four outbred Swiss mice, seven-weeks old female, were immunised subcutaneously three times with 1.5 µg HA or 0.5 µg NA VLPs adjuvanted with Montanide ISA 51 (VG, Seppic, France) at four-week intervals.

Three bleeding points, at day 0 to confirm flu sero-negativity, at day 42 for intermediate evaluation and at day 70 for the final bleed were performed. Pooled sera from mice immunised with the five selected HA variants from each subtype (H1, H3, B) and with the three selected NA variants, were analysed for specific HA / NA IgG quantity using ELISA and ADAMSEL FPL, a free-software for non-commercial users. This application converts the Optical Density (OD) readings obtained

from ELISA plate readers into concentrations by four-parameter fitting. With this system, a value of 1 OD over background has been considered as 1 AU/mL.

To reduce potential cross-reactivity with impurities in ELISA that can arise if coating and immunising antigens are the same, antigens produced from a different expression system were needed to coat the ELISA plates. Although it was not initially foreseen in the proposal, egg-derived whole purified viruses for the five variants of each sub type were produced by Zydus International Ltd (that acquired ETNA Biotech in 2008) and were used as coating antigens for all planned ELISA assays.

Pilot mouse study

Exploratory experiments were conducted (without adjuvant) with all five HA variants per (sub)type (H1, H3, B and Group 2 HA) and with the three selected NA variants to define the vaccine dose/regimen and to verify, compare and establish kinetics as well as assay conditions. Groups of five outbred Swiss mice, six-week old female, were subcutaneously immunised with i) monovalent HA VLPs as well as with a mixture of all five variants per (sub) type (H1, H3, B and six group 2 HAs) (total HA dose per vaccination was kept at 1.5 μg) or ii) monovalent NA, as well as with a mixture of three N1 VLPs (at a 0.5 μg NA dose). Three bleeding points, at day 0 to confirm flu sero-negativity, at day 42 for intermediate evaluation and at day 70 for the final bleeding were performed. The individually collected sera (300 μl sera/mouse, shared between ETNA, BPRC and DH-MHRA) were used to fine-tune the ELISA assays and MN assay and tested for specific HA or NA IgG production.

Dose-range study of the VLPs

Although not initially planned, a dose-range experiment comparing 0.3 and 1.5 μg doses of VLPs from a single strain was performed in order to confirm that the lowest dosage is not in the saturation part of the dose response curve. Two groups of eight outbred Swiss mice, six-week old female, were immunised three times at four-week interval with A/Texas/36/91 (H1N1) strain at two different concentrations: 0.3 or 1.5 μg.

Mice immunised with the high dose had about twice the IgG levels (0.91 to 4.61) as mice immunised with the low dose (**Figure 6**). Due to the limited sample size, the 95% confidence interval is not significant ($p=0.07$) but close to. This is sufficient to show that equal titres one may achieve with a mix of monovalent VLPs and pentavalent VLPs for a specific subtype is not due to a saturating vaccine dose.

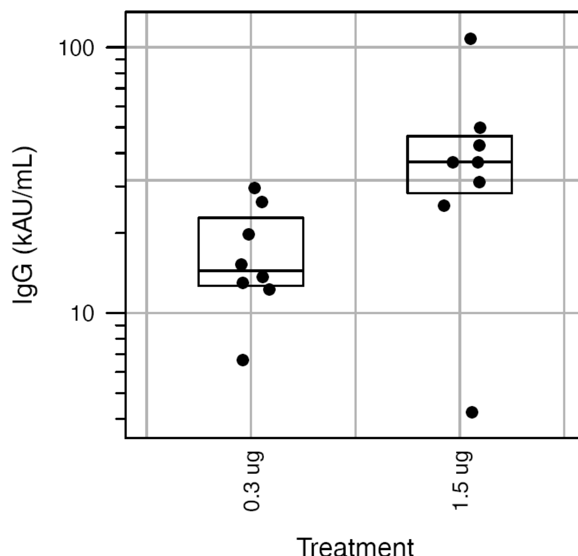

Figure 6: IgG titres for the dose-range study. *Mice were immunised with H1 VLP from the A/Texas/36/91 (H1N1) strain at two different concentrations: 0.3 or 1.5 µg*

Mouse vaccination study: seasonal influenza vaccine

The aims of this study were:

1. To determine whether immunisation with polyvalent VLPs expressing different HA antigens from the same subtype can induce broad antibody responses within that subtype.
2. To determine the minimum number of components required.
3. To establish whether multivalent VLPs of H1, H3 and B can be administered simultaneously without loss of immunogenicity and neutralising capacity.

Groups of eight outbred Swiss mice, six-week old female, were immunised with 1.5 µg HA of monovalent, trivalent and pentavalent VLPs for each sub type (H1, H3 and B) as well as with a mixture of monovalent H1, H3 and B subtypes (4.5 µg total HA/dose). For HA/NA competition studies, eight mice were immunised with 0.5 µg of polyvalent N1 VLP expressing three N1 variants on each VLP or combined with H1 polyvalent VLP (HA dose 1.5µg) expressing three H1 antigens. Blood samples for analysis were collected on day 70. The sera generated were individually collected (300 µl sera/mouse) and used for ELISA and MN assays.

The MN assay (performed at DH-MHRA) was used as the main decision making assay for down selection of vaccine candidates, measuring the ability of sera to neutralise both homologous and heterologous sera. cELISA (performed at BPRC) and HI assay are secondary evaluation assays to assess the breadth of the antibody response. A second criterion for the selection of the lead vaccine candidate(s) was the multivalency: it was agreed to go for the simplest formulation that gives broadening of the immune response.

Main conclusions: The results obtained clearly indicate that broadening of anti-influenza responses can occur and requires polyvalent VLPs with at least five components. For the H3 and B strains the response was broadened beyond the vaccine composition, i.e. strains isolated ~10 years after the last strain included in the vaccine were neutralised by sera from mice immunised with pentavalent VLPs. The H3N2 and B strain data show that at least five components on a

single VLP induce broadened responses. The trivalent formulations were reactive to the vaccine components, but responses were not broadened.

For H1N1 the data show that he mix of five as well as the pentavalent covered all vaccine components (i.e. neutralising and IgG titres were induced). None of the vaccination regimes, however, induced significant MN or IgG titres to the A/California/7/2009 (ACal09) antigen. This may be because the distance between the vaccine component and the ACal09 is too large with a difference of over 90 amino acids. This may suggest that more vaccine components are required to cover this large difference. Alternatively, it may be that the MN assay lacks sensitivity to detect ACal09 responses in mouse serum as previously observed with mini HA stem antigen constructs (Impagliazzo et al. 2015 Science 349(6254), 101-6).

Based on these data, the pentavalent VLPs showing the best broadening of the immune response were selected to proceed as vaccine candidates in the ferret (H1, H3) and NHP (H1) challenge studies.

Challenge studies in ferrets and NHPs

Proof of concept studies to evaluate whether immunisation with multivalent VLPs expressing HAs from divergent influenza strains of the same subtype can induce broad responses that afford protection against heterologous viral challenge were performed in ferrets and NHPs.

Prior to performing these efficacy studies, influenza virus infection models were set-up for H1N1pdm09 (A/California/04/2009) and H3N2 in ferrets and for H1N1pdm09 in macaques and characterised in regard to clinical, immunological and pathological criteria. These infection models allow for the appropriate evaluation of the H1 and H3 influenza virus vaccines developed by the consortium. To be able to demonstrate the concept of the vaccines, the selection of the challenge strains for the infection models was based on amino acid/antigenic difference from the H1 and H3 HAs present in the vaccines

Efficacy studies in the ferret model

Two vaccine efficacy and immunogenicity studies were carried out in ferrets. In one study vaccines containing pentavalent VLPs with five variants of H1 HA were compared with a monovalent VLP vaccine containing an H1 HA; in a second study pentavalent VLPs containing five variants of H3 HA were compared with a mixture of five monovalent H3 VLPs and additionally with a monovalent VLP containing an H3 HA. Vaccination with pentavalent H1 VLPs did not induce a broadening of the serological immune response measured by antibody activity against a pandemic H1N1 2009 strain and did also not lead to protection after challenge. In contrast, vaccination with a mixture of monovalent H3 VLPs and the pentavalent H3 VLPs induced a serological and cellular immune response against heterologous H3N2 strain and led to reduction in clinical severity and a lower virus load after challenge.

Efficacy studies in the NHP model

The main objectives of this study were to evaluate safety, immunogenicity and efficacy of the new influenza virus vaccines in NHPs. In more detail, we wanted to induce broadening of influenza virus specific immunity by including different HA antigens of 5 different seasonal H1N1 influenza virus strains in the vaccine (pentavalent HA) compared to monovalent HA, to induce protection against infection with pandemic H1N1 influenza virus A/California/04/2009.

Cynomolgus macaques (*Macaca fascicularis*) were chosen as the NHP model, since this species is more susceptible to H1N1 influenza virus infection and develops more fever upon infection than rhesus macaques (31).

Two groups of six cynomolgus macaques each were immunised four times (weeks 0, 6, 12, and 21) with either the monovalent VLP vaccine (expressing HA of influenza virus A/Brisbane/59/2007 (H1N1)) or with the pentavalent VLP vaccine. Four animals were mock vaccinated (NaCl), and served as controls. Four weeks after the final immunisation all animals were challenged with influenza virus A/California/04/2009 (H1N1) via a combined infection route; intra-bronchial (2ml) intranasal (0.5ml per nostril), conjunctival (0.1ml per eyelid) and oral (1ml) with a suspension containing 10^6 $TCID_{50}$/ml influenza virus, total amount of virus was $6.2x10^6$ $TCID_{50}$. B- and T-cell immune responses were measured after each immunisation and after challenge. Virus load was measured in nose, throat and lung after challenge

Main conclusions: Immunisation with a pentavalent HA VLP vaccine broadened the ELISA antibody response to all five different HA vaccine components as compared to the monovalent HA VLP vaccine, while responses to the challenge influenza virus strain A/Ca/04/09 were modest. HI and MN antibodies were induced, but only against the components included in the vaccine preparations and not against other seasonal H1N1 or pandemic H1N1 strains.

Immunisation with both mono- and pentavalent HA VLP vaccines induced modest T-cell responses (measured by IFN-γ ELISpot assay and Intracellular Cytokine Staining (ICS)), that were too low to determine epitope specificity.

Upon challenge with influenza virus A/Ca/04/09 (H1N1)pdm09, all animals became infected and vaccine-induced reduction of virus load in nose/throat/lung or decrease of clinical symptoms was not observed. The antibody, memory B-cell and T-cell responses were increased clearing the virus from nose and lung by day 14. However, there was no significant difference in the magnitude or specificity of the responses between the monovalent and pentavalent vaccine groups.

In conclusion, there is no indication that immunisation with pentavalent VLPs in comparison to monovalent HA VLPs leads to a broadening of the antibody or T-cell response to the heterologous challenge virus A/California/04/2009 (H1N1)pdm09.

Vaccination did not protect from infection with the heterologous challenge virus pH1N1 Ca/04/2007 and did not reduce virus load in nose/throat/lung or decrease of clinical symptoms. Since no protection was induced, the correlates of protection against infection could not be identified.

Dissemination activities & workshop organisation

The EDUFLUVAC partners, as part of their work programme on the development of a broadly reactive influenza vaccine, have organised a workshop series with the aim to bring together the relevant and current efforts on universal influenza vaccine development and address critical topics in the field.

The first workshop took place on 18-19 June 2015 at the National Institute for Biological Standards and Control (NIBSC), UK. It focused on the standardisation of immunoassays that can be used to assess broadly reactive or universal influenza vaccines and was co-organised and co-sponsored by the National Institutes of Health (NIH) - National Institute of Allergy and Infectious Diseases (NIAID), USA. The agenda encompassed a wide range of immunoassays that can be used to assess immune responses to broad (or 'universal') influenza vaccines, from classical serological assays to assays measuring cell mediated immunity. A manuscript summarising the outcome of the workshop was published in 2017 in the journal Influenza and Other Respiratory Viruses (32).

A second workshop on experimental animal models for preclinical evaluation of universal influenza vaccines was organised on 23-24 June 2016 at the Biomedical Primate Research Center. The

programme included a diversity of influenza models from well established, widely accepted models to cutting edge, newly developed animal models as well as *ex-vivo* approaches and human models. The workshop's audience concluded that animal models need to be chosen carefully, depending on the questions asked, and that no one animal model could be considered the best for all purposes.

The third and last workshop organised by the consortium took place in Brussels on 12-13 June 2017 and was entitled: "Four years of European research on the development of universal influenza vaccines: what have we learnt and how can we move forward?". The aim of this workshop was to bring together the five consortia that received funds by the EC FP7 programme in 2013 and to discuss what has been achieved, what can be saved/maintained after the projects come to an end and how to strengthen the European vaccine development landscape. This workshop has been a great opportunity for the five consortia to discuss the products and technologies developed, and to present the results of their research to stakeholders and several funding organisations.

The vaccine development pipeline in Europe is well populated with promising vaccine candidates from the preclinical stage all the way to comparative proof-of-concept clinical trials. Furthermore, the five EU consortia have developed successful technology platforms, to allow smooth process development and GMP production, and have created an efficient clinical development infrastructure.

The European influenza community agreed that building on the existing consortia and knowledge generated by creating a common portfolio management would greatly increase the chance of success to bring the products further into the pipeline of vaccine development. The community will have to work together to:

- Define the Preferred Product Characteristics for broadly reactive influenza vaccines
- Agree on the go-no go criteria for selecting, assessing and advancing vaccine candidates
- Propose a regulatory and clinical strategy
- Put in place harmonisation efforts (standards and assays) as well as comparative platforms

References

1. Carter DM et al. J Virol. (2012)
2. Wei CJ et al. Science 329(5995), 1060-1064 (2010)
3. Weaver EA et al. PLoS One 6(3), e18314 (2011)
4. Huber VC et al. Vaccine 27(8), 1192-1200 (2009).
5. Wang TT et al. PLoS.Pathog. 6(2), e1000796 (2010).
6. Prabakaran M et al. J Virol 84(22), 11822-11830 (2010).
7. Ekiert DC et al. Science 324(5924), 246-251 (2009).
8. Ekiert DC et al. Science 333(6044), 843-850 (2011).
9. Dreyfus C et al. Science 337(6100), 1343-1348 (2012).
10. Kusi KA et al. PLoS One 4(12), e8110 (2009).
11. Kusi KA et al. PLoS One 5(11), e15391 (2010).
12. Remarque EJ et al. Infection and Immunity 76(6), 2660-2670 (2008).
13. Remarque EJ et al. Trends Parasitol. 24(2), 74-84 (2008).
14. Duan J et al. Proc Natl Acad Sci U S A 105(22), 7857- 7862 (2008).
15. Kusi KA et al. PLoS One 5(11), e15391 (2010).
16. Kusi KA et al. Malar.J 10, 40 (2011).
17. Drew DR et al. PLoS One. 7(12), e51023 (2012).

18. Bachmann MF et al. Kaufmann SHE (Ed. Wiley-VCH Verlag, 415-431 (2004).
19. Russell SM, Liew FY. Nature 280(5718), 147-148 (1979).
20. Jennings GT et al. Biol Chem 389(5), 521-536 (2008).
21. Braun M et al. European journal of immunology 42(2), 330-340 (2012).
22. Song H et al. Vaccine 28(34), 5524-5532 (2010).
23. Grgacic EV, Anderson DA.. Methods. 40(1), 60-65 (2006).
24. Quan FS, Huang C, Compans RW, Kang SM. J Virol 81(7), 3514-3524 (2007)
25. Smith DJ et al. Science 305(5682), 371-376 (2004).
26. Huber VC et al. Vaccine 27(8), 1192-1200 (2009).
27. Sequeira et al. Vaccine, S0264-410X(17)30246-3 (2017)
28. Carvalho et al. J Chem Technol Biotechnol (accepted author manuscript) (2017)
29. Carvalho et al. Bioconjugate Chemistry (2016). DOI: 10.1021/acs.bioconjchem.6b00372
30. Carvalho et al. Biotechnology J (2017). DOI: 10.1002/biot.201700031
31. Mooij et al, 2015, PlosOne 2015 May 6;10(5):e0126132.
32. Pavlova et al. IORV Journal (2017) DOI: 10.1111/irv.12445

Potential impact of the project

Global health impact of a broadly reactive influenza vaccine. The insecurity caused by the unpredictable nature of the influenza virus that causes influenza epidemics and pandemics and subsequent significant mortality has led to the urgent need to develop broadly-protective influenza vaccines. A significant advance in human infectious diseases research would be the development of a new generation influenza vaccine that stimulates production of a robust, broadly neutralising antibody response not only to emerging drift variants of seasonal influenza viruses, but preferably also to different influenza A virus subtypes that regularly infect birds and mammals and may be the basis of future influenza pandemics.

The innovation brought by EDUFLUVAC lay in the development of a combinatorial immunisation strategy, which aims at "educating" the immune system to cross-recognise common epitopes within multiple influenza virus strains, and which is expected to confer protection against epidemic influenza. This new vaccine concept would help reduce the burden of disease that influenza imposes across Europe and other regions on an annual basis. The development of a vaccine that elicits broad long-lasting defence would allow considerably longer intervals between re-vaccination than at present, avoid vaccine failures due to drifted seasonal variants, and provide protection against pandemic viruses. Furthermore, it would facilitate vaccination campaigns in low and middle-income countries and thereby also confer protection against influenza in hitherto untargeted groups with limited health care programmes.

Importantly, the knowledge gathered from manufacturing (i.e. production and purification) and characterising (i.e. quality control) over 30 different influenza VLPs within EDUFLUVAC project can provide the basis to develop a production platform that can easily be transferred into pilot- and full-scale cGMP clinical manufacturing, and ultimately capable of, in the event of a pandemic, manufacturing high quantities of vaccine doses.

As described in 'Section A' of this document, the results of EDUFLUVAC have been disseminated at conferences, as poster and oral communications, and in form of scientific peer-reviewed publications. Additional publications (e.g. present the latest pre-clinical data) are in preparation. A patent application has also been submitted to the Patent Office of Luxembourg to cover the algorithm employed to select the HA and NA vaccine strains based on the Epitope Dilution Phenomenon.

The workshop series organised by the project's partners were a great opportunity to bring together experts from the influenza community and to reach out to the other EC-funded consortia developing broadly reactive influenza vaccines trying to identify common interests and synergies. In particular, one of the major conclusions of the last EDUFLUVAC workshop was that the European influenza community should profit from the expertise available (in terms of formulation and delivery systems, immunological assays, animal models and process development) and build upon the latest achievements to speed up the development of the next generation of influenza vaccines. The establishment of a collaborative platform in Europe with a portfolio management approach would allow a head-to-head comparison to select the best vaccine candidates.

European and International added value and impact on SMEs. The EDUFLUVAC consortium included seven research groups operating in four public and three private organisations, including two Research Intensive SMEs (iBET and Redbiotec) with unique and complementary technological expertise in vaccine development. The research conducted within the EDUFLUVAC project has led to enhancement of already established networks of collaboration within the European and Global Vaccine development field (e.g. iBET-Redbiotec-EVI) as well as the creation of new collaborations (i) between European Institutions (e.g. iBET-Instituto Gulbenkian de Ciência (Portugal), iBET-Instituto de Medicina Molecular (Portugal), iBET-Max Planck Institut Magdeburg (Germany)) and (ii) between European Institutions and International Biopharmaceutical companies (e.g. iBET-Merck Millipore, iBET-Pall Life Sciences). The work developed under these collaborations has led to improved manufacturing processes (i.e. production and purification of VLPs using the insect cells-baculovirus expression system) and innovative quality control methods (i.e. characterisation of multivalent VLPs). The knowledge and tools gathered in EDUFLUVAC will allow IBET to (i) improve its competitiveness and attractiveness as contract manufacturing or research organisation, and (ii) target new prophylactic and/or therapeutic vaccines against other viral and even non-viral diseases with significant impact on Human Health. Besides the operational and technical advantage brought to iBET, EDUFLUVAC project was also capable of indirectly generating new jobs, either via successful admission of students to Portuguese PhD Programs (e.g. MIT-Portugal, MolBioS) or via graduation of MSc students that are now in renowned international companies (e.g. Lonza, Novasep).

Project progress and major achievements were published annually in the EVI annual report for donors and stakeholders thus giving visibility of EDUFLUVAC to other potential donors and funding agencies.

Please find further information about the EDUFLUVAC project at www.edufluvac.eu

EDUFLUVAC Project Management Team
(edufluvacinfo@euvaccine.eu)
European Vaccine Initiative, UniversitätsKlinikum Heidelberg,
Voßstraße 2, Geb. 4040, 69115 Heidelberg, Germany

Use and dissemination of foreground

Section A

TEMPLATE A1: LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS; STARTING WITH THE MOST IMPORTANT ONES										
NO.	Title	Main author	Title of periodical series	Number, date or frequency	Publisher	Place of Publication	Year of Publication	Relevant pages	Permanent identifiers[1] (if available)	Is/Will open access[2] provided to this publication?
1.	Universal label-free in-process quantification of influenza virus-like particles. DOI: 10.1002/biot.201700031	Carvalho SB.; Alves PM	Biotechnology Journal				2017			YES
2.	Workshop report: Immunoassay standardisation for "universal" influenza vaccines. DOI: 10.1111/irv.12445	Pavlova S.; Engelhardt OG	Influenza and other Respiratory Viruse				2017			YES
3.	Combining stable insect cell lines with baculovirus-mediated expression for multi-HA influenza VLP production. DOI: 10.1016/j.vaccine.2017.02.043	Sequeira DP.; Alves PM	Vaccine				2017			YES
4.	Bioorthogonal Strategy for Bioprocessing of Specific-Site-Functionalized Enveloped Influenza-Virus-Like Particles.	Carvalho SB.; Alves PM	Bioconjugate Chem				2016			YES

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

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

	DOI: 10.1021/acs.bioconjchem.6b00372									
5.	Influenza Vaccine Research funded by the European Commission FP7-Health-2013-Innovation-1 project. DOI: 10.1016/j.vaccine.2016.10.040	Liu H.; Hak E.	Vaccine				2016			
6.	Purification of influenza virus-like particles using sulfated cellulose membrane adsorbers. DOI:10.1002/jctb.5474	Carvalho SB.; Alves PM	J Chem Technol Biotechnol.				2017			YES

TEMPLATE A2: LIST OF DISSEMINATION ACTIVITIES								
NO.	Type of activities	Main leader	Title	Date/Period	Place	Type of audience	Size of audience	Countries addressed
1.	Website/applications	EVI	EDUFLUVAC - Educate the immune system to recognise common influenza epitope	19 November 2013	www.edufluvac.eu	Scientific community - Industry - Civil society - Policy makers - Medias		International
2.	Flyers	EVI	EDUFLUVAC - Educate the immune system to recognise common influenza epitope	19 November 2013	Distributed at conference & meetings	Scientific community Industry - Policy makers		International
3.	Press release	EVI	EDUFLUVAC: Universal flu vaccine based of diversity covering approach	20 November 2013	Cordis / Marketwired/EDUFLUVAC and partners websites	Scientific community - Industry - Civil society - Policy makers - Medias		International
4	Oral presentation at EVI Rendez-Vous 2013	BPRC	EDUFLUVAC: Universal flu vaccine based of diversity covering approach	4 December 2013	Heidelberg, Germany	Scientific community	100	International
5.	Articles published in the popular press	BPRC	Searching for a universal flu vaccine	17 February 2014	Horizon	Scientific community - Industry - Civil society - Policy		International

						makers - Medias		
6.	Oral presentation at the 16th European Congress on Biotechnology	iBET	Insect cell technology as a vaccine producing platform	13–16 July 2014	Edinburgh, Scotland	Scientific community	1400	International
7.	Oral presentation at the EVI Rendez-Vous 2014	iBET	Universal flu vaccine based on diversity covering approach: antigen selection and production	3 December 2014	Paris, France	Scientific community	100	International
8.	Oral presentation at the brainstorming meeting with other EC-funded consortia on universal flu vaccine organised by the UNISEC consortium	BPRC	Introduction and overview of the EDUFLUVAC project	6 March 2015.	Copenhagen, Denmark	Scientific community	30	International
9.	Oral presentation at the Modern Vaccines Adjuvants & Delivery Systems meeting	BPRC	EDUFLUVAC: Universal flu vaccine based of diversity covering approach	18-20 May 2015	Leiden, The Netherland	Scientific community	200	International
10.	Organisation of workshops	DH-MHRA	Workshop on immunoassay standardisation for universal flu vaccines	18 June 2015	NIBSC, UK	Scientific community	60	International
11.	Oral presentation at the 5th Influenza Vaccines for the World conference	BPRC	Application of lessons learnt from a polymorphic malaria vaccine antigen to influenza: the EDUFLUVAC EC-FP7 Project	06-09 October 2015	Albufeira, Portugal	Scientific community	100	International
12.	Poster at the 5th Influenza Vaccine for the World conference	iBET	A click chemistry approach to monitor and improve influenza VLPs downstream processing	06-09 October 2015	Albufeira, Portugal	Scientific community	100	International
13	Poster at the 5th Influenza Vaccine for the World conference	iBET	Combining stable insect cell lines with baculovirus-mediated expression for production of multi-HA influenza VLPs	06-09 October 2015	Albufeira, Portugal	Scientific community	100	International
14	Poster at the 21st Biennial Meeting of the International Society for Molecular Recognition	iBET	A click chemistry approach to monitor and improve influenza VLPs downstream processing	27 September - 01 October 2015	Puerto Vallarta, Mexico	Scientific community	100	International

15	Oral presentation at the 21st Biennial Meeting of the International Society for Molecular Recognition	iBET	Universal label-free in-process analytical tool for influenza VLPs quantification	27 September - 01 October 2015	Puerto Vallarta, Mexico	Scientific community	100	International
16	Oral presentation at the EVI Rendez-Vous	BPRC	Diversity Covering approach for universal influenza vaccines: the EDUFLUVAC EC-FP7 Project	09 December 2015	Paris, France	Scientific community	100	International
17	Oral presentation at the EVI Rendez-Vous	DH-MHRA	Harmonisation of immunoassays for universal influenza vaccines	09 December 2015	Paris, France	Scientific community	100	International
18	Oral presentation at the ISBioTech 6th Spring Meeting	iBET	Improving and Monitoring an Influenza VLP Downstream Process using a Click Chemistry Strategy	07-09 March 2016	Washington, USA	Scientific community	150	International
19	Poster at the Vaccine Technology VI meeting	iBET	Combining stable insect cell lines with baculovirus-mediated expression for production of multi-HA influenza VLPs	12-17 June 2016	Albufeira, Portugal	Scientific community	200	International
20	Oral presentation at the Vaccine Technology VI meeting	iBET	Insect cells platform for fast production of Pseudo-Typed VLPs for drug and vaccine development	12-17 June 2016	Albufeira, Portugal	Scientific community	200	International
21	Poster at the Vaccine Technology VI meeting	iBET	A click chemistry strategy to specifically monitor and improve purification of influenza virus-like particles	12-17 June 2016	Albufeira, Portugal	Scientific community	200	International
22	Oral presentation at the Vaccine Technology VI meeting	iBET	Universal and in-process analytical tool for influenza quantification using a label-free technology	12-17 June 2016	Albufeira, Portugal	Scientific community	200	International
23	Oral presentation at the Virus-Like Particle & Nano-Particle Vaccines meeting	iBET	Combining stable and baculovirus-mediated expression towards production of an universal influenza VLP-based vaccine	22-24 June 2016	Leiden, The Netherlands	Scientific community	200	International

24	Oral presentation at the Virus-Like Particle & Nano-Particle Vaccines meeting	iBET	Universal label-free in-process analytical tool for Influenza virus-like particles quantification	22-24 June 2016	Leiden, The Netherlands	Scientific community	200	International
25	Organisation of workshops	DH-MHRA	Experimental animal models for universal influenza vaccines	23 June 2016	BPRC, The Netherlands	Scientific community	60	International
26	Poster presentation at the PREP 2016 meeting	iBET	Process Design Strategy to Monitor and Improve Purification of Influenza Virus-like Particles using Click Chemistry	17-20 July 2016	Philadelphia, USA	Scientific community	300	International
27	Oral presentation at the Eighth WHO meeting on development of influenza vaccines that induce broadly protective and long-lasting immune responses	DH-MHRA	Immunological evaluation of next-generation influenza vaccines	23 – 24 August 2016	Chicago, USA	Scientific community, policy makers	50	International
28	Oral presentation at the 10th Vaccine Congress	iBET	Universal influenza VLP-based vaccine: What can we learn from producing over thirty different VLPs	17-20 September 2016	Amsterdam, The Netherlands	Scientific community	300	International
29	Oral presentation at the PALL FortéBio User Meeting	iBET	Universal and in-process influenza virus-like particles quantification tool using Octet technology	29 September 2016	Lyon, France	Scientific community	50	International
30	Oral presentation at the meeting in Luxembourg with the other EC-funded consortia on universal flu vaccine and representatives of DG-Health	EVI	Overview of the EDUFLUVAC project	07 October 2016	Luxembourg	Scientific community, policy makers	10	International
31	Poster at the SPICA 16th meeting	iBET	Process design strategy to monitor and improve purification of influenza virus-like particles using click chemistry	09-12 October 2016	Vienna, Austria	Scientific community	250	International

32	Poster at the SPICA 16th meeting	iBET	In-process label-free analytical tool for influenza quantification	09-12 October 2016	Vienna, Austria	Scientific community	250	International
33	Oral presentation at the World Vaccine Congress Europe	iBET	Lessons learnt on producing and purifying over thirty different VLPs for a universal influenza vaccine	10-12 October 2016	Barcelona, Spain	Scientific community	400	International
34	Oral presentation at the ISPPP 2016 meeting	iBET	Monitoring and improving influenza Virus-like particles downstream processing using a click chemistry approach	06-09 November 2016	Salzburg, Austria	Scientific community	350	International
35	Oral presentation at the EVI Rendez-Vous 2016	BPRC	Diversity Covering approach for universal influenza vaccines: the EDUFLUVAC EC-FP7 Project	14 December 2016	Paris, France	Scientific community	50	International
36	Oral presentation at the Influenza Vaccine for the World Conference	iBET	Challenges in influenza virus-like particles production: New analytical tools and downstream process optimization	19-21 April 2017	Lausanne, Switzerland	Scientific community	100	International
37	Poster at the 25th ESACT meeting	iBET	A modular strategy for multi-HA influenza VLPs production: combining stable and baculovirus-mediated expression in insect cells	May 14-17 2017	Lausanne, Switzerland	Scientific community	1000	International
38	Poster at the 25th ESACT meeting	iBET	Adaptive Evolutionary Engineering of Insect Cells for Improved Influenza HA VLPs Production	May 14-17 2017	Lausanne, Switzerland	Scientific community	1000	International
39	Poster at the Single-use Technologies II: Bridging Polymer Science to Biotechnology Applications meeting	iBET	Production and purification of influenza virus-like particles using single-use technologies	May 07-10 2017	Tomar, Portugal	Scientific community	250	International
40	Organisation of workshops	DH-MHRA	Four years of European research on the development of universal influenza vaccines: What have	12 June 2017	Brussels, Belgium	Scientific Community, Policy makers.	50	International

			we learnt, and how can we move forward?					
41	Oral presentations at the third EDUFLUVAC workshop "Four years of European research on the development of universal influenza vaccines: what have we learnt and how can we move forward?"	EVI	Introduction and overview of the EDUFLUVAC project	12-13 June 2017	Brussels, Belgium	Scientific community	50	International
42	Oral presentations at the third EDUFLUVAC workshop "Four years of European research on the development of universal influenza vaccines: what have we learnt and how can we move forward?"	BPRC	Polyvalent VLP: expressing multiple HA's on a single particle	12-13 June 2017	Brussels, Belgium	Scientific community	50	International
43	Oral presentations at the third EDUFLUVAC workshop "Four years of European research on the development of universal influenza vaccines: what have we learnt and how can we move forward?"	iBET	Lessons learnt on producing and purifying over thirty different VLPs for a "universal" influenza vaccine	12-13 June 2017	Brussels, Belgium	Scientific community	50	International
44	Oral presentations at the third EDUFLUVAC workshop "Four years of European research on the development of universal influenza vaccines: what have we learnt and how can we move forward?"	BPRC	Animal models in influenza vaccine evaluation	12-13 June 2017	Brussels, Belgium	Scientific community	50	International
45	Oral presentation at the workshop organised by the European Commission on the development of broadly reactive universal flu vaccines	EVI	Introduction and overview of the EDUFLUVAC project	14 June 2017	Brussels, Belgium	Scientific community, Policy makers	50	International

46	Poster at the PREP 2017 meeting	iBET	Improving Downstream Processing of Influenza Virus-like Particles using Multi-column Chromatography	July 16-19 2017	Philadelphia, USA	Scientific community	300	International
47	Oral presentation at the Affinity 2017 meeting	iBET	Improving purification of influenza virus-like particles using a pseudo-affinity strategy	June 26-29 2017	Paris, France	Scientific community	200	International
48	Oral presentation at the ISPP 2017 meeting	iBET	Novel Pseudo-affinity Strategy for the Purification of Influenza Virus-like Particles	July 19-21 2017	Philadelphia, USA	Scientific community	150	International
49	Oral presentation at the Biochemical and Molecular Engineering XX meeting	iBET	Bioprocess Engineering of Insect Cells for Accelerating Vaccines Development	July 16-20 2017	Newport Beach, CA, USA	Scientific community	250	International
50	Oral presentation at ICB2017	iBET	Multi-column chromatographic purification of influenza virus-like particles	September 17-21 2017	Cascais, Portugal	Scientific community	200	International
51	Oral presentation at 9th Annual PEGS Europe Summit	iBET	Bioprocess engineering of insect cells for accelerating vaccines development	November 16 2017	Lisbon, Portugal	Scientific community	300	International

Section B (Confidential or public: confidential information to be marked clearly)

Part B1

TEMPLATE B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.					
Type of IP rights	Confidential Yes/No	Foreseen embargo date dd/mm/yyyy	Application reference(s) (e.g. EP123456)	Subject or title of application	Applicant(s) as on the application
Patent Application No. 93267 in Luxembourg	Yes	Priority deadline 19.10.2017	93267	Influenza virus vaccine	Redbiotec AG ETNA S.r.l. EVI BPRC WBVR iBET
International Patent Application No. PCT/EP2017/076705	Yes	19.04.2017 - 30 months of the earliest priority date (19.10.2016)	PCT/EP2017/076705	Influenza virus vaccine	Redbiotec AG ETNA S.r.l. EVI BPRC WBVR iBET

Part B2

Type of Exploitable Foreground	**Description of Exploitable Foreground**	**Confidential Yes/No**	**Foreseen embargo date dd/mm/yyyy**	**Exploitable product(s) or measure(s)**	**Sector(s) of application**	**Timetable, commercial or any other use**	**Patents or other IPR exploitation (licenses)**	**Owner & Other Beneficiary(s) involved**
Patent application	Antigen selection for a broadly	Yes	19/10/2016	Algorithm to select the antigen strains	Vaccine development	2-5 years	Patent application	Redbiotec AG ETNA S.r.l. EVI

	reactive influenza vaccine						filed in October 2016	BPRC WBVR iBET
International Patent Application	Antigen selection for a broadly reactive influenza vaccine	Yes	19/10/2017	Algorithm to select the antigen strains	Vaccine development	2-5 years	Patent application filed in October 2017	Redbiotec AG ETNA S.r.l. EVI BPRC WBVR iBET

Antigen selection for a broadly reactive influenza vaccine.
A patent claim has been filed at the Patent Office in Luxembourg which include the algorithm used to select the HA and NA vaccine strains developed by the EDUFLUVAC consortium and the preliminary pre-clinical data obtained in mice. The selection of the antigen strains is based on the Epitope Dilution Phenomenon which has already been demonstrated in the malaria vaccine field by the vaccine inventor, Edmond Remarque (BPRC). Further research is needed to optimise the antigen selection and the distance between the vaccine components in order to be able to show protection.

4.3 Report on social implications

A General Information	
Grant agreement number:	**602640**
Title of Project:	**EDUFLUVAC**
Name and Title of Coordinator:	**Dr Odile Leroy**

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

• Did the project involve the use of local resources (genetic, animal, plant etc)?	**Yes**
• Was the project of benefit to the local community (capacity building, access to healthcare, education ect)?	**No**
DUAL USE	
• Research having direct military use	**No**
• Research having the potential for terrorist abuse	**No**

C Workforce Statistics

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

Type of position	Number of Women	Number of Men
Scientific Coordinator	1	
Work package leaders	2	3
Experienced researchers (i.e. PhD holders)	12	11
PhD Students	3	3
Other	12	13

4. How many additional researchers (in companies and universities) were recruited specifically for this project?	5
Of which, indicate the number of men:	2

D Gender Aspects

5. Did you carry out specific Gender Equality Actions under the project?

☑ Yes
☐ No

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

Action	Not at all effective				Very effective
☑ Design and implement an equal opportunity policy	☐	☐	☐	☑	☐
☐ Set targets to achieve a gender balance in the workforce	☐	☐	☐	☐	☐
☐ Organise conferences and workshops on gender	☐	☐	☐	☐	☐
☑ Actions to improve work-life balance	☐	☐	☐	☑	☐

○ Other:

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

○ Yes- please specify

x No

E Synergies with Science Education

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

- ○ Yes- please specify
- x No

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

- ○ Yes- please specify
- x No

F Interdisciplinarity

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

- x Main discipline[3]: 1.5 and 3.1
- ○ Associated discipline: ○

G Engaging with Civil society and policy makers

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

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

- ○ No
- ○ Yes- in determining what research should be performed
- ○ Yes - in implementing the research
- ○ Yes, in communicating /disseminating / using the results of the project

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

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

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

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

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

13b If Yes, in which fields?

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

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

13c If Yes, at which level?

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

H Use and dissemination

14. How many Articles were published/accepted for publication in peer-reviewed journals?	6
To how many of these is open access[4] provided?	5
How many of these are published in open access journals?	5
How many of these are published in open repositories?	
To how many of these is open access not provided?	1
Please check all applicable reasons for not providing open access:	
☐ publisher's licensing agreement would not permit publishing in a repository ☐ no suitable repository available ☐ no suitable open access journal available ☐ no funds available to publish in an open access journal ☐ lack of time and resources ☐ lack of information on open access ☒ other[5]: ……………	
15. How many new patent applications ('priority filings') have been made? *("Technologically unique": multiple applications for the same invention in different jurisdictions should be counted as just one application of grant).*	1

16. Indicate how many of the following Intellectual Property Rights were applied for (give number in each box).	Trademark	
	Registered design	
	Other	

[4] Open Access is defined as free of charge for anyone via Internet

[5] For instance: classification for security projects

17. How many spin-off companies were created / are planned as a direct result of the project?	0
Indicate the approximate number of additional jobs in these companies:	

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

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

19. For your project partnership please estimate the employment effect resulting directly from your participation in Full Time Equivalent (*FTE = one person working fulltime for a year*) jobs:	*Indicate figure:*
Difficult to estimate / not possible to quantify	☒

I Media and Communication to the general public

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

○ Yes x No

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

○ Yes x No

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

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

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

☐	Language of the coordinator	☒	English
☐	Other language(s)		

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

FIELDS OF SCIENCE AND TECHNOLOGY

1. NATURAL SCIENCES

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

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

1.3 Chemical sciences (chemistry, other allied subjects)

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

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

2. ENGINEERING AND TECHNOLOGY

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

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

2.3. Other engineering sciences (such as chemical, aeronautical and space, mechanical, metallurgical and materials engineering, and their specialised subdivisions; forest products; applied sciences such as geodesy, industrial chemistry, etc.; the science and technology of food production; specialised technologies of interdisciplinary fields, e.g. systems analysis, metallurgy, mining, textile technology and other applied subjects)

3. MEDICAL SCIENCES

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

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

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

4. AGRICULTURAL SCIENCES

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

4.2 Veterinary medicine

5. SOCIAL SCIENCES

5.1 Psychology

5.2 Economics

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

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

6. HUMANITIES

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

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