

IMI1 Final Project Report Public Summary

Project Acronym: FLUCOP

Project Title: Standardization and development of assays for assessment of influenza vaccine correlates of protection

Grant Agreement: 115672

Project Duration: 01/03/2015 – 28/02/2022

1. Executive summary

1.1 Project rationale and overall objectives of the project

Vaccination remains the most effective method to control Influenza seasonal infections and the most important strategy to prepare for a possible pandemic. FLUCOP aim was to provide the seasonal influenza vaccine community with improved or new tools to evaluate the vaccine's ability to raise an immune response in humans and pave the way for establishing novel correlates of protection. The project included consultation with partners from regulatory agencies to ensure that the regulatory aspects of the standardization efforts are addressed and duly considered.

These goals have been achieved by targeting three practical objectives:

- Standardization of Hemagglutination Inhibition (HAI) and Virus Neutralization (VN) assays (WP1), as primary goal
- Advancing the understanding and application of Cell-Mediated Immunity (CMI, WP2) and Neuraminidase assays (WP3) as tools for evaluating influenza vaccine performance, as secondary goal
- Exploration of new technologies that could be applied to investigate Correlates of Protection (CoP) and population-based evaluations of influenza vaccines (WP4), as an exploratory goal.

1.2 Overall deliverables of the project

The overall deliverables of the project are the following:

- Harmonized protocols for the most common tests used in the private and public sector, when evaluating influenza vaccines in humans namely: Hemagglutination Inhibition (HAI) and Virus Neutralization (VN) assays.
- Consensus on the most effective methodology to measure neuraminidase inhibiting (NI) antibodies and a robust assay methodology that can be applied successfully to future clinical trials with seasonal influenza vaccines
- Consensus on the most effective methodology to measure cell-mediated responses in humans against influenza vaccines antibodies and a standardized assay(s) protocol(s) that can be deployed to future clinical trials with seasonal influenza vaccines
- Development of additional laboratory or mathematical tools that can be applied to the evaluation seasonal influenza vaccines in humans, beyond state of the art.

1.3 Summary of progress versus plan since last period

During the seventh reporting period the project work tasks have progressed on schedule and objectives have been made in a timely manner. Progress was made in all WPs with the consortium getting to the Standardization/ Harmonization of the various antibody- and cell-based assays through several collaborative studies.

Most notably, in WP1 "Standardisation HAI & VN assays" was designed a large study to assess selected parameters for their contribution to the hemagglutination inhibition (HAI) assay variability and was developed a standard protocol to promote consistent HAI testing methods across laboratories. The use of this protocol and common reagents resulted in lower levels of variability in results between participating laboratories than achieved using in-house HAI testing. Human sera sourced from vaccination campaigns over several years, and thus including antibody to different influenza vaccine strains, served as effective assay standards. Based on our findings, we recommend the use of a common protocol and/or human serum standards, if available, for testing human sera for the presence of antibodies against seasonal influenza using

turkey red blood cells. Results of the HAI pilot studies were published with the acknowledgment to the FLUCOP project in the manuscript: “Assay Harmonization and Use of Biological Standards to Improve the Reproducibility of the Hemagglutination Inhibition Assay: a FLUCOP Collaborative Study”. mSphere. 2021 ;6(4) DOI: [10.1128/mSphere.00567-21](https://doi.org/10.1128/mSphere.00567-21).

Additionally, an external quality assessment (EQA) to further assess the variability of HAI and VN methods across a wider range of laboratories including both FLUCOP and non-FLUCOP partners was conducted. For that purpose, participating laboratories were asked to test a serum panel of 30 samples with four seasonal influenza viruses. Data were collected from 13 laboratories for HAI and from 7 laboratories for VN.

In WP2 “Advancing the understanding and application of CMI”, two proficiency tests have been completed. Samples were analyzed by 5 partners by applying the harmonized IFN- γ ELISpot and by 7 partners applying the harmonized ICS protocol. Data have been collected, analyzed and reported.

In WP3 “Assays to detect NA-specific antibody responses for evaluating influenza vaccines Immunogenicity”, additional experiments to analyze the ELLA consensus protocol against other human influenza virus neuraminidases such as B neuraminidases have been conducted. These results were collected, analyzed, and compiled with the previous results obtained with N1 neuraminidase into a manuscript.

In WP4 “Consideration of new technologies yet to be applied to population-based evaluations of influenza vaccines”, A no-cost extension of program into Year 7, has allowed the partners to make arrangements for extended collaborative studies, validation and proficiency testing activities, as well as for dissemination of results through a number of scientific publications that are already in preparation.

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1.4 Significant achievements since last report

The main achievements of this year are:

WP1: Standardization of HAI and VN

A standard protocol to promote consistent HAI testing methods across laboratories was developed and results were published in mSphere. 2021. 25;6(4) DOI: [10.1128/mSphere.00567-21](https://doi.org/10.1128/mSphere.00567-21)). For both HAI and VN methods, an external quality assessment (EQA) was performed to further assess the variability of HAI and VN methods across a wider range of laboratories including both FLUCOP and non-FLUCOP partners. For that purpose, participating laboratories were asked to test a serum panel of 30 samples with four seasonal influenza viruses. Data were collected from 13 laboratories for HAI and from 7 laboratories for VN. Results obtained are in agreement with previous FLUCOP VN and HAI studies, i.e. they showed that the use of standards (calibrators, reference reagents) is beneficial and reduced variation between laboratories consistently across assays (HAI and VN) and between virus types and subtypes. However, combining standard(s) with harmonized protocols had no added benefit.

WP2: Advancing the understanding and application of CMI

The majority of the partners participating in the IFN- γ ELISpot and/or ICS proficiency tests performed the testing in 2021. Data was collected throughout the year and analyses have been initiated in Q4 2021. Data have been presented at the final FLUCOP Annual Meeting held on-line on 1 and 2 February 2022.

WP3: Assays to detect NA-specific antibody responses for evaluating influenza vaccine immunogenicity

Following the scientific advisory board recommendations, the ELLA consensus protocol was adapted to ensure analyze of other human Influenza virus neuraminidase subtypes, such as B neuraminidase. For this purpose, B/Victoria and B/Yamagata NA mismatched viruses were designed and shared along with a common serum panel to Flucop partners.

Additional experiments were performed to test an additional source of antigen such as recombinant B neuraminidases as well as the specificity of the consensus protocol.

WP3 successfully achieved all tests and combined all data obtained over the last 7 years (including the validation data and use of a calibrator) into a manuscript.

The final document was reviewed by FLUCOP members and was submitted to *Frontiers in Immunology* as part of a special issue on the theme of “Development and standardization of assays to assess immunogenicity and correlates of protection of vaccines against respiratory viral infections”.

WP4: Consideration of new technologies yet to be applied to population-based evaluations of influenza vaccines

In WP4, there were no activities in this reporting period. All WP4 activities were successfully completed in previous years and the related Deliverables and Milestones submitted to IMI, as indicated in the table "Summary of Progress".

WP5: Project Management, Impact Assessment, Dissemination and Ethics

The Scientific and Administrative Management of the project has been successfully conducted in Year 7. Efforts focused on mitigating the effect of the COVID pandemic on the work plan and in reorganizing the activities of the Consortium to ensure final objectives are fully met despite the significant impact. The FLUCOP Scientific and Administrative report related to Year 6 was prepared and submitted in due time to the EC project officer. The seventh Annual Meeting was successfully organized in February 2022 and an open Conference to broadly disseminate the public results of the FLUCOP Project has been organized in February 2022. Dissemination activities have been conducted: the FLUCOP website was continuously updated, seven newsletters have been published and several manuscripts published.

1.5 Scientific and technical results/foregrounds of the project

During the life of the Project relevant scientific and technical results have been achieved for each Work Package.

A standard protocol to promote consistent HAI testing methods across laboratories was developed and published (DOI: 10.1128/mSphere.00567-21).

Human pre- and postvaccination sera from individuals ($n = 30$) vaccinated against influenza were tested across six laboratories. We used a design of experiment (DOE) method to evaluate the impact of assay parameters on interlaboratory HAI assay variability. Statistical and mathematical approaches were used for data analysis. We developed a consensus protocol and assessed its performance against in-house HAI testing. We additionally tested the performance of several potential biological standards. In-house testing with four reassorting viruses showed considerable interlaboratory variation (geometric coefficient of variation [GCV] range of 50% to 117%). The age, concentration of turkey red blood cells, incubation duration, and temperature were key assay parameters affecting variability. Use of a consensus protocol with common reagents, including viruses, significantly reduced GCV between laboratories to 22% to 54%. Pooled postvaccination human sera from different vaccination campaigns were effective as biological standards. Our results demonstrate that the harmonization of protocols and critical reagents is effective in reducing interlaboratory variability in HAI assay results and that pools of postvaccination human sera have potential as biological standards that can be used over multiple vaccination campaigns. Moreover, the use of standards

together with in-house protocols is as potent as the use of common protocols and reagents in reducing interlaboratory variability

The EQA study for the HAI and VN confirmed earlier results obtained in WP1: they showed that the use of standards (calibrators, reference reagents) is beneficial and reduced variation between laboratories consistently across assays (HAI and VN) and between virus types and subtypes. However, combining standard(s) with harmonized protocols had no added benefit.

Concerning the Work Package 2, the partners involved have made Standardized Operating Procedures (SOPs) for the isolation and cryopreservation of PBMC, for the execution of influenza-specific Intracellular Cytokine Staining (ICS) and interferon- γ (IFN- γ) Enzyme Linked Spot (ELISpot) assays. The two CMI assays have been qualified and assay characteristics have been defined (e.g. specificity, limits of linearity, limits of reproducibility, limits of quantifications). Additionally, proficiency tests have been organized to assess the quality of performing these CMI assays and to examine the effect of harmonization by introducing these SOPs. Data has shown that the level of interlaboratory variation decreased from 64%, 63% and 34% to 52%, 19% and 16% for unstimulated and stimulated conditions with A/California and B/Phuket split vaccine, respectively. Therefore, we were able to demonstrate that harmonization of CMI assays can allow for evaluating CMI data in a multi-laboratory setting. This will contribute to a better evaluation of influenza vaccine performance and the identification of correlates of protection.

During this FLUCOP project, WP3 has successfully determined which method would be the best to measure NA antibody in human sera. The selection of the ELLA protocol led to the validation of an approved SOP in a multi-laboratory collaborative study. WP3 partners have also successfully demonstrated the specificity of our protocol, including differentiation between B Yamagata and B Victoria lineages. Additional sources of antigen were also selected to challenge our SOP using either mismatched viruses or recombinant proteins. Finally, WP3 partners could demonstrate the importance of a calibrator in improving both inter-laboratory results and difference sources of antigens. The compilation of all these data has led to the submission of a manuscript: Validation of a harmonized Enzyme-Linked-Lectin-Assay based Neuraminidase Inhibition Assay (ELLA-NI) standard operating procedure (SOP) for quantification of N1 influenza antibodies and the use of a calibrator to improve the reproducibility of the ELLA-NI with reverse genetics viral and recombinant neuraminidase antigens: a FLUCOP collaborative study.

1.6 Potential impact and main dissemination activities and exploitation of results

The scope of the FLUCOP project was to improve and standardize the existing immunological assays applicable for the definition of correlates of protection in future efficacy trials and, whenever feasible, to develop new assays to better evaluate influenza vaccine immunogenicity.

FLUCOP results demonstrate that the harmonization of protocols and critical reagents are effective in reducing interlaboratory variability in HAI assay results and that pools of postvaccination human sera have potential as biological standards that can be used over multiple vaccination campaigns. The harmonization of the hemagglutination inhibition (HAI) assay (WP1) through the use of the consensus FLUCOP protocol and common critical reagents significantly reduces interlaboratory variation, as reported in the paper Waldock J. 2021 (mSphere 2021 Aug 25;6(4):e0056721. doi: 10.1128/mSphere.00567-21.).

The standardization of the harmonized serology assays, done in the context of the FLUCOP Project, once implemented by vaccine manufacturers, may lead to acceleration of development of new/updated/improved influenza vaccines through the better ability to collaborate and to compare results between studies.

The results achieved during the project by Work Package 2 complement and strengthen those made in other work packages. The standard operation procedures that have been drafted for “isolation and cryopreservation of peripheral blood mononuclear cells (PBMC)”, the performance of “interferon-gamma ELISpot” and “Intracellular cytokine staining/Flow cytometry” represents a useful tool for all involved in measuring the strength and quality of immune responses induced by influenza-infections and vaccines. These

procedures will serve the European R&D community in the development of new vaccines and the evaluation of their immunogenicity in a harmonized manner. The development of vaccines against influenza and SARS-CoV-2 that have a broader protection than today's vaccine is highly desirable. Ideally vaccines can be developed that can elicit protection against (severe) disease caused by present and future variant strains (SARS-CoV-2) or drift and shift strains (influenza). It is very likely that protective efficacy of these vaccines will not only rely on the production of protective antibodies but also on the induction of a robust, cross-protective cellular immune response. The instruments created in the context of WP2 will contribute to the development of these new vaccines against influenza and by extension to other respiratory pathogens such as RSV, SARS-CoV-2. A fast development of high-quality vaccines will benefit the health of EU citizens.

Concerning the work carried out from Work Package 3, due to lack of standardization of currently used serological assays, the use of threshold titers for protection against influenza is difficult. This led to the urgent need of improving international standardization and/or harmonization of serological assays to reduce the variability among laboratories. Serological techniques commonly used to quantify influenza-specific antibodies include the Hemagglutination Inhibition (HAI), Single Radial Haemolysis (SRH) and Virus Neutralization (VN) tests. Due to the prominent functional role of HA in influenza virus infection (receptor binding, fusion mediation) anti-HA antibodies have been the prime target parameter for evaluating immunity following infection or vaccination. However, it is known that NA-specific humoral responses can contribute considerably to immune protection. Further, cross-protection mediated by anti-NA antibodies against viral strains of heterologous HA-subtypes but with (partially) matching NA antigens has been reported. Several assays have been developed for the determination of NA-specific antibody responses, but most have not been harmonized or evaluated for their applicability to reliably determine NA-specific antibodies in a controlled and regulated environment.

The identification and implementation of validated assay qualified for the routine determination of anti-NA responses and for the generation of data that might eventually be applicable as supplementary immunogenicity parameter for the regulatory evaluation of influenza vaccines was therefore important. The goal of WP3 was to advance the understanding and application NA assays as tools for evaluating influenza vaccine performance.

For this purpose, WP3 succeeded in the validation of a common protocol for ELLA-NI that can be used and will be freely available to all groups, private and public, when testing influenza vaccines in humans.

The work performed by WP3 enable private and public to reach a consensus on the way to measure neuraminidase-specific functional antibodies which could possibly applied to future clinical trials. WP3 project has now collected and compiled all data into a manuscript with a detailed harmonized SOP. This manuscript will be peer-reviewed and freely available to the scientific and industrial community.

During the Project several workshops has been organized on the biopharmaceutical research and development that contributes to training young scientists.

Two Assay Validation workshops were organized. The objective of the validation workshops was to raise awareness about the critical aspects of assay development, validation and life cycle and to align the partners on assay development and validation essentials (including statistical perspective). The first in GSK, Belgium, Wavre, on 7th and 8th of June 2016 and the second at the University of Siena, Italy, on the 5th of April 2017.

Moreover, WP2 has focused on developing standard protocols for two assays to measure cell-mediated responses generated by influenza vaccination, namely Intracellular Cytokine Staining (ICS-flow) and ELISpot. In project year 4 protocols for both assays were validated with reports on both assays finalized in year 5. The next, and final, task of WP2 is to conduct a quality assessment of these two assay protocols via proficiency testing. Preparations for these proficiency tests were initiated with a training session on the standardized operating procedures (SOPs) generated in the preceding project years (held on 13-14 November 2019 in Ghent, Belgium).

1.7 Lesson learned and further opportunities for research

The collaboration between private and public organizations has led to different added values along the last 7 years of the project.

Without the close cooperation between industry and public health/academic laboratories, the work could not have been carried out. Both industry and academic/public health laboratories shared protocols and materials, designed studies together and participated in these studies. Each partner brought the own expertise and life experience to the project, which led to a strong and fast collaboration.

Most notably it has created new or strengthened existing partnerships, it has brought together complementary knowledge and skills leading to results that would not have been achieved if partners would have worked separately.

FLUCOP project demonstrated that basic analytic tools used for vaccine evaluation, such as serological tests and assays to measure cell-mediated immunity, are still prone to great variability. Harmonization of procedures is possible and reduces precision and inter-laboratory comparability. One of the most important key points observed during the FLUCOP project was the organization of a validation seminar to align the partners on assay development and validation essentials (including statistical perspective) and to raise awareness about the critical aspects of assay development, validation and life cycle. This was an opportunity to share manufacturer's expertise and understanding/interpretation of current assay validation guidelines with real-life examples from Flu field, as well as bring public partners to a common definition of validation process. This workshop covered the basics of assay development until validation and assay maintenance. A special focus was given to statistical essentials and to real-life examples.

Standardized assays, or other ways to harmonize the results of common assays, such as the use of standards (reference reagents), are a prerequisite to the determination of generic correlates of protection (CoPs). Thus, with the progress made by FLUCOP, future research could focus on finding CoPs for seasonal influenza vaccines.

Regarding potential new research for further advancement in the field, it could be set up a prospective clinical trial (Phase 3 type) to evaluate the protective efficacy of (an) influenza vaccine(s): this would require a large enough number of participants and sufficiently high attack rate, in order to capture a sufficient number of cases (infections) to compare with non-infected (protected) participants. Other potential new research would be to collect biological material (serum, cells, mRNA, saliva) during this study to evaluate humoral and cellular immune responses using the assays harmonized in FLUCOP, and also examine other biological markers that may lead to the discovery of one or more or a set of markers that represent a correlate of protection.

Additionally, the importance of the antigen source was demonstrated. Flucop work showed the applicability of both mismatched viruses and recombinant proteins to ELLA-NI. The application of such alternative sources could also be evaluated for other functional assays as well as the build-up of a platform to create such mismatched viruses following the strain adaptation of vaccines.

Finally, the major importance of a standard has been highlighted all FLUCOP work packages. A new research field on standard applicable not only for HAI or VN, but also for CMI and ELLA is one straightforward upcoming goal. This is one critical part to limit inter-laboratories variation and enable a strong comparison of data.