**ISPRI-HCP: CHO protein impurity immunogenicity risk prediction for improving biosimilar product development and assessing product interchangeability**

## I. OVERVIEW of PROJECT AIMS/MILESTONES AND TIMELINE

### SPECIFIC AIMS

**Overview.** The identification and removal of host cell proteins (HCP) from biologic products is a critical step in biosimilar drug development. While the sequence of the biosimilar may be identical to the innovator, the process used to produce the biosimilar will be different, and as a result, new HCPs may be introduced into the product. HCPs have been linked to off-target effects and immunogenicity, raising concern about drug safety. Despite recent improvements to purification processes, biologics that are manufactured in different cell lines and purified using different processes contain variable HCP impurities, making it necessary to identify and quantify impurities for each product, be it a reference innovator product or a proposed biosimilar product. The significance is underscored by recent reports of unwanted immunogenicity and loss of efficacy linked to HCPs in monoclonal antibody and recombinant protein products manufactured in the cells most commonly used to produce biologics, Chinese hamster ovary (CHO) cells.

The goal of this program is to improve methods for assessing HCP immunogenicity risk, making it possible to evaluate whether a biosimilar and an innovator product are interchangeable, without significant safety risk (due to HCP immunogenicity) for the patient.

Well-established methods for quantifying and identifying HCPs, such as ELISA assays and two-dimensional western blots using non-human species' anti-HCP antibodies, may fail to identify immunogenic HCPs. More recently, drug developers have turned to identification and evaluation of individual impurities by LC-MS, with the goal of selectively removing the most immunogenic impurities. To facilitate this task, developers and regulators need a rapid means of assessing the risk of individual impurities using in silico tools, which would ultimately reduce the number of HCPs to be removed, improving safety and speeding the process workflow. **We propose to develop a predictive model for HCP immunogenicity that can facilitate assessment of clinically meaningful immunogenicity risk for biologics and assess interchangeability risk between a biosimilar and an innovator product.**

We have developed a web-based tool called ISPRI-HCP that predicts the immunogenic potential of HCP sequences by evaluating T cell epitope count and density, and relative conservation with other epitopes in the human genome. Building on previous studies of monoclonal antibody and biologic protein immunogenicity using silico methods and our FDA generic peptide immunogenicity research experience, we hypothesize that ISPRI-HCP can accurately classify candidate HCP impurities according to their immunogenicity risk. The EpiMatrix and JanusMatrix core algorithms that are integrated into ISPRI-HCP have been well established.

Originally, Aim 1 was: "Develop an ISPRI-HCP immunogenicity prediction model that is trained on the T cell immunogenicity of CHO HCPs", and Aim 2 was "Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro." In Aim 1, 87 HCP were to be evaluated. In the interim between proposal generation and receipt of funding, we had identified significant issues with overlapping peptide arrays that had traditionally been used by researchers. These arrays contain repeated epitopes, truncated epitopes, and epitopes that are improperly centered.

To more accurately evaluate the potential for immunogenicity we proposed to re-order the aims so as to determine whether a computationally designed peptide array that reduced the repetition of epitopes, and properly centers the epitopes, would improve the accuracy of the results. The modified array format would also be less expensive and less wasteful of resources, leading to cost reductions and, potentially, the publication of a new and improved method for peptide arrays that could be used by other researchers. Once the array format is validated, then, the full complement of HCP could be evaluated in vitro. We anticipate that the PeptiCAD approach will be more accurate and less costly, which would be a significant advance for the field.

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***SPECIFIC AIM 1: Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro.*** The suitability of overlapping peptides to model T cell responses of whole HCP proteins that would be encountered by patients in a biosimilar or innovator product will be assessed. We will also explore the suitability of a focused set of peptides that is computationally selected (PeptiCAD) in comparison with recombinant protein and overlapping peptides. T cell immunogenicity equivalence will be evaluated by the method used Wullner et al. 2010 to stimulate de novo T cell responses for a subset of CHO HCPs. Six commonly found CHO protein impurities covering a wide range of immunogenicity risk will be tested. Expected outcome: Methods to be made public about a more efficient way to screen for potential immunogenicity risk using peptide arrays.

***SPECIFIC AIM 2: Develop an ISPRI-HCP immunogenicity prediction model that is trained on the T cell immunogenicity of CHO HCPs.*** We will generate a T cell immunogenicity dataset for 87 commonly found CHO HCP impurities in licensed monoclonal antibodies that were defined by a 26-company collaboration. Based on ISPRI-HCP sequence analysis, these proteins span a wide range of immunogenic potential, from high- to low-risk. For each CHO protein, we will stimulate de novo immune responses in vitro for an HLA-diverse cohort and measure T cell immunogenicity of the HCP in dose ranging studies, by FluoroSpot assay. The experimental dataset generated for all the proteins will be used to evaluate performance of ISPRI-HCP risk classification by cross-validation methods to estimate the performance of the ISPRI-HCP machine learning model.

This research program will improve the accuracy of the ISPRI-HCP platform, providing drug developers with a rapid and efficient means to reduce HCP-associated immunogenicity and evaluate whether a biosimilar and an innovator product are interchangeable. In **future work**, we will use ISPRI-HCP to perform side-by-side predictions and in vitro assessments of the immunogenicity of CHO HCP impurities found in innovator versus biosimilar products.

## MILESTONES AND TIMELINE

The overall goal of the research program is to sharpen the predictive accuracy of ISPRI-HCP and demonstrate that ISPRI-HCP is a reliable tool for assessing innovator versus biosimilar immunogenicity risk. In Aim 1, we will first evaluate immunogenic similarity of variable antigen formats using in vitro T cell assays (Year 1). The antigen format that performs best will be selected to go forward to Aim 2 studies that are scheduled to begin in Year 2 and continue to the end of the project.

Note: Percent completion values below reflect work completed from the award date, August 24, 2022 through the reporting date, July 1, 2023.

### AIM 1: Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro. (Year 1)

**Objective 1.1: Select CHO HCP test articles for antigen format comparison.**

- **Milestone 1.1:** Selection of six commonly found CHO HCP impurities (PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2) with ISPRI-HCP scores that cover a wide range of immunogenicity risk.
- **Timeline:** Year 1: start – end of Month 2
- **Percent completion:** 100%


**Objective 1.2: Design PeptiCAD and peptide arrays for six CHO HCPs.**

**Milestone 1.2:** Production of PeptiCAD and peptide array designs for PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO HCPs.

- **Timeline:** Year 1: end of Month 2 – start of Month 4
- **Percent completion:** 100%

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**Objective 1.3: Obtain PeptiCAD, peptide array, and whole protein test articles for: PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO proteins.**

- **Milestone 1.3:** Production of PeptiCAD and peptide arrays for six CHO HCP test articles. Successful production of whole proteins for four out of the six HCPs.
- **Timeline:** Year 1: start of Month 4 – end of Month 8
- **Percent completion:** 100%


**Objective 1.4: Determine the appropriate dose for Aim 1 T cell assays**

- **Milestone 1.4:** Selection of the optimal dose for AIM 1 T cell assays. Complete a dose ranging study using PeptiCAD, peptide array and whole protein PLBL2 antigens.
- **Timeline:** Year 1: start of Month 5 – middle of month 8
- **Percent completion:** 100%

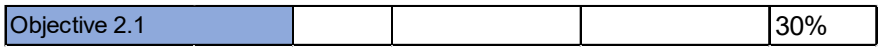

**Objective 1.5: Complete AIM 1 T cell assays for PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO proteins and determine the best antigen format for AIM 2.**

- **Milestone 1.5:** Determine the best HCP antigen format for AIM 2 T cell assays .
- **Timeline:** Year 1: end of Month 8 – end of Year 1
- **Percent completion:** 25%


**AIM 2: Develop an ISPRI-HCP immunogenicity prediction model that is trained on the T cell immunogenicity of CHO HCPs. (Years 1-2)**

**Objective 2.1: Design peptide arrays for commonly found CHO HCP impurities.**

- **Milestone 2.1:** Design peptide arrays for 87 commonly found CHO HCP impurities
- **Timeline:** Year 1: start of Month 11 – Year 2: start of Month 1
- **Percent completion:** 30%




**Objective 2.2: Generation of T cell immunogenicity dataset for 87 commonly found CHO HCP impurities.**

- **Milestone 2.2:** Perform in-vitro immunogenicity assessment of 87 commonly found CHO HCP impurities, for 30-40 donors, based on production of IFNγ and IL-10 cytokines.
- **Timeline:** Year 2: start of Month 1 – end of Month 10
- **Percent completion:** 0%

**Objective 2.3: ISPRI-HCP Classification model development.**

- **Milestone 2.3:** Develop a classification model that will assign immunogenicity risk to CHO HCP impurities using ISPRI-HCP from the analysis of in-vitro experimental data.
- **Timeline:** Year 2: start of Month 2 – end of Year 2
- **Percent completion:** 0%
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## II. PROJECT ACTIVITIES AND ACCOMPLISHMENTS

Objectives 1.1, 1.2, 1.3 and 1.4 are completed and Objective 1.5 will be completed by the end of Year 1. Aim 2 objectives are not scheduled to begin until Year 2.

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**Objective 1.1: Select CHO HCP test articles for antigen format comparison.** To assist with our selection of HCPs for Aim 1, we first used ISPRI-HCP to evaluate the immunogenic potential of the 143 CHO HCPs that are frequently found to co-purify with mAbs. Shown in **Figure. 1** is a subset of these proteins plotted on the Y axis by their EpiMatrix Protein Score and on the X axis by their JanusMatrix Human Homology Score. Several of these commonly identified HCPs with their EpiMatrix and JanusMatrix Scores are illustrated on the accompanying Quadrant plot (**Fig. 1**). The bubble plot shows each of the HCP in a quadrant that is used for classifying their immunogenicity risk based on EpiMatrix (EMX) and JanusMatrix (JMX) thresholds. We find that the predicted immunogenic potential of CHO HCPs covers a wide range of scores, both in terms of epitope content and "human-ness" as defined by JanusMatrix. Using this data, we selected six HCPs that cover a wide range of immunogenicity, and they are identified by name in **Figure 1** (PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2) and their scores are listed in **Table 1**.

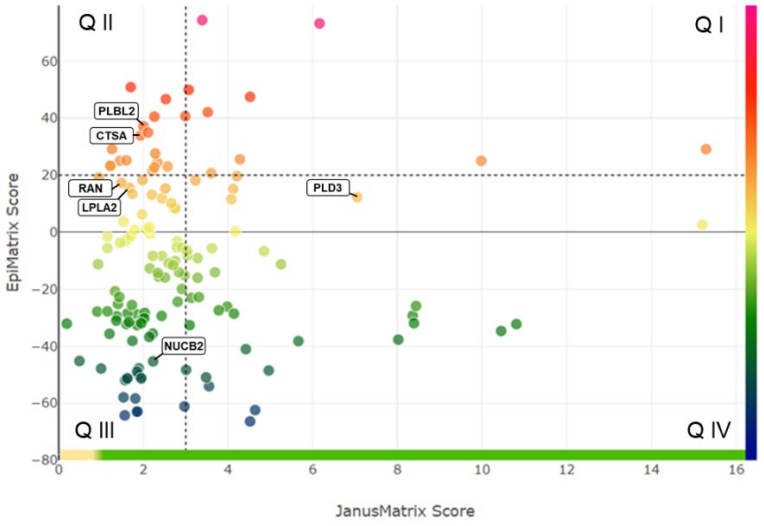


**Fig. 1. ISPRI-HCP analysis of CHO HCP Landscape.** Using a list of commonly found CHO protein impurities, we calculated the EpiMatrix (EMX) and JanusMatrix (JMX) scores for each protein. Shown here are 143 CHO HCP impurities and selected proteins. Proteins with high (orange), medium (yellow), and low (green) immunogenicity potential are shown. Proteins with EMX greater than 20 and JMX scores less than 3 are predicted to be high risk (Q II). Proteins with EMX less than 20 and JMX greater than 3 are predicted to be low risk (Q IV). The list of commonly found HCPs was sourced from: Jones, M. et al. "High-risk" host cell proteins (HCPs): A multi-company collaborative view. Biotechnology and Bioengineering vol. 118 2870–2885 (2021) and Molden et al. (2021), Host cell protein profiling of commercial therapeutic protein drugs as a benchmark for monoclonal antibody-based therapeutic protein development.

**Table 1. CHO HCP test articles for antigen format comparison**

| Protein Name | Label | Risk Group | EpiMatrix Score | JanusMatrix Score |
|---|---|---|---|---|
| Phospholipase B-like 2 | PLBL2 | High | 37.12 | 2 |
| Lysosomal protective protein | CTSA | High | 33.93 | 1.93 |
| GTP-binding nuclear protein | RAN | Intermediate | 13.46 | 1.74 |
| Phospholipase D3 | PLD3 | Intermediate | 12.23 | 7.05 |
| Lysosomal Phospholipase A2 | LPLA2 | Intermediate | 11.89 | 2.44 |
| Nucleobindin-2 | NUCB2 | Low | -45.32 | 2.23 |

**Objective 1.4: Determine the appropriate dose for Aim 1 T cell assays.** To determine the optimal dosage for evaluating CHO HCP immunogenicity, the immunogenic potential of PLBL2, a commonly found CHO HCP impurity was assessed using EpiVax's In Vitro Immunization Protocol (IVIP). IVIP is performed as follows: Peripheral blood mononuclear cell (PBMC) samples are isolated from de-identified whole blood filters obtained from the Rhode Island Blood Center. Each donor is screened for HCP immunogenicity. Based on a probabilistic model, we calculated that blood samples collected from 30 randomly selected individuals will cover each HLA class II DR supertype allele two times with a probability of 85%; the probability of covering each allele once is >99%. Male and female donors are equally weighted for consideration of sex as a biological variable. Cells are cultured in 96-well U-bottom plates at a density of 2.5 x $10^5$ cells/well in complete human RPMI media (chRPMI) supplemented with IL-2 and IL-7 growth cytokines. Cells are stimulated with test articles (pooled peptides) over a range of concentrations from 1-20 µg/ml, an immunogenic protein positive control (KLH; 10 µg/ml), a non-immunogenic protein negative control (HSA; 10 µg/ml), an antigenic peptide pool positive control (CEFTA; 2 µg/ml), or media only. The test article concentration maximum is 20 µg/ml. Control concentrations are based on in-house assay development. All PBMCs are cultured for 14 days at 37 ºC with chRPMI media exchanges and growth cytokine supplementation on Days 4, 7, and 11. Per sample, 10 million PBMCs are taken for HLA typing to four-digit resolution by the sequence-specific oligonucleotide method at the American Red Cross. The dose response of the whole proteins, standard peptide arrays or peptide epitope Computer Assisted Design (PeptiCAD) PLBL2 antigen formats is compared. In this experiment PBMCs from normal healthy donors, with no

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known previous exposure to CHO HCPs were cultured in the presence or absence of PLBL2 antigens with cytokine support for 14 days alongside the appropriate controls. The interferon-gamma (IFNγ) response to PLBL2 antigens was measured by Fluorospot (**Fig.2**). We find that the IFNγ responses to the peptide array and PeptiCAD was greater than whole proteins. Note that the PLBL2 PeptiCAD design has half the number of peptides as the peptide array and produced similar IFNγ responses. To determine the dose response to PLBL2 antigens, we exposed PBMCs to 1, 5, 10 and 20 µg/mL of each antigen format (**Fig. 3**). On average, 20 µg/mL produced the highest PLBL2 specific IFNγ responses. Unlike whole proteins, a dose dependant relationship was observed for the peptide array and PetiCAD antigens. The responses to whole proteins was varied and is potentially due to incomplete antigen processing and presentation. Based on these results, we determined the optimal dose for testing the CHO HCPs to be 20 µg/mL.

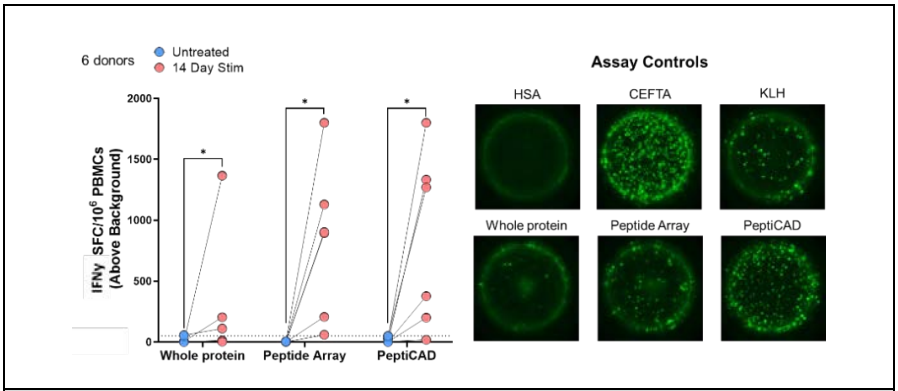


**Fig. 2. CHO PLBL2 antigens induces INFγ in human PBMC .** Shown is the INFγ induced after 14 day exposure (14 Day Stim) and without 14 day exposure (Untreated) to whole protein, peptide array, and PeptiCAD PLBL2 antigens. Representative figure with assay controls are shown: HSA (negative control), CEFTA and KLH (positive controls).

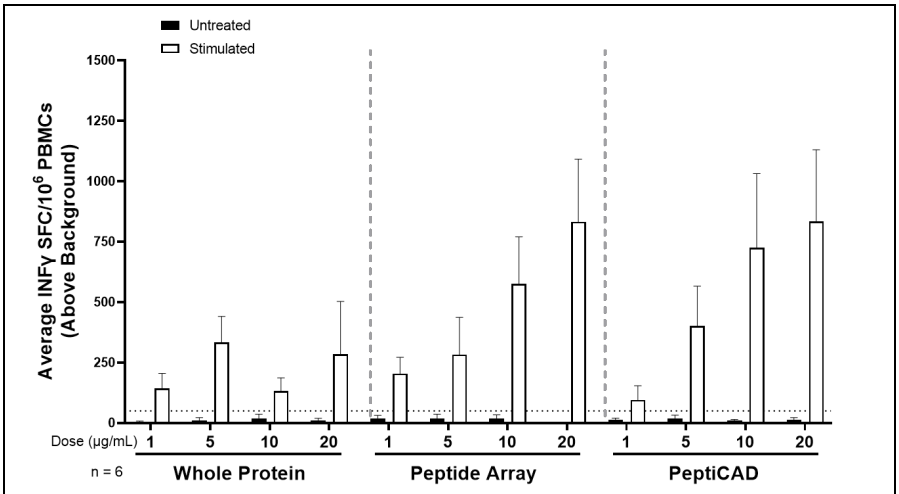


**Fig. 3. Human PBMC dose response to PLBL2 antigens.** Shown is the average INFγ SFC per million PBMC after 14 day exposure (Stimulated) with whole protein, peptide array, or PeptiCAD PLBL2 antigens. Without 14 day exposure (Untreated).

**Objective 1.5: Complete AIM 1 T cell assays for PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO proteins and determine the best antigen format for AIM 2.** Using EpiVax's In Vitro Immunization Protocol (IVIP) assay, 30 donors will be tested per protein (six proteins total) to broadly cover HLA class II DR diversity. Data analysis will include a comparison of three antigen formats (whole proteins, standard peptide arrays, and PeptiCAD) for magnitude of cytokine responses and frequency of donors responding. The antigen format that performs the best and is most cost efficient will be selected for AIM 2 studies. We have setup experiments for 16 donors and will have the data processed for 6 donors by the end of July 1st. Objective 1.5 is expected to be completed by the end of Year 1 .

## III. REGULATORY IMPACT OF RESULTS

In silico immunogenicity assessment of host cell protein (HCP) impurities could impact biosimilar development by streamlining the clinical data needed to support the designation of a proposed biosimilar product to be interchangeable with the reference listed drug (RLD). In 2017, EpiVax programmers developed a toolkit comprised of several integrated algorithms for immunogenicity screening of host cell proteins, known as ISPRI-HCP (Interactive Screening and Protein Reengineering Interface for Host Cell Proteins). In this project, in vitro T cell immunogenicity data with be generated from screening commonly found CHO HCP impurities. These

results will be used to further validate and refine the immunogenicity assessments made by ISPRI-HCP, providing a valuable resource for biosimilar development.

## IV. PLANS FOR NEXT REPORTING PERIOD

During Year 2, we will complete Aim 2 objectives. The large majority of effort will go toward the generation of T cell immunogenicity dataset for 87 commonly found CHO HCP impurities (Objective 2.2), and the ISPRI-HCP risk classification model devepment (Objective 2.3).

**Objective 2.2: Generation of T cell immunogenicity dataset for 87 commonly found CHO HCP impurities.** We will perform in vitro immunogenicity assessments of over 80 commonly found CHO HCPs for 30-40 donors. We will use PBMC production of IFNγ and IL-10 as biomarkers of immune response. IFNγ is a signature marker of immunogenicity and IL-10 is an important anti-inflammatory cytokine that can regulate immune responses to CHO HCP impurities due to Treg recognition of epitopes with human sequence homology. This experimental data will be used in Objective 2.3 to inform the current ISPRI-HCP four-quadrant classification model by fine-tuning the thresholds set for EpiMatrix and JanusMatrix scores.

**Objective 2.3: ISPRI-HCP Classification model development.** We will develop a classification model to assign immunogenicity risk to CHO HCP impurities. Our task is to approximate a mapping function from predictive scores (EpiMatrix, JanusMatrix, or a combination thereof) to FluoroSpot data (EpiMatrix scores to IFNγ data and JanusMatrix scores to IL-10 data). Predictive scores will be mapped to FluoroSpot data using machine learning techniques with FluoroSpot data split into training and validation sets. To avoid overfitting, we will use a cross-validation method. Cross-validation evaluates machine learning models by training several models on subsets of the input data and evaluating them on a complementary subset of data. We will utilize the k-fold cross-validation procedure to evaluate our machine learning model. k-fold validation splits input data into k subsets, or folds, trains a model on k-1 (all but one) subsets, and evaluates the model on the subset that is not used for training. The process is repeated k times, each time with a different subset used for evaluation and excluded from training. Each model is trained and evaluated using complementary data sources. The data in the evaluation data source includes all data that is not in the training data source. An area under the curve performance metric will be calculated for each of k evaluations and the overall model performance is the average of the area under the curve values.

We will begin modeling with the simplest approach and implement mapping methods with increasing complexity to increase predictive accuracy, if needed. Immunogenicity risk will be initially modeled as a binary variable (immunogenic or non-immunogenic HCPs) using EpiMatrix and JanusMatrix scores as predictor variables. Various HCP immunogenicity risk definitions will be explored, including frequency of positive donor responses (positive response defined above) and mean, median, or inter-quartile range spot forming cell magnitude. A logistic regression model with k-fold cross-validation using a typical k = 5 value will be used as it yields test error rate estimates that do not suffer from high bias and high variance.

If modeling by the initial approach does not generalize well and/or overfits the training data, we will try more complex mapping algorithms for classification (i.e., random forest, stochastic gradient descent, etc.), try different regularization techniques to reduce overfitting, and/or try different predictor variables (epitope cluster count, or ratio of predicted immunogenic to tolerogenic clusters, for example). We will also consider multi-class classification with HCPs assigned as low, intermediate, or high risk for immunogenicity over a range of IFNγ and IL-10 response frequencies, and combinations thereof.

We expect this Aim will yield an accurate CHO HCP immunogenicity predictor that is ready for prospective testing for biosimilar (and innovator) development and evaluation of interchangeability of reference and biosimilar products.

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## V. PUBLIC COMMUNICATIONS OF RESULTS

### Conference Presentations

Haltaufderhyde K., Roberts B., Terry F., Boyle C., William W. M., De Groot A. (2023) Comprehensive assessment of immunogenicity risk of host cell proteins in biologics using in silico and in vitro methods. *BioPharmaceutical Emerging Best Practices Association (BEBPA) HCP Conference.* Oral Presentation. Dubrovnik, Croatia.

### Websites

Riley Nolan. EpiVax Secures Additional Funding from FDA for Immunogenicity Risk Assessment for Biosimilar Products. Retrieved from https://epivax.com/news/epivax-secures-additional-funding-from-fda-for-immunogenicity-risk-assessment-for-biosimilar-products, September 12, 2022.

Elena Iemma. EpiVax: 25 years of Fearless Science. Retrieved from https://epivax.com/news/epivax-25-years-of-fearless-science, May 16, 2023.