“HIGH-RISK” HOST CELL PROTEINS (HCPs): A MULTI-COMPANY COLLABORATIVE VIEW

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About Biophorum Development Group (BPDG): Since its inception in 2004, BioPhorum has become a trusted environment in which senior leaders of the biopharmaceutical industry come together to share and discuss openly the emerging trends and challenges facing their industry. BioPhorum currently comprises more than 3,800 active participants in seven “phorums” covering cell and gene therapy, drug substance, development, fill–finish, a technology roadmap, information technology, and supply partners. The Host Cell Protein (HCP) Workstream is part of the Development Group (BPDG). This article is a composite view of

opinions shared by the whole of the BPDG-HCP Workstream and should not be attributed to the individual positions of the participating companies.

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ABSTRACT

Host cell proteins (HCPs) are process-related impurities that may co-purify with biopharmaceutical drug products. Within this class of impurities there are some that are more problematic. These problematic HCPs can be considered high-risk and can include those that are immunogenic, biologically active, or enzymatically active with the potential to degrade either product molecules or excipients used in formulation, and often are difficult-to-purify. Why should the biopharmaceutical industry worry about these high-risk host cell proteins? What approach could be taken to understand the origin of this co-purification and to deal with these high-risk HCPs? To answer these questions, the BioPhorum Development Group (BPDG) HCP Workstream initiated a collaboration among its 26-company team with the goal of industry alignment around high-risk HCPs. A sub team was formed, in which the members performed literature searches and discussed the information available around this topic. A survey to the BPDG HCP Workstream team members led to team discussions and insights into a list of frequently seen problematic HCPs. These HCPs were further classified based on their potential impact into different risk categories that could be beneficial to the biopharmaceutical industry for targeted monitoring of those HCP impurities in CHO-produced biologics to minimize risk to product quality, safety, and efficacy.

Key Words:

Host Cell Proteins, Chinese Hamster Ovary, high-risk HCPs, Problematic HCPs, Protein A, PLBL2, Cathepsins, Lipase(s)

Introduction

Host cell proteins (HCPs) are process-related impurities derived from host cells that may co-purify with a biopharmaceutical drug product. Within this class of impurities, some are more problematic as reported in literatures. These problematic HCPs can be considered high-risk and can include those that are immunogenic, biologically active, or enzymatically active with the potential to degrade either product molecules or excipients used in the product formulation, and often difficult to purify (Bee et al., 2015; Chiu et al.; Vanderlaan et al., 2018). Although there are only very few cases known with direct impact of HCPs on patient´s safety, the biopharmaceutical industry is concerned about these high-risk HCPs as shown through several recent publications (Cheung et al., 2016; Jawa et al., 2016; Reijers et al., 2019). The BioPhorum Development Group (BPDG) Host Cell Proteins (HCP) Workstream initiated a collaboration among its 26-company team with the goal of industry alignment around high-risk HCPs. The BioPhorum ™ is a powerful cross industry collaboration process for Biopharmaceutical Drug Developers and Manufacturers. The HCP Workstream aims for industry alignment, building a common understanding of agency requirements for HCPs through benchmarking and gap analysis of guidances. A sub team of members performed literature searches to understand the impact of high-risk HCPs and discussed each member companies' collective experiences. These discussions focused on biopharmaceuticals produced in Chinese Hamster Ovary (CHO) cells and purified by a Protein A affinity column and additional polishing steps. Protein A affinity purification has been a widely used robust process purification step for mAbs. Most sub- team members had direct experience with CHO cell line products and Protein A purification. All HCP Workstream team members were surveyed, which led to team discussions and insights into a

list of frequently seen problematic HCPs. These HCPs can be classified based on reported biologic functions, potential for immunogenicity and impacts on therapeutic drug or formulation into different risk categories. This publication will provide recommendations to the industry to aid the impact evaluation of the individual HCPs identified in specific processes. Tiered immunogenicity risk assessments including in silico method, followed by in vitro comparative immunogenicity assessment (IVCIA), immunotoxicological evaluation in vivo, and data from clinical studies could help establish the possible link between high-risk HCPs and patient safety. Although each individual company may deal with unique circumstances during their product and process development and need to perform HCP risk assessment on a case-by-case basis, we hope this BPDG cross-industry collaboration in reviewing historic problematic HCPs and the high-risk HCPs listed in this paper will guide the industry on targeted high-risk HCP characterization and proactively mitigate the risk posed by potential presence of those HCPs in biological products.

Host Cell Proteins Frequently Seen in Downstream Processing

The CHO expression system has been one of the most commonly used mammalian systems in the production of biopharmaceuticals due to its ability to produce complex proteins with post-translational modifications similar to those produced in humans. Over 6000 CHO HCPs were identified through glycoproteome and proteome analysis (Baycin-Hizal et al., 2012). A subset of these CHO proteins along with the expressed recombinant drug substance are found in the harvest material. Although most of these HCPs are cleared to a minimum through multiple downstream processing steps, there are still some HCPs that escape clearance. A literature search done on the HCPs present after Protein A chromatography revealed that many of the

same HCPs are found across the biopharmaceutical industry. The HCPs identified were similar despite differences in CHO cell lines, upstream processes or downstream processes used (Yuk et al., 2015). The BPDG HCP Workstream group compiled a list of these common HCPs from previously published literature to be used as a reference for HCPs found throughout different processes (Table 1). The identities of the different HCPs could be used by companies as a guide in the development of a downstream process or possibly a refinement of existing purification platforms.

Current Understanding of the Mechanisms for HCP Co-purifications and the Tools to Investigate

In the production of mAbs or Fc fusion proteins, downstream processing often includes Protein A affinity purification followed by one or two additional polishing steps to further remove aggregates, charge variants, HCP, and host cell DNA (Shukla & Thommes, 2010). Although HCPs are cleared to a minimum through the downstream process, there are still some HCPs that escape clearance due to two main routes. One is the HCP's ability to associate with the product via specific or non-specific interactions and to be thus carried through the process (Clavier et al., 2020). The second route is the HCP's ability to interact with protein A chromatography resin, resulting in its co-elution with the product (Levy, Valente, Choe, Lee, & Lenhoff, 2014).

In mAbs, where protein A affinity purification is used, most of the persistent HCP impurities are associated with the mAb itself (Aboulaich et al., 2014; Levy et al., 2014; Shukla et al., 2008; Valente, Levy, Lee, & Lenhoff, 2018). The interaction between a HCP and a mAb is difficult to study as the abundance of the HCP is usually very low in comparison with the mAb. It was found that using washing additives can disrupt mAb-HCP interaction and thus improve HCP

clearance (Aboulaich et al., 2014). The HCP-mAb association can be specific to the mAb product due to the presence of a CHO homolog to the targeted human protein of mAb such as anti-Tissue Plasminogen Activator (tPA) antibody (Valente et al., 2018) or it can be a non-specific interaction with the mAb such as in the case with PLBL2 (Tran et al., 2016). Cross-interaction chromatography (CIC) in conjunction with ELISA and LC-MS has been used to understand the HCPs that often bind to mAbs or hitch-hike (Aboulaich et al., 2014). This approach involves immobilization of purified mAb onto chromatography resin via cross-linking, followed by incubation with HCPs obtained from supernatant of non-mAb producing cells (null cells).

The following types of interaction between HCP and mAb products are important to HCP co-purification, 1) hydrophobic interactions; 2) electrostatic repulsion; 3) hydrogen bond; 4) Van der Waal's force; 5) ionic interaction; 6) presence of immunoglobulin-like domains (for protein A purification). The presence of hydrophobic zones and charge distribution on the surface of the HCP is often the key for HCP co-elution (Clavier et al., 2020; Goey, Alhuthali, & Kontoravdi, 2018). Certain HCPs, such as Clusterin, PRDX1, GAPDH, actin, elongation factor EF-1 alpha often appear in protein A step with PRDX1 often persisting through polishing steps

(Falkenberg et al., 2019). Clusterin is known to interact with mAbs (Wilson & Easterbrook-Smith, 1992). To investigate the interaction type, such as hydrogen bonds, Van der Waals forces, ionic or hydrophobic interactions, different resins relying on distinct purification mechanism can be tested and coupled with a screen of wash conditions with varying pH, salt concentration, or percentage of organic solvent (Joucla et al., 2013).

To characterize the interaction dynamic between a given HCP and a mAb, surface plasmon resonance (SPR) is a method of choice as it can aid in determining specificity, affinity, and

kinetic parameters of the binding. However, this technique requires having the HCP of interest available in sufficient amounts to immobilize it on the SPR chip surface. To do so one can either use a recombinantly produced HCP or when possible purify the HCP from the cell culture fluid (Tran et al., 2016). This approach was done with cathepsin D using a pepstatin A affinity resin (Ranjan et al., 2019). To determine the interaction zone, one approach consists of performing SPR or affinity purifications with Fc and Fab'2 mAb fragments (Zhang et al., 2016). To further define the interaction zone, chemical crosslinking allowing covalent bond formation between HCP and the mAb of interest is an option (Ranjan et al., 2019). It however relies on the use of high-resolution tandem mass spectrometers with sophisticated software for data processing and determination of the residues involved in the interactions. Modeling information might also be needed to obtain a 3D image of the contact region. Studies were conducted to determine the interaction zone and amino acids important for the interaction of cathepsin D with mAbs, including screening of 13 distinct mAbs and mutagenesis analysis (Bee et al., 2015). With lipases such as PLBL2, LPLA2, LPL that are also known to be high-risk and problematic, it will be useful to determine the interaction of those enzymes with product through modeling studies.

For non-specific interaction with products, increased HCP load due to upstream production titer increase and cell viability decrease also contribute to the co-purification of HCP with mAb product, due to the increased impurity load to downstream purification and the potential binding to product when the product has basic pI. In these cases, acidification of HCCF before protein A column or using AEX hybrid purifier has demonstrated capability to reduce co-purification of high-risk HCPs through protein A column (Khanal et al., 2018; Yigzaw, Piper, Tran, & Shukla, 2006).

Although some HCPs may interact with protein A resins, causing co-purification during protein A chromatography steps, most of those proteins that persist through protein A are often those abundant proteins in harvested cell culture fluid (HCCF), and can mostly be removed during polishing steps.

Identifying and understanding the origin of an HCP co-purification is key to find adequate ways to remove it.

BPDG HCP Workstream Survey Results

After thorough literature searches and BPDG team discussions, the team was surveyed to develop an understanding of the collective experience of the member companies with problematic or high-risk HCPs. Of the 26 companies surveyed, 18 companies responded to questions that were developed from the literature review discussions. The survey consisted of 10 questions and the findings are summarized in this section to provide practical insights into industry trends and opinions.

As in the literature searches, the survey focused on high-risk HCPs identified mainly in CHO-expressed products that included a Protein A affinity capture chromatography stage. Though the survey responses are from multiple company members, they do not necessarily reflect the views of an individual company and no firm conclusions should be drawn from this data. A majority of the companies (69%) indicated that they had experienced issues with individual HCPs during drug production. It was surprising to note that 31% of respondents had not experienced issues: this could be partially due to some companies not characterizing their products for individual HCP identification or due to limited product experience.

Companies were asked if they detected or identified certain HCPs in their final product and if they did detect these HCPs, were they able to demonstrate clearance after detection (Figure 1). The results highlight PLBL2 as the HCP with the highest response for both identification/detection and for clearance after detection. It was interesting to note that the number of responses were higher for PLBL2 for clearance after detection compared to identification; this could be due to some companies detecting PLBL2 elsewhere in their process, but not in Drug Substance or final product. Other HCPs identified included LPL, Cathepsins (D, B and L) and LPLA2. The majority of respondents indicated that they were able to show clearance of these HCPs through their process. Companies also included 'other' in their response, which included HCPs such as Clusterin, Flagellin and an unknown lipase. Only 40% of these 'other' HCPs could be successfully cleared in the process. During the discussion on the clearance-focused survey question, an additional question came up; which was whether or not companies added additional purification steps to demonstrate clearance. The idea being that once the HCP was identified and the company reworked their process, the HCP was then removed. The team's response varied depending on their own personal experience; however, in some cases additional purification steps were added to their process.

The survey aimed to understand how companies responded to the detection of individual HCPs. Since there are several ways to identify and quantitate individual HCPs that may be present in drug product, companies were initially asked to identify the analytical methods and technologies used. Companies could choose more than one response and the results are shown in Figure 2. The majority of companies surveyed use either mass spectrometry (MS) (13 of 18) or ELISAs specific to the particular HCP (11 of 18) to identify and measure these individual HCPs.

Fewer respondents (4 of 18) use enzyme/activity assays and one respondent indicated the use of gel excision followed by LC-MS. It was interesting that the majority of member companies were aligned in their strategy for detecting and quantifying individual HCPs.

Companies were also asked about the type of analytical method and/or technologies used for (a) quantitation of total HCPs for DS release and (b) characterization of total HCPs (Figure 3). A majority of respondents used one or more HCP ELISA methods for release testing to quantify total HCP in DS. These included commercial (or generic) kits (13 of 18) and process specific (13 of 18) or platform ELISAs (10 of 18) developed internally by the company. A few respondents also used technologies such as MS quantitation, Gyros and MSD. Gyros and MSD are types of technologies that facilitate higher throughput and/or faster turnaround compared to the more traditional ELISA technology. Subsequent to the initial survey responses, some companies have implemented the use of the Ella (another rapid turnaround technology) for sample testing. The same ELISA methods (commercial, process specific and platform) were also used to characterize total HCPs (9-12 each). In addition, many respondents (15 of 18) reported the use of mass spectrometry techniques and/or orthogonal gel techniques to characterize HCPs. While most companies used 2D gels to characterize coverage by their anti-HCP reagents, 1 company also shared the use of these techniques to detect specific individual HCP on 1D gels.

The survey had several questions related to mass spectrometry. The first question asked about the strategy for the use of MS for HCP identification and quantification. The majority of respondents (15) said that they use MS for process development support. Others responded that they use MS for clinical (6) and non-clinical DS/DP samples (7). There were 3 respondents who selected ‘other’. The write-in responses to ‘other’ included no routine strategy, to support

process validation or investigation. When asked if using relative or absolute quantification, 64% of the results were relative quantification, whereas 36% of respondents use absolute quantification (Figure 4).

The next question about MS methodologies was related to sample preparation before digestion. 67% of those surveyed did not enrich for HCPs prior to analysis, while 17% of those surveyed do enrich for HCPs. An additional 17% of respondents chose 'other' and wrote in that they use precipitation. In terms of digestion methods, 13 of those surveyed use trypsin, while 4 companies use trypsin + Lysine C. When asked what type of MS method companies are using to quantitate HCP, 6 respondents said that they use data dependent acquisition, while 6 respondents said that their company uses data independent acquisition.

The final question of the survey asked whether companies have received regulatory feedback about their analytical testing strategy for total HCP and individual HCPs. Interestingly, 45% of people responded 'no', while 36% of people responded 'yes'. 18% of people responded that 2D coverage was requested as part of regulatory feedback. For the 36% who responded 'yes', there were a variety of examples of feedback received, ranging from a request for a description of the HCP assay and 2D coverage for early phase, to a process-specific or platform HCP assay being recommended for late phase development.

Classification of Host Cell Proteins

The classification of an individual HCP to be either high or low risk for immunogenicity can be difficult. The risk can vary based on many factors including the drug indication, the route of administration, the frequency of administration, the amount of HCP per drug dose administered, the patient population, the homology of HCPs with their human counterparts,

and prior non-clinical and/or clinical experience. An approach to classifying these HCPs to be high-risk or problematic could be based on an individual HCP's ability to co-purify with the product, the frequency at which it is seen in downstream processing, its ability to modify or degrade the drug and/or the excipient, together with its potential for immunogenicity. Using this approach, HCPs can be considered high-risk or problematic and classified into four major categories: Those that impact Product Quality, Formulation, Direct Biological Function in humans and Immunogenicity. Through extensive literature searches and working experiences of the BPDG HCP Workstream, a list of high-risk HCPs was compiled and categorized based on their impact (Table 2).

Many HCPs have been identified from various product processes that could impact drug quality. HCPs with enzymatic activity, even though not immunogenic, have been shown to impact drug quality at very low levels and may not get detected by traditional ELISAs. Proteases like cathepsins, metalloproteinases and serine protease (HTRA1) have been shown to degrade monoclonal antibodies (mAbs). Specific sequences within monoclonal antibodies have been suggested to have a higher propensity to bind cathepsins. Cathepsin D, B, H, L and Z have all been reported to degrade mAbs (Bee et al., 2015; Luo et al., 2019). Another class of HCPs that are abundant in the CHO proteome and potentially problematic are the chaperone proteins like 78 kDa glucose-regulated protein (GRP78). They are important for the proper folding of the recombinant product. GRP78 also referred to as immunoglobulin heavy chain-binding protein (BiP), have strongly been correlated with the amount of secreted protein and is observed to be non-covalently associated with the unfolded antibody chains within the endoplasmic reticulum (Joucla et al., 2013). Elevated expression of these proteins during biotherapeutic development

can lead to intracellular aggregation of the recombinant product (Goey, Alhuthali, & Kontoravdi, 2018).

Degradation of polysorbates during storage have been observed for drugs formulated with polysorbate 20 and polysorbate 80. Group of Host cell proteins belonging to the lipase family, specifically Lipoprotein Lipase (LPL), LPLA2 (group XV lysosomal phospholipase A2 isomer X1) and Putative phospholipase B like 2 (PLBL2), have been identified to be primarily responsible for impacting the formulation. LPL hydrolyzes ester bonds in triglycerides. Due to structural similarity between polysorbates and triglycerides, it was also shown to degrade polysorbate 80 and polysorbate 20 in product formulations (Chiu et al., 2017). In addition, CHO cells with LPL knockouts were shown to have significantly reduced polysorbate degradation confirming the role of LPL in polysorbate degradation. Endogenous LPLA2 (group XV lysosomal phospholipase A2 isomer X1) is another lipase, when detected at ≤ 1ppm in antibody formulations demonstrated polysorbate hydrolysis while in contrast the antibody without detectable LPLA2 did not show polysorbate hydrolysis. Comparison of polysorbate degradation products generated from the formulated antibody and samples of polysorbate incubated with recombinant LPLA2 resulted in similar elution profiles suggesting that LPLA2 plays a key role in polysorbate degradation in some antibody preparations (Hall, Sandefur, Frye, Tuley, & Huang, 2016).

Host cell protein impurities that are cytokines and are functional in humans can cause biological impact like histamine release in patients that could be fatal. Monocyte Chemoattractant Protein-1 (MCP-1), a highly glycosylated 22 kDa chemokine that is biologically active across species was identified in two different product processes and in one case led to clinical hold of

the product. Transforming Growth Factor-β1 (TGF-β1) is another biologically active protein in humans that is constitutively secreted from CHO Cells. TGF-β1 is reported in literature to be co-purified with an Fc-fusion protein and was detected before any clinical exposure. However, these findings suggest that process developers need to be alerted to the potential that highly conserved cytokines in HCPs could be active in humans (Vanderlaan et al., 2018).

Some HCPs can cause unwanted immune response. There are immunogenicity prediction models like CHO Protein Predicted Immunogenicity (CHOPPI) (Bailey-Kellogg et al., 2014) and the Immune Epitope Data Base (IEDB) (Jawa et al., 2016) that can calculate the potential risk scores of an HCP to elicit a human response but are unable to predict the threshold above which it can cause immunogenic response. In addition, these in silico tools are limited and can only evaluate the HCP primary sequence and T cell epitopes. CHOPPI and IEDB predicted HCPs like glutathione-S-transferase P, peroxiredoxin-1, procollagen lysine 2-oxoglutarate 5-dioxygenase 1 and PLBL2 with high immunogenicity scores (Jawa et al., 2016). On the other hand, there are in vitro assays, such as reactive naïve T cells, affinity of novel T cell epitopes for MHC, and MHC-associated peptide proteomics, and B cell activation, that can measure overall immunogenic effect of a drug formulation but are unable to attribute to a single HCP and suffer from high donor-donor variability and limited MHC of the population. It would be beneficial to combine these in silico and in vitro methodologies to determine the immunogenicity potential of a known HCP contaminant in the drug substance in the process that is being developed.

Recommendations for the Industry in Monitoring and Controlling High-Risk Host Cell Protein (HCPs)

Various approaches are available to identify and quantify the level of total and individual HCPs residing within drug processes. Each method has its own benefits and drawbacks / weaknesses. Additionally, establishing a link between the identified HCP and its impact to the process or the patient can be challenging. Current state of the industry in detecting, identifying, and quantifying individual HCPs is presented along with recommendations for the industry in establishing proper measures for continuous monitoring and/or elimination of the known contaminant if identified as a high-risk HCP.

Total HCP measurement by ELISA-based methods is the major workhorse to determine residual HCP levels to guide process development, as well as to support GMP batch release testing (Tscheliessnig, Konrath, Bates, & Jungbauer, 2013). The ELISA-based methods usually rely on sufficient coverage from anti-HCP polyclonal antibody reagents during method qualification and validation. ELISA methods have the advantage of high throughput in supporting process development and are amenable to qualification and/or validation. Careful generation and characterization of the critical reagents are highly recommended. Assay sensitivity, precision, accuracy and linearity need to be well assessed during assay qualification or validation. 2D-gels with sensitive staining methods and western blots or 2D-DIGE/DIBE are most commonly used to characterize the antibody coverage (USP Chapter <1132>) and (Ph. Eur. 2.6.34) 1D or 2D gels are also employed by some companies to monitor individual HCPs, as this method provides the advantage of visualizing the clearance of the HCP(s) bands/spots.

Individual HCP ELISA is a powerful technique to quantify specific high-risk HCPs that might come through a given process, but only a few are commercially available today. In addition, antibodies for the individual HCPs detection may have been developed using peptides or recombinant protein orthologs that may have wide-ranging affinity towards the target HCP and should be well characterized for their ability to detect the specific CHO protein. In-house critical reagents generation and careful screening can help build a highly sensitive ELISA assay (Vanderlaan et al., 2015). Developing an individual HCP ELISA to support frequently seen high-risk HCPs requires significant time and resources investment due to the need to generate recombinant protein and corresponding antibody. Data obtained from the individual HCP ELISAs can aid assessing the risk of the identified HCP based on the levels measured and the impact to determine if continuous monitoring or further downstream process optimizations are necessary. An approach may be to develop individual HCP ELISAs to those HCPs that are frequently seen in DS based on LC-MS/MS analysis.

LC-MS/MS methods have several advantages. It can provide information regarding a product's HCP profile including identification early in product development. By doing so, potential high-risk HCPs can be identified and further assessed to determine associated risks, which can lead to additional downstream process optimizations before the GMP manufacture of a product batch (Farrell et al., 2015; Huang et al., 2017; Levy et al., 2014; Zhang et al., 2014). LC-MS methods can also determine relative levels of individual HCPs leading to identification of those that are not detected and/or fully quantitated by the total HCP ELISA method due to lack of and/or limitation in the antibody to the specific HCP. Although current LC-MS/MS together with proteomics technology and CHO protein database are advancing the sensitivity and detection

limit of these methods, it might still be challenging for some companies to generate an adequate HCP profile from drug substance due to the very low levels of individual HCPs in the final product. Moreover, sub femtomolar levels of enzymatically active HCPs may still have the ability to affect product quality in many ways thus influencing the product efficacy, stability, and potency without having an impact to patient safety (Gao et al., 2011).

For those enzymatic HCPs an activity assay would be beneficial in determining the residual HCP's potential impact to the formulation excipients (e.g. lipase(s) and hydrolase(s) known to degrade polysorbates), such as Polysorbate-20 and Polysobate-80, (Dixit, Salamat-Miller, Salinas, Taylor, & Basu, 2016; Hall et al., 2016); or to the therapeutic protein itself (HCPs with protease activity like Cathepsins) (Bee et al., 2015). These assays need to be highly sensitive, reliable and robust in determining the potential impact to the drug and/or the formulation. Setting up reliable enzymatic activity assays early in development will be important to address these product stability-related issues.

Developing a strategy to assess and mitigate the risks associated with the individual HCPs identified within a drug substance to patient safety is a major challenge due to lack of clinical data, and unclear regulatory guidance. A tiered immunogenicity risk assessment could be developed to assess the potential impact caused by specific high-risk HCPs identified within a process. The assessment could be initiated with an in silico method, followed by in vitro comparative immunogenicity assessments (IVCIA). Based on the information gathered, toxicology experts could be engaged to further assess the risk. Data from clinical trials could ultimately help establish a possible link between high-risk HCPs and patient safety, if one existed.

For in silico assessment, some companies have established an in-house software system to evaluate immunogenicity risk based on known protein sequence, while others have applied commercial software for the same purpose, for example, CHOPPI system from Epivax (De Groot, McMurry, & Moise, 2008). The in silico methods are hard to validate and only provide an immunogenicity score, which relatively ranks proteins for their potential to cause human immune response. More clinical data are needed to understand the correlation between an HCP scored high (i.e. score above 20 in CHOPPI from Epivax) from in silico assessments and its risk level for potential immunogenicity (Bailey-Kellogg et al., 2014).

In the absence of clinical data, in vitro comparative immunogenicity assessments could be employed by industry to gather information on the levels of a given HCP that could potentially cause human immune response. The individual HCP levels determined from either ELISA and or LC-MS method will be required to set up these in vitro assays. This information will be helpful to develop appropriate cell-based assays to evaluate specific high-risk HCP's dose related ability to elicit T cell functional response. For example, multiplex cytokine analysis and T-cell proliferation assays with spiking known concentrations of HCPs could provide useful information for a therapeutic protein's potential risk for immunogenicity caused by those HCPs (Jawa et al., 2016). A well-developed IVCIA could help set up recommendations for an individual HCP threshold to avoid T-cell activations.

Recent presentation from regulatory representatives included recommendations for developing assays to monitor antibodies specific to host cell proteins from clinical samples (Khrenov & Friedl, 2019). This type of clinical assays is difficult to develop with a desirable sensitivity due to complicated matrix effects. However, some combination of HCP profiling, quantitation,

enzymatic assays, in silico, or IVCIA methods could provide a good assessment of dose-related risks to drug quality and/or immunogenicity caused by high-risk HCPs.

Conclusions

Host cell protein detection, quantitation and removal from the final biotherapeutic process can be complex. The high-risk HCP list provided can be a resource for companies developing biotherapeutics in CHO cells. This whitepaper not only provides a list of HCPs that are considered high-risk or problematic, but it also classifies them into four major categories: Those that impact Product Quality, Formulation, Direct Biological Function in humans and Immunogenicity.

If a host cell protein on the high-risk list was to be identified in a given process, it is the recommendation that a risk assessment is performed for that individual HCP. Several recent publications have touched upon HCP risk assessment from both CMC product quality perspective and clinical safety risk perspective (Bracewell, Francis, & Smales, 2015; Clavier et al., 2020; de Zafra, Quarmby, Francissen, Vanderlaan, & Zhu-Shimoni, 2015; Jawa et al.; Wang et al., 2018a, 2018b). However, a case-by-case risk assessment and mitigation discussion within the company or between companies, including analytical, process development and toxicology groups would be a meaningful approach. Information gathered from literature surveys, LC-MS/MS HCP profiling, individual HCP quantification and enzymatic activity assays can help determine those that impact product quality or formation. In silico immunogenicity assessments, and/or IVCIA assays can provide additional information about those high-risk HCPs that have direct biological function in humans and immunogenicity. The combined information will be valuable in determining if certain HCPs remaining in drug product would

have significant risks to product stability or clinical safety and if further purification or formulation changes are necessary.

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Table 1: A Comprehensive List of Host Cell Proteins Frequently seen in Downstream Processing

	Identified HCP	References
1	40S ribosomal protein	(Aboulaich et al., 2014; Liu et al., 2019; Migani et al., 2017)
2	60S acidic ribosomal protein P0	(Aboulaich et al., 2014; Liu et al., 2019; Zhang et al., 2014)
3	60S ribosomal protein	(Aboulaich et al., 2014; Gilgunn et al., 2019)
4	78 kDa glucose-regulated protein	(Aboulaich et al., 2014; Albrecht et al., 2018b; Chiverton et al., 2016; Farrell et al., 2015; Jawa et al., 2016; Kreimer et al., 2017; Liu et al., 2019; Migani et al., 2017; Zhang et al., 2014)
5	Actin, alpha	(Aboulaich et al., 2014; Falkenberg et al., 2019; Farrell et al., 2015; Jawa et al., 2016; Liu et al., 2019; Zhang et al., 2014)
6	Alpha-enolase	(Aboulaich et al., 2014; Liu et al., 2019; Valente et al., 2014; Zhang et al., 2014)
7	Annexin A2	(Aboulaich et al., 2014; Falkenberg et al., 2019; Fukuda et al., 2019)
8	**Annexin A5**	(Farrell et al., 2015; Gilgunn & Bones, 2018)
9	**BiP (78 kDa glucose regulated protein)**	(Joucla et al., 2013; Levy et al., 2016; Liu et al., 2019)
10	**C-X-C motif chemokine 3**	(Farrell et al., 2015; Gilgunn & Bones, 2018; Gilgunn et al., 2019)
11	Calmodulin	(Albrecht et al., 2018b; Liu et al., 2019)

12	Calreticulin	(Farrell et al., 2015; Gilgunn et al., 2019; Zhang et al., 2014)
13	**Carboxypeptidase D**	(Hu et al., 2016; Dick et al., 2008) (Dick, Qiu, Mahon, Adamo, & Cheng, 2008)
14	Catalase	(Ahluwalia et al., 2017; Gilgunn et al., 2019; Liu et al., 2019)
15	**Cathepsin B**	(Aboulaich et al., 2014; Albrecht et al., 2018b; Gilgunn et al., 2019; Levy et al., 2016; Migani et al., 2017; Gao et al., 2011; Yang et al., 2019; Zhang et al., 2016; Zhang et al., 2019)
16	**Cathepsin D**	Albrecht et al., 2018b; Bee et al., 2017; Bee et al., 2015; Fukuda et al., 2019; Gilgunn et al., 2019; Park et al., 2017; Ranjan et al., 2019; Robert et al., 2009; Singh et al., 2020; Zhang et al., 2016(Singh, Mishra, Yadav, Budholiya, & Rathore, 2020)
17	**Cathepsin E**	(Vanderlaan et al., 2018; Yang et al., 2019)
18	**Cathepsin L**	Gao et al., 2011; Luo et al., 2018; Park et al., 2017
19	**Cathepsin Z**	Chiverton et al., 2016; Gao et al., 2011; Park et al., 2017
20	Chondroitin sulfate proteoglycan 4	(Falkenberg et al., 2019; Levy et al., 2016)
21	**Clusterin**	Aboulaich et al., 2014; Albrecht et al., 2018a; Farrell et al., 2015; Gilgunn et al., 2019; Jawa et al., 2016; Kreimer et al., 2017; Levy et al., 2016; Migani et al., 2017; Singh et al., 2020; Zhang et al., 2014; Doneanu et al., 2012; Levy et al., 2014; Vanderlaan et al., 2018; Wilson & Easterbrook-Smith, 1992
22	Cofilin-1	(Aboulaich et al., 2014; Albrecht et al., 2018a; Albrecht et al., 2018b;

		Gilgunn et al., 2019; Liu et al., 2019)
23	DnaK-type molecular chaperone GRP78 precursor	(Falkenberg et al., 2019; Levy et al., 2016)
24	Elongation factor 1-alpha	(Falkenberg et al., 2019; Gilgunn et al., 2019; Jawa et al., 2016; Liu et al., 2019; Zhang et al., 2014)
25	Elongation factor 2	(Aboulaich et al., 2014; Albrecht et al., 2018b; Gilgunn et al., 2019; Jawa et al., 2016; Liu et al., 2019; Migani et al., 2017; Zhang et al., 2014)
26	Endoplasmin	(Albrecht et al., 2018b; Falkenberg et al., 2019; Gilgunn et al., 2019; Jawa et al., 2016; Zhang et al., 2014)
27	ERP57 protein	(Levy et al., 2016; Zhang et al., 2014)
28	Follistatin-related protein 1	(Levy et al., 2016; Migani et al., 2017; Zhang et al., 2014)
29	Fructose-bisphosphate aldolase	(Chiverton et al., 2016; Liu et al., 2019; Zhang et al., 2014)
30	Galectin 3 binding protein	(Gilgunn et al., 2019; Levy et al., 2016; Singh et al., 2020; Zhang et al., 2014)
31	Galectin-1	(Albrecht et al., 2018a; Albrecht et al., 2018b)
32	Galectin-3	(Aboulaich et al., 2014; Liu et al., 2019)
33	Glutathione S-transferase P 1	(Aboulaich et al., 2014; Gilgunn & Bones, 2018; Jawa et al., 2016; Zhang et al., 2014) (Albrecht et al., 2018b)
34	**Glutathione-S-Transferase (GST)**	(Goey et al., 2018; Hall et al., 2016; Jawa et al., 2016; Zhang et al., 2016; Zhang et al., 2014)
35	Glyceraldehyde-3-phosphate	(Aboulaich et al., 2014; Albrecht et

	dehydrogenase	al., 2018a; Albrecht et al., 2018b; Falkenberg et al., 2019; Farrell et al., 2015; Gilgunn et al., 2019; Jawa et al., 2016; Levy et al., 2016; Liu et al., 2019; Zhang et al., 2014)
36	Granulins	(Jawa et al., 2016; Zhang et al., 2014)
37	GTP-binding nuclear protein Ran	(Aboulaich et al., 2014; Gilgunn et al., 2019; Jawa et al., 2016)
38	Guanine nucleotide-binding protein beta-2-like 1	(Aboulaich et al., 2014; Gilgunn et al., 2019)
39	Heat shock cognate 71 kDa protein	(Aboulaich et al., 2014; Gilgunn et al., 2019; Jawa et al., 2016; Liu et al., 2019; Migani et al., 2017)
40	**Heat Shock Protein (DNAK)**	(Goey et al., 2018; Ratanji et al., 2017)
41	Heat shock protein HSP 90-alpha	(Albrecht et al., 2018b; de Zafra et al., 2015; Gilgunn et al., 2019; Jawa et al., 2016; Zhang et al., 2014)
42	Heat shock protein HSP 90-beta	(Albrecht et al., 2018b; Gilgunn et al., 2019; Jawa et al., 2016; Liu et al., 2019; Zhang et al., 2014)
43	Histone H2A	(Aboulaich et al., 2014; Gilgunn et al., 2019; Liu et al., 2019)
44	Histone H2B	(Aboulaich et al., 2014; Gilgunn et al., 2019)
45	Histone H3	(Gilgunn et al., 2019; Liu et al., 2019)
46	Inter-alpha-trypsin inhibitor heavy chain H5 isoform X2	(Falkenberg et al., 2019; Levy et al., 2016)
47	L-lactate dehydrogenase A chain	(Aboulaich et al., 2014; Albrecht et al., 2018b)

48	Lactadherin	(Aboulaich et al., 2014; Farrell et al., 2015; Levy et al., 2016)
49	lactotransferrin	(Kreimer et al., 2017; Liu et al., 2019)
50	Laminin subunit beta-1	(Falkenberg et al., 2019; Gilgunn et al., 2019; Levy et al., 2016; Zhang et al., 2014)
51	Laminin subunit gamma-1	(Gilgunn et al., 2019; Levy et al., 2016; Zhang et al., 2014)
52	Legumain	(Albrecht et al., 2018b; Levy et al., 2016; Migani et al., 2017)
53	**Lipoprotein Lipase (LPL)**	(Chiu et al., 2017; Levy et al., 2014; McShan et al., 2016; Gilgunn et al., 2019; Levy et al., 2016; Singh et al., 2020)
54	**Lysosomal Acid Lipase (LAL)**	(Levy et al., 2014; Liu et al., 2019; McShan et al., 2016)
55	**Lysosomal Phospholipase A2 (LPLA2)**	(Chiu et al., 2017; Hall et al., 2016; Levy et al., 2013; McShan et al., 2016; Shayman et al., 2011)
56	Lysosomal protective protein	Levy et al, 2014, Migani et al., 2017, Valente et al., 2015
57	Malate dehydrogenase, cytoplasmic	(Albrecht et al., 2018a; Albrecht et al., 2018b; Gilgunn et al., 2019)
58	**Matrix metalloproteinase-19**	(Aboulaich et al., 2014; Farrell et al., 2015; Gilgunn & Bones, 2018; Gilgunn et al., 2019; Levy et al., 2016; Migani et al., 2017)
59	Metalloproteinase inhibitor $_1$	(Aboulaich et al., 2014; Albrecht et al., 2018b; Levy et al., 2016)
60	**Monocyte Chemoattractant Protein-1 (MCP-1)**	(Leister et al., 2019; Vanderlaan et al., 2018; Yoshimura & Leonard, 1989)
61	Myosin-9	(Gilgunn et al., 2019; Liu et al., 2019)
62	Nidogen-1	(Aboulaich et al., 2014; Farrell et al., 2015; Gilgunn et al., 2019; Levy et al., 2016; Migani et al., 2017; (Singh et al., 2020)

63	Nucleobindin-2	(Levy et al., 2016; Migani et al., 2017)
64	Nucleoside diphosphate kinase B	(Aboulaich et al., 2014; Albrecht et al., 2018b)
65	**Peptidyl-prolyl cis-trans isomerase**	Aboulaich et al., 2014; Albrecht et al., 2018b; Falkenberg et al., 2019; Gilgunn et al., 2019; Jawa et al., 2016; Kreimer et al., 2017; Liu et al., 2019; Zhang et al., 2016
66	Peroxiredoxin-1	(Aboulaich et al., 2014; Albrecht et al., 2018b; Chiverton et al., 2016; Falkenberg et al., 2019; Farrell et al., 2015; Gilgunn et al., 2019; Jawa et al., 2016; Kreimer et al., 2017; Liu et al., 2019; Zhang et al., 2014)
67	Phosphoglycerate kinase	(Falkenberg et al., 2019; Gilgunn et al., 2019; Jawa et al., 2016; Liu et al., 2019; Zhang et al., 2014)
68	Phosphoglycerate mutase 1	(Aboulaich et al., 2014; Gilgunn et al., 2019; Liu et al., 2019; Zhang et al., 2014)
69	**Phospholipase B - like 2 (PLBL2)**	(Ahluwalia et al., 2017; de Zafra et al., 2015; Dixit et al., 2016; Fischer et al., 2017; Hanania et al., 2015; Hogwood, Ahmad, Tarrant, Bracewell, & Smales, 2016; Jawa et al., 2016; McShan, Kei, Ji, Kim, & Wang, 2016)
70	**Phospholipase D3**	(McShan et al., 2016; Zhang et al., 2020)
71	Phospholipid transfer protein	(Gilgunn et al., 2019; Zhang et al., 2014)
72	Plectin-1	(Aboulaich et al., 2014; Falkenberg et al., 2019)
73	Procollagen C-endopeptidase enhancer	(Farrell et al., 2015; Levy et al., 2016; Migani et al., 2017; Zhang et al., 2014)
74	**Procollagen-lysine 2-oxoglutarate 5-deoxygenase_1**	(Hogwood et al., 2016; Jawa et al., 2016; Zhang et al., 2014)

75	Proteasome subunit alpha type-7	(Aboulaich et al., 2014; Liu et al., 2019)
76	**Protein disulfide isomerase (PDI)**	Aboulaich et al., 2014; Kreimer et al., 2017; Migani et al., 2017; Gilgunn & Bones, 2018; Gilgunn et al., 2019; Goey et al., 2018; Maeda et al., 2007
77	**Protein S100-A6**	(Aboulaich et al., 2014; Gilgunn & Bones, 2018; Gilgunn et al., 2019; Paintlia et al., 2004)
78	Putative phospholipase B-like 2	(Aboulaich et al., 2014; de Zafra et al., 2015; Fischer et al., 2017; Jawa et al., 2016; Kreimer et al., 2017; Migani et al., 2017)
79	**Pyruvate Kinase**	Chiverton et al., 2016; Gilgunn et al., 2019; Jawa et al., 2016; Zhang et al., 2014Goey et al., 2018; Zhang et al., 2014
80	**Serine protease HTRA1**	Aboulaich et al., 2014; Dorai et al., 2011; Falkenberg et al., 2019; Farrell et al., 2015; Gilgunn & Bones, 2018; Gilgunn et al., 2019; Goey et al., 2018; Jawa et al., 2016; Zhang et al., 2014
81	Sulfated glycoprotein	(Aboulaich et al., 2014; Singh et al., 2020; Zhang et al., 2014)
82	T-complex protein 1 subunits	(Aboulaich et al., 2014; Gilgunn et al., 2019; Jawa et al., 2016; Zhang et al., 2014)
83	**Transforming Growth Factor-b1 (TGF-b1)**	(Beatson et al., 2011; Vanderlaan et al., 2018)
84	Transgelin-2	(Albrecht et al., 2018a; Albrecht et al., 2018b)
85	Transketolase	(Gilgunn et al., 2019; Zhang et al., 2014)
86	Triosephosphate isomerase	(Aboulaich et al., 2014; Gilgunn et al., 2019)

87	Tubulin alpha-1A chain	(Gilgunn et al., 2019; Zhang et al., 2014)
88	ubiquitin	(Kreimer et al., 2017; Liu et al., 2019; Zhang et al., 2014)
89	V-type proton ATPase subunit C $_1$	(Albrecht et al., 2018a; Liu et al., 2019)
90	Vimentin	(Aboulaich et al., 2014; Gilgunn et al., 2019)

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Table 2: Through extensive literature searches and working experiences of the BPOG HCP workstream, a list of high-risk HCPs was compiled and categorized based on their impact

Protein Name	Function	Impact	Type of Impact	References
Alpha-enolase	glycolytic enzyme that catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate	Drug Quality	modification of drug	(Valente et al., 2014; Zhang et al., 2014)
Annexin A5	apoptosis, plasma membrane derived microparticles	Immunogenicity	may cause an immunogenic response	(Farrell et al., 2015; Gilgunn & Bones, 2018)
BiP (78 kDa glucose regulated protein)	protein folding and quality control in the endoplasmic reticulum lumen	Drug Quality	Aggregation of drugs	(Joucla et al., 2013; Levy et al., 2016; Liu et al., 2019)
C-X-C motif chemokine 3	cytokine with potential oncogenic properties	Biological Function in Humans	potentially cause immunogenic response	(Farrell et al., 2015; Gilgunn & Bones, 2018; Gilgunn et al., 2019)
Carboxypeptidase D	Serine exopeptidase that release C-terminal amino acids.	Drug Quality	C-terminal truncation	(Hu et al., 2016; Dick et al., 2008)
Cathepsin B	lysosomal cysteine Protease responsible for intracellular proteolysis	Drug Quality	fragmentation of Drug	(Aboulaich et al., 2014; Gao et al., 2011; Yang et al., 2019; Zhang et al., 2016; Zhang et al., 2019)
Cathepsin D	active aspartyl protease with activity in both acidic and neutral pH	Drug quality	fragmentation of Drug	(Bee et al., 2017; Bee et al., 2015; Robert et al., 2009; Zhang et al., 2016 Ranjan et al., 2019)
Cathepsin E	aspartic protease with a vital role in protein	Drug Quality	fragmentation of Drug	(Vanderlaan et al., 2018; Yang et al., 2019)

	degradation and antigen processing via the MHC class II pathway			
Cathepsin L	Cysteine Protease	Drug Quality	fragmentation of Drug	Gao et al., 2011; Luo et al., 2018; Park et al., 2017
Cathepsin Z	Cysteine Protease responsible for protein degradation and turnover in the lysosome.	Drug Quality	fragmentation of Drug	Chiverton et al., 2016; Gao et al., 2011; Park et al., 2017
Clusterin	multifunctional disulfide-linked heterodimeric glycoprotein associated with clearance of celluar debris and apoptosis.	Immunogenicity	extracellular chaperone that prevents aggregation of non native proteins	(Doneanu et al., 2012; Levy et al., 2014; Vanderlaan et al., 2018; Wilson & Easterbrook-Smith, 1992)
Glutathione-S-Transferase (GST)	Conjugates reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles	Immunogenicity	may cause an immunogenic response	(Goey et al., 2018; Hall et al., 2016; Jawa et al., 2016; Zhang et al., 2016; Zhang et al., 2014)
Heat Shock Protein (DNAK)	binds hydrophobic regions and potentially cause protein aggregation	Drug Quality	Aggregation of drugs	(Goey et al., 2018; Ratanji et al., 2017)
Lipoprotein Lipase (LPL)	Catalyzes the hydrolysis of triacylglycerol of LDL and regulates the plasma concentrations of triglycerides and HDL.	Formulation	degradation of polysorbates	(Chiu et al., 2017; Levy et al., 2014; McShan et al., 2016)
Lysosomal Acid Lipase (LAL)	hydrolyzes cholesteryl esters and triglycerides	Formulation	degradation of polysorbates	(Levy et al., 2014; Liu et al., 2019; McShan et al., 2016)
Lysosomal	cleaves the acyl	Formulation	degradation of	(Chiu et al.,

Phospholipase A2 (LPLA2)	ester bonds of glycerophospholipids and produce free fatty acid and the corresponding lyso-glycerophospholipid		polysorbates	2017; Hall et al., 2016; Levy et al., 2013; McShan et al., 2016; Shayman et al., 2011)
Matrix Metalloproteinase	Calcium-dependent zinc-containing endopeptidases responsible for the degradation of extracellular matrix proteins	Drug Quality	degradation of drug	(Gilgunn & Bones, 2018; Gilgunn et al., 2019)
Monocyte Chemoattractant Protein-1 (MCP-1)	Acts as a ligand for C-C chemokine receptor	Biological Function in Humans	cytokine release	(Leister et al., 2019; Vanderlaan et al., 2018; Yoshimura & Leonard, 1989)
Peptidyl-prolyl cis-trans isomerase	folding and assembly in the ER	Drug Quality	Aggregation of drugs	(Zhang et al., 2016; Jawa et al., 2013)
Phospholipase B - like 2 (PLBL2)	Putative phospholipase	Drug Quality and immunogenicity	degradation of polysorbates	(Vanderlaan et al., 2015;Tran et al., 2016;Fischer et al., 2017 ;Dixit et al., 2016;Jawa et al., 2016;Zhang et al., 2020 ;McShan et al., 2016 ;Hanania et al., 2015)
Phospholipase D3	degrades polysorbates	Formulation	degradation of polysorbates	(McShan et al., 2016)
Procollagen-lysine 2-oxoglutarate 5-deoxygenase_1	catalyzes hydroxylation of lysine residues in collagen alpha chains and is required for normal assembly and cross-linking of	Immunogenicity	may cause an immunogenic response	(Hogwood et al., 2016; Jawa et al., 2016; Zhang et al., 2014)

	collagen fibrils			
Protein disulfide isomerase (PDI)	catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins	Drug Quality	reduction of disulfide bonds	(Gilgunn & Bones, 2018; Gilgunn et al., 2019; Goey et al., 2018; Maeda et al., 2007)
Protein S100-A6	Potential influence on Cell membrane	Immunogenicity	may cause an immunogenic response	(Aboulaich et al., 2014; Gilgunn & Bones, 2018; Gilgunn et al., 2019; Paintlia et al., 2004)
Pyruvate Kinase	Glycolytic enzyme	Immunogenicity	may cause an immunogenic response	(Goey et al., 2018; Zhang et al., 2016; Zhang et al., 2014)
Serine Protease HTRA1	Serine protease that can degrade proteoglycans	Drug Quality	Clipping of N-terminus	(Dorai et al., 2011; Gilgunn & Bones, 2018; Gilgunn et al., 2019; Goey et al., 2018)
Transforming Growth Factor-b1 (TGF-β1)	Maintaining immune homeostasis and immune suppression	Biological Function in Humans	cytokine release	(Beatson et al., 2011; Vanderlaan et al., 2018)

FIGURE LEGENDS:

Figure 1: HCPs detected/identified in final product and the demonstration of clearance of those HCPs

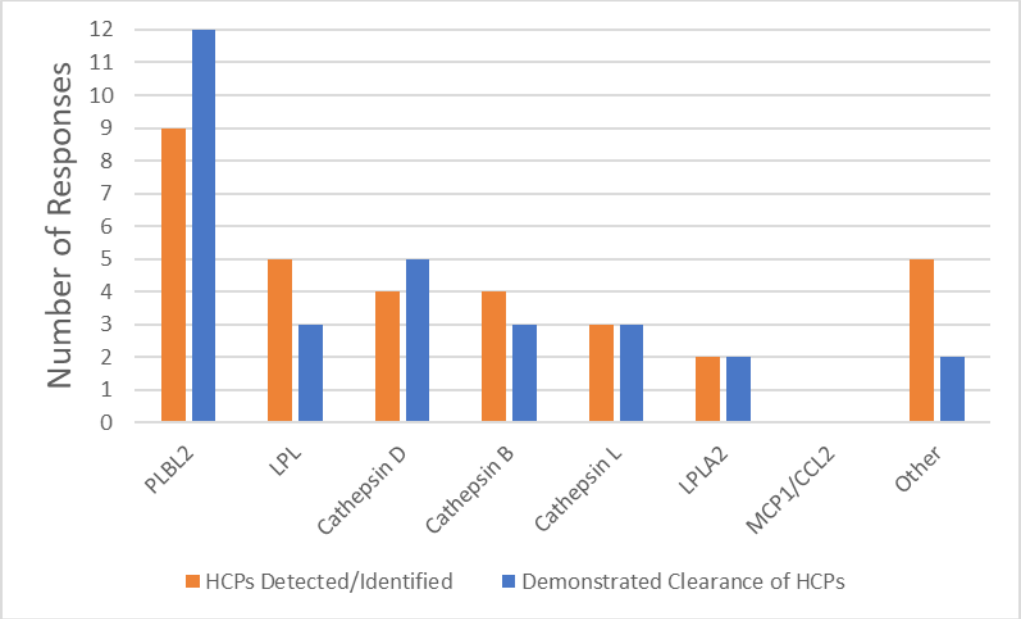

Figure 2: Methods used to identify and quantitate specific HCPs in drug product samples

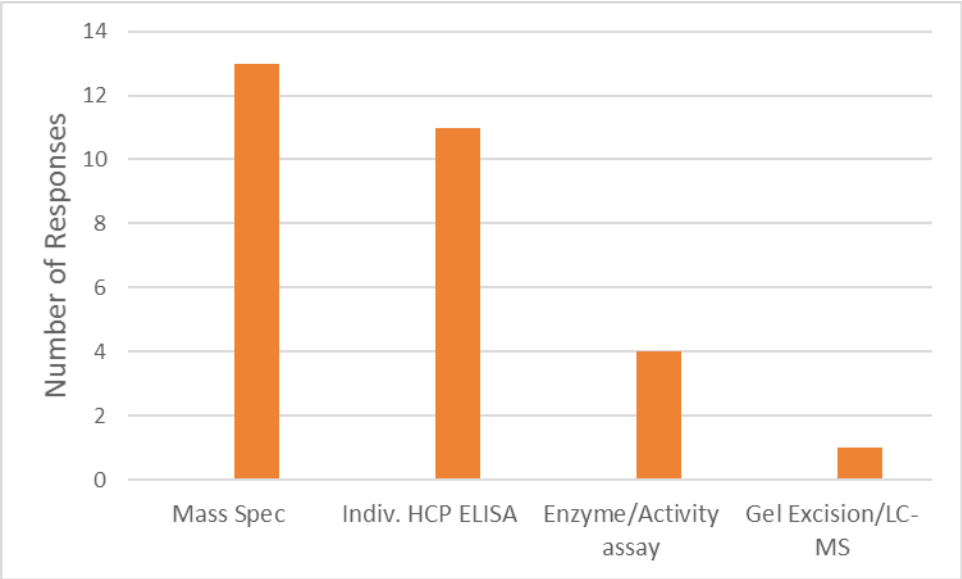

Figure 3: Analytical methods and/or technologies used to quantitate and characterize HCPs

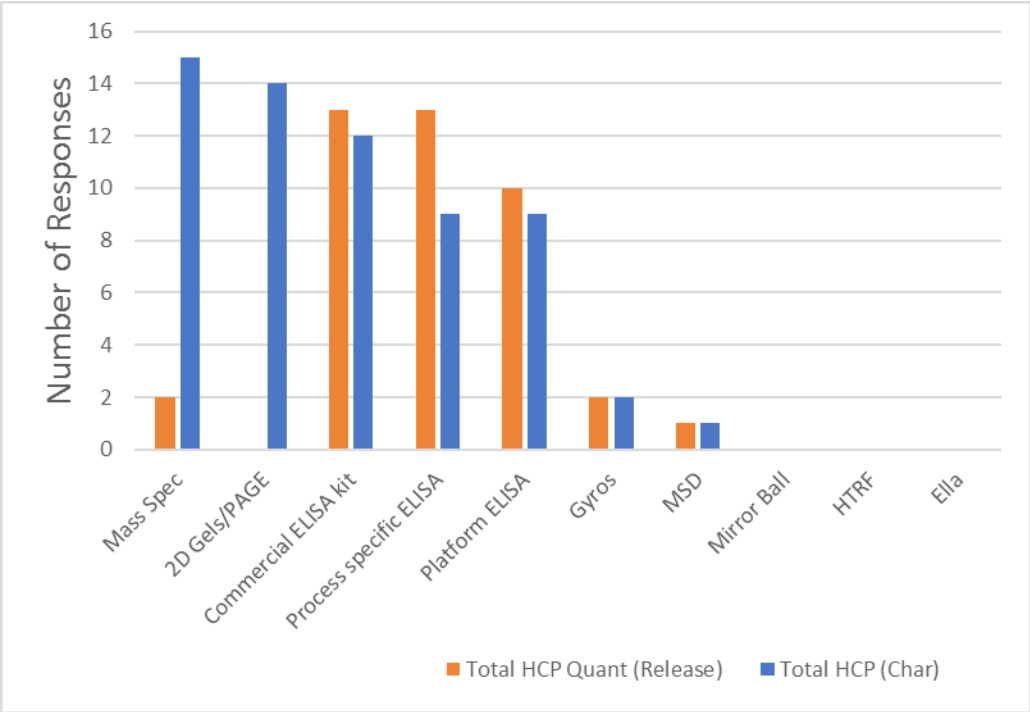

Figure 4: Application of mass spectrometry for HCP detection and quantitation. (**A**) Types of samples tested by MS analysis (**B**) Types of quantification used for MS analysis