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New insights into Factor VIII immunogenicity – an innovative method for personalized Hemophilia diagnostics

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Abstract

Therapeutic protein drugs are widely used to treat a variety of diseases. Although, they offer a favorable benefit-risk ratio, one key hurdle for the maintenance of clinical safety and efficacy has been the development of unwanted immune responses against them. While some patients develop harmless non-neutralizing antibodies, others develop pathogenic antibodies which attenuate or neutralize the biological activity of the protein drug or cause devastating health problems. Therefore, regulatory authorities demand immunogenicity risk assessments for new biological drug candidates during preclinical and clinical development. The establishment and validation of analytical methods for immunogenicity risk assessments of new drug candidates, is one of the main focus areas of the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems. One prominent example for pathogenic anti-drug antibodies is the development of neutralizing antibodies against blood coagulation Factor VIII in the course of replacement therapy of Hemophilia A patients with human Factor VIII products. These antibodies may neutralize the biological activity of Factor VIII and render replacement therapies ineffective resulting in life-threatening bleeding complications. Therefore, it is of utmost importance to distinguish non-neutralizing from neutralizing antibodies against Factor VIII and unravel nature and evolution of these. In close cooperation with the Research division of Baxalta Innovations GmbH, part of Shire plc, the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems is dedicated to generate novel insights into the evolution of antibodies against Factor VIII. For this purpose, two different strategies, which are based on state-of-the-art ELISA-based platform technologies that were validated/qualified according to current regulatory guidelines of EU- and US-regulatory authorities, are followed: (1) the analysis of plasma samples obtained during clinical studies involving Hemophilia A patients treated with Factor VIII; (2) initiation of an inhouse research project to improve the understanding of immune regulation and the nature of harmless and pathogenic anti-drug antibodies. Particularly the latter is intended to establish a scientific basis for the development of novel, patient-specific diagnostics with the intention to enable early personalized therapies.

Keywords:

Blood coagulation, Hemophilia A, Factor VIII, Immunogenicity, Anti-Drug Antibodies

Since the approval of the first recombinant, therapeutic protein drug, recombinant human Insulin in 1982, more than 200 therapeutic protein drugs have entered the market (Dewan 2016). Therapeutic protein drugs have been widely used to treat a variety of diseases including cancer, autoimmune diseases, neurological diseases, metabolic diseases, bleeding disorders and others. The huge success of these drugs is related to their high specificity and low intrinsic toxicity. Although most protein drugs offer a favorable benefit-risk ratio, one key hurdle for the maintenance of clinical efficacy and safety has been the development of unwanted immune responses against protein drugs, in particular the development of anti-drug antibodies (Koren et al. 2002, Schellekens / Casadevall 2004, Yin et al. 2015). The root cause for the development of anti-drug antibodies is not well understood. Some patients develop harmless antibodies which resemble natural low-affinity autoantibodies found in healthy individuals, other patients develop pathogenic antibodies which attenuate or neutralize the biological activity of the exogenously supplied protein or cause devastating health problems such as anaphylaxis or autoimmune pathologies (Gunn et al. 2016). Therefore, regulatory authorities demand immunogenicity risk assessments for biological drugs and new biological drug candidates during preclinical and clinical development. The establishment and validation of analytical methods for immunogenicity risk assessments of new drug candidates, is one of the main focus areas of the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems. New analytical methods are designed and applied to identify, characterize and quantify potential adverse immune reactions such as the development of anti-drug antibodies but also the potential to induce the release of inflammatory cytokines or the potential to activate the complement system.

One prominent example for pathogenic anti-drug antibodies is the development of neutralizing antibodies against blood coagulation Factor VIII (FVIII) in the course of replacement therapy of Hemophilia A patients with human FVIII products (DiMichele 2013). About 20-30% of patients with severe Hemophilia A (plasma FVIII activities <1%) and about 3-13% of patients with moderate (plasma FVIII activity 1-5%) and mild (plasma FVIII activity 5-25%) Hemophilia A develop neutralizing antibodies (Gouw et al 2013, Hay 1998). Anti-drug antibodies against FVIII may neutralize its biological activity and render replacement therapies ineffective resulting in life-threatening bleeding complications. The development of neutralizing antibodies is therefore associated with an increased morbidity and a decreased health-related quality of life (HRQoL) for patients (Gringeri et al 2003). Immune tolerance induction (ITI) therapy using regular high-dose applications of FVIII over a period of up to two or more years is the only strategy that has been proven to successfully eradicate neutralizing antibodies and enable normal therapy with FVIII products. Besides being a huge burden for patients and their care givers, ITI is only successful in 60-85% of patients with neutralizing antibodies (DiMichele 2013). Therefore, it is of utmost importance to unravel nature and evolution of pathogenic neutralizing antibodies against FVIII in order to facilitate the development of advanced diagnostic tools which would allow for early personalized therapies to prevent the development of antibodies interfering with FVIII replacement therapy in Hemophilia A patients.

In close cooperation with the Research division of Baxalta Innovations GmbH, now part of Shire plc, the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems is dedicated to generate novel insights into the evolution of pathogenic antibodies against FVIII in patients with Hemophilia A. For this purpose, we have followed two different strategies: (1) support the Research division of Baxalta Innovations GmbH, part of Shire plc, with the analysis of plasma samples obtained during clinical studies involving Hemophilia A patients treated with FVIII; (2)

initiation of an inhouse research project which is run in close cooperation with the Research division of Baxalta Innovations GmbH, part of Shire plc.

In the last two years, we established and optimized two ELISA-based platform technologies for the identification, quantification and characterization of FVIII-specific antibodies: a FVIII-binding antibody ELISA platform and a competition based apparent affinity ELISA platform.

The FVIII-binding antibody ELISA platform enables the discrimination, semi-quantitative characterization and FVIII-specificity testing of a broad spectrum of FVIII-binding antibodies of different isotypes (IgM, IgG, IgA) and IgG subclasses (IgG1, 2, 3, 4) (Whelan et al. 2012). The technology platform involves three sequential analytical steps:

- 1.) Ig isotype- and IgG subclass-specific screening ELISAs to identify samples with FVIII-specific antibodies
- 2.) Ig isotype- and IgG subclass-specific titration ELISAs to determine the corresponding titer levels to the detected antibodies.
- 3.) Ig isotype- and IgG subclass-specific competition ELISAs to confirm FVIII-specificity of the identified and quantified anti-FVIII antibodies.

All individual assays were established and validated according to current regulatory guidelines of the European Medicines Agency (EMA) and United States' Food and Drug Administration (FDA) which makes them applicable for sample analysis during clinical assessment of FVIII products and new FVIII product candidates (EMA/CHMP/BMWP 2015, FDA/CDER/CBER/CDRH 2016).

The second platform technology, a competition based apparent affinity ELISA platform, was established and optimized for FVIII-specific IgG subclasses (IgG 1-4), and for IgA. This platform is based on modified competition ELISAs according to Hofbauer et al 2015. Therefore, a defined series of FVIII-concentrations is incubated with a plasma sample to be analyzed. After incubation, unbound antibodies within the sample are detected by ELISA. By blotting the ELISA read-outs as a function of the different FVIII competition concentrations, a sample-specific competition function is obtained. Upon analysis with a non-linear regression model, which is based on previous publications by Stevens and Bobrovnik (Stevens / Bobrovnik 2007, Bobrovnik et al. 2010), apparent affinity parameters for up to two different antibody populations in the same plasma sample can be determined (Hofbauer et al. 2015). In order to demonstrate reliability of the modified competition ELISAs, test parameters such as precision (inter- and intra-assay variability) and robustness, as defined in current EMA and FDA regulatory guidelines (EMA/CHMP/BMWP 2016, FDA/CDER/CBER/CDRH 2016), were assessed during assay qualification.

The major advantage of the two platform technologies established at the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems is the utilization of very small volumes of native, human plasma without the need for antibody purification. This is essential for the target population of previously untreated Hemophilia A patients. The patients are born with the disease and usually receive their first dose of FVIII during the first 18 months of their life. The other advantage of using native human plasma samples rather than purified antibody preparations is the prevention of artefacts which might be introduced during the purification procedure.

After establishment and validation respectively qualification of all assays, which are part of the two platform technologies, a cross-qualification study with 21 blinded, human plasma samples was performed at the two research sites: the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems and the research labs of Baxalta Innovations GmbH, part of

Shire plc, in Vienna. Both, validation/qualification and cross-qualification studies delivered subclass/isotype-specific FVIII-binding Ab-titers and associated apparent affinity values as expected. Hence, performance similarity of the two ELISA platforms could be demonstrated at both laboratory sites.

Using these two platform technologies we have participated in the analysis of human plasma samples obtained during the Hemophilia Inhibitor PUP (previously untreated patients) study (HIPS, clinicaltrials.gov NCT01652027). HIPS is a prospective multicenter observational study with the primary objective to elucidate immune system changes in severe Hemophilia A patients during their first 50 exposure days to a FVIII product. So far, 25 patients in 19 study centers in the US and Europe have been enrolled in this study. Interim data coming out of this study suggested that the development of neutralizing antibodies against FVIII is preceded by the detection of high-affinity FVIII-specific antibodies which undergo class switch from IgG1 and IgG3 to IgG4 (Reipert et al. 2016). The findings confirm affinity maturation of FVIII-specific antibodies which require T-cell dependent antibody responses resulting from germinal center reactions. While low affinity IgG1 antibodies were sometimes seen in subjects without neutralizing antibodies, high-affinity antibodies were only seen in subjects who developed neutralizing antibodies. These data indicate that high affinity FVIII-specific antibodies preceded the clinical diagnosis of neutralizing antibodies and may serve as early biomarkers for the development of neutralizing antibodies against FVIII facilitating early immune intervention (Reipert et al. 2016).

In addition to neutralizing antibodies against FVIII, there have also been reports on the development of non-neutralizing antibodies in patients undergoing FVIII replacement therapy. The functional activity of these antibodies is not fully understood (Whelan et al. 2012, Klintman et al. 2013). Some of these antibodies have similar characteristics as low-affinity autoantibodies against FVIII which are found in some healthy individuals (Whelan et al. 2012). These antibodies are mostly of low-affinity and can persist long-term without any pathology (Hofbauer et al. 2015). Other non-neutralizing antibodies reduce the half-life of FVIII in patients which could result in the requirement for more frequent treatments and higher doses of FVIII, or increase its clearance rate (Batsuli et al. 2016). These findings are supported by clinical reports of patients with undetectable neutralizing antibodies who have severe bleeding episodes despite infusions of high doses of FVIII (Kempton et al. 2011).

Earlier studies done at the research labs of Baxalta Innovations GmbH, part of Shire plc, had indicated that Hemophilia A patients with neutralizing antibodies have a distinct spectrum of FVIII-specific IgG subclasses when compared to patients with non-neutralizing antibodies. (Whelan et al 2012). Moreover, FVIII-specific antibodies found in patients with neutralizing antibodies demonstrated an up to 100-fold higher apparent affinity than antibodies found in patients with non-neutralizing antibodies (Hofbauer et al. 2015).

These results demonstrate that differentiating neutralizing from non-neutralizing antibodies is evidently not sufficient to understand and predict the pathogenic nature of FVIII-specific antibodies in patients. Up to now, no universal diagnostic tool to foresee and distinguish the development of harmless and/or pathogenic antibodies in an individual patient is available. Therefore, research efforts are required to improve the understanding of immune regulation and the nature of harmless and pathogenic anti-drug antibodies as well as to establish a scientific basis for the development of novel, patient-specific diagnostics. Based on the two ELISA-based platform technologies, the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems in cooperation with Baxalta Innovations GmbH, part of Shire plc, has initiated a new research project with the aim to close a major gap in understanding the evolution of pathogenic antibodies against FVIII in patients with Hemophilia A. Particularly, novel insights on the temporal association between epitope specificity, affinity, isotype/IgG subclass profiles and functional activities of antibodies against FVIII, which develop in patients following replacement therapy with FVIII products, are to be generated.

For this purpose, the following questions will be addressed:

- 1.) Is the antibody response to FVIII restricted to protein epitopes or might carbohydrate residues also be targeted?
- 2.) Is there a correlation between the nature of the epitopes and their capacity to interfere with the biological function of FVIII?
- 3.) Is there a difference in the affinity and/or the isotype/IgG subclass profile between antibodies directed against carbohydrate structures or protein epitopes?
- 4.) Is there a temporal association between epitope specificity, affinity and isotype/IgG subclass profile of FVIII-specific antibodies which develop in patients undergoing replacement therapy with FVIII products?

In order to address questions 1 and 3, the nucleotide sequences encoding FVIII-protein domains have been cloned into mammalian expression vectors, host cells were transfected and best-expressing clones were selected and cell banked. In parallel, deglycosylation studies are ongoing with the intention to remove sugar moieties from purified recombinant FVIII and FVIII-domains to compare them to their glycosylated pendants upon their use in the ELISA-based platform technologies and further affinity evaluation technologies (e.g. Surface Plasmon Resonance (SPR) and Biolayer Interferometry (BLI)). Similarly, commercially available monoclonal anti-FVIII antibodies, artificially synthesized published FVIII epitopes and FVIII-epitopes identified by peptide mapping technologies will be included in the studies. Furthermore, the establishment of functional assays was started to evaluate how antibodies against carbohydrate structures and antibodies against protein epitopes of FVIII interfere with the biological function of FVIII once they are identified (question 2). Based on these outcomes, a microarray containing protein and carbohydrate epitopes of FVIII will be rationally designed and tested to address question 4. The microarray will be tested using plasma samples from patients with neutralizing or non-neutralizing antibodies against FVIII. Finally, longitudinal samples obtained from Hemophilia A patients undergoing FVIII therapy will be tested. Thereby, novel research findings could be directly translated into clinical application. The results of this applied research project might provide the scientific basis to differentiate patient-specific characteristics, design personalized treatment approaches and ultimately improve patient outcomes by an early recognition of evolving pathogenic antibodies against FVIII.

The following figure (Figure 1) provides the workflow of the research project which was initiated at the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems.

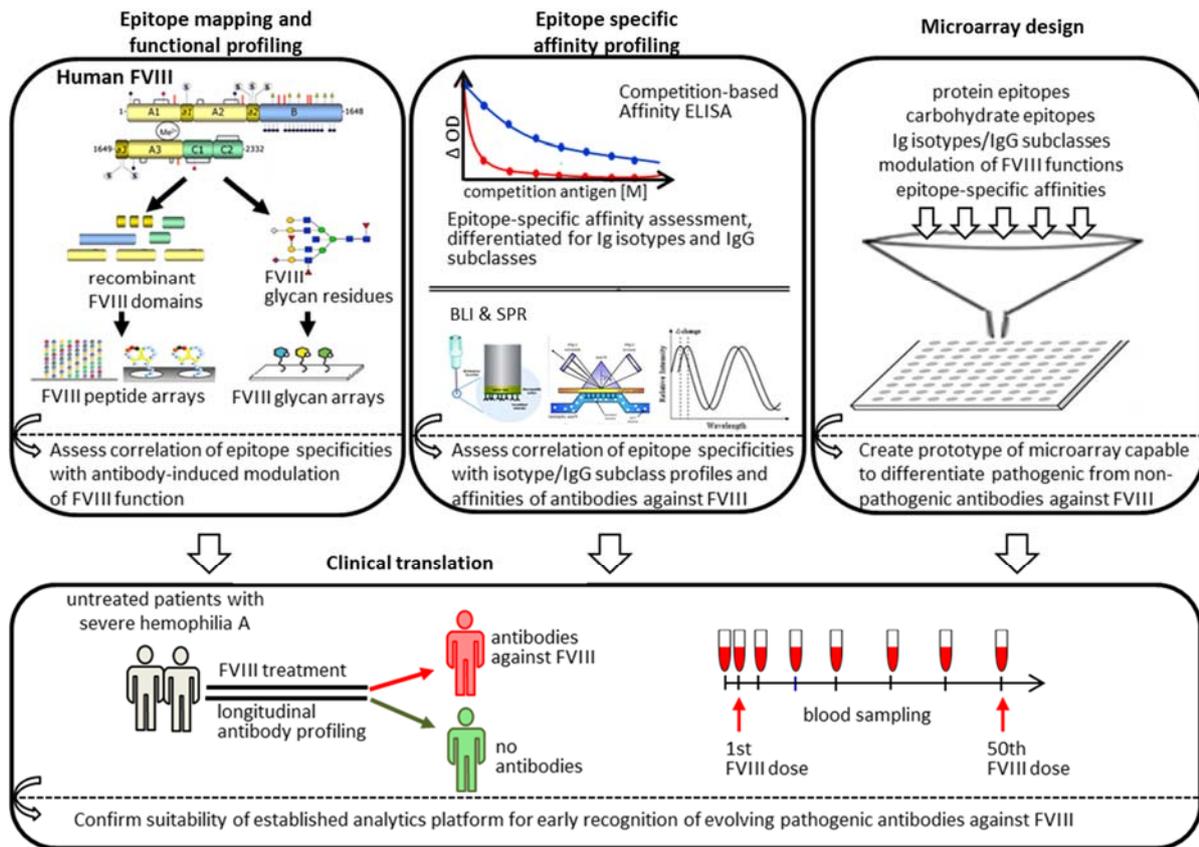


Figure 1: Work flow for the research project which was initiated at the Research Institute for Applied Bioanalytics and Drug Development at the IMC University of Applied Sciences Krems.

References

- Dewan, Shalini Shahani (2016): Global Markets and Manufacturing Technologies for Protein Drugs. In: BCC Research Report BIO021E
- Koren, E./Zuckerman, L.A./Mire-Sluis, A.R. (2002): Immune responses to therapeutic proteins in humans – clinical significance, assessment and prediction. In: *Curr Pharm Biotechnol* 3:2002, 349–60.
- Schellekens, H./Casadevall N. (2004): Immunogenicity of recombinant human proteins: causes and consequences. In: *J Neurol* 251 (Suppl. 2), II4–9.
- Yin, L./Chen, X./Vicini, P./Rup, B./Hickling, T.P. (2015): Therapeutic outcomes, assessments, risk factors and mitigation efforts of immunogenicity of therapeutic protein products. In: *Cell Immunol* 295, 118-126.
- Gunn, G.R./Sealey, D.C.F./Jamali, F./Meibohm, B./Ghosh, S./Shankar, G. (2016): From the bench to clinical practice: understanding the challenges and uncertainties in immunogenicity testing for biopharmaceuticals. In: *Clin Exp Immunol* 184, 137–146.
- DiMichele, D.M. (2013): Inhibitors in childhood hemophilia A: genetic and treatment-related risk factors for development and eradication. In: *Pediatr Blood Cancer* 60 Suppl 1, 30-3
- Gringeri, A./Mantovani, L.G./Scalone, L./Mannucci, P.M. (2003): COCIS Study group. Cost of care and quality of life for patients with hemophilia complicated by inhibitors: the COCIS Study Group. In: *Blood* 102, 2358–63.
- Gouw, S.C./van der Bom, J.G./Ljung, R./Escriola, C./Cid, A.R./Claeyssens-Donadel, S./van Geet, C./Kenet, G./Mäkipernaa, A./Molinari, A.C./Muntean, W./Kobelt, R./Rivard, G./Santagostino, E./Thomas, A./van den Berg, H.M. (2013): PedNet and RODIN Study Group. Factor VIII products and inhibitor development in severe hemophilia A. In: *N Engl J Med*. 368(3), 231-239
- Hay, C.R. (1998): Factor VIII inhibitors in mild and moderate-severity haemophilia A. In: *Haemophilia* 4(4), 558-563.
- Whelan, S.F.J./Hofbauer, C.J./Horling, F.M./Allacher, P./Wolfsegger, M.J./Oldenburg, J./Male, C./Windyga, J./Tiede, A./Schwarz, H.P./Scheiflinger, F./Reipert, B.M. (2012): Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophilia A patients. In: *Blood* 121(6), 1039-48.
- Klintman, J./Hillarp, A./Donfield, S./Berntorp, E./Astermark, J. (2013): Antibody formation and specificity in Bethesda-negative brother pairs with haemophilia A. In: *Haemophilia* 19(1), 106-12
- Hofbauer, C.J./Whelan, S.F.J./Hirschler, M./Allacher, P./Horling, F.M./Lawo, J.P./Oldenburg, J./Tiede, A./Male, C./Windyga, J./Greinacher, A./Knöbl, P.N./Schrenk, G./Koehn, J./Scheiflinger, F./Reipert, B.M. (2015): Affinity of FVIII-specific antibodies reveals major differences between neutralizing and nonneutralizing antibodies in humans. In: *Blood* 125(7), 1180-8.
- Batsuli, G./Wei, D./Healey, J.F./Parker, E.T./Baldwin, W.H./Cox, C./Nguyen, B./Kahle, J./Königs, C./Li, R./Lollar, P./Meeks, S.L. (2016): High-affinity, non-inhibitory pathogenic C1 domain antibodies are present in patients with hemophilia A and inhibitors. In: *Blood* 128, 2055-2067.

Kempton, C.L./Meeks, S.L./Donald Harvey, R./Abshire T.C. (2011): Evaluation of factor VIII pharmacokinetics and anti-factor VIII antibodies in four boys with haemophilia A and a poor clinical response to factor VIII. In: Haemophilia 17(1), 155-156.

European Medicines Agency (EMA), Committee for Medicinal Products for Human Use (CHMP), Biosimilar Medicinal Products Working Party (BMWP) (2016): Guideline on Immunogenicity assessment of biotechnology-derived therapeutic proteins. Reference document: EMEA/CHMP/BMWP/14327/2006 Rev. 1

U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Center for Devices and Radiological Health (CDRH) (2016): Draft Guidance for Industry: Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products

Stevens, F.J./Bobrovnik, S.A. (2007): Deconvolution of antibody affinities and concentrations by non-linear regression analysis of competitive ELISA data. In: J Immunol Methods 328(1-2), 53-8

Bobrovnik, S.A./Demchenko, M./Komisarenko, S./Stevens, F. (2010): Traditional ELISA methods for antibody affinity determination fail to reveal the presence of low affinity antibodies in antisera: an alternative approach. In: J Mol Recognit 23(5), 448-56

Reipert BM, Gangadharan B, Hofbauer CJ, Scheifflinger F, Bowen J, Donnachie E, Fijvandraat K, Gruppo R, Klintman J, Male C, McGuinn C, Meeks S, Recht M, Ragni MV, Yaish HM, Santagostino E, Brown DL. Appearance of High-Affinity Antibodies Precedes Clinical Diagnosis of FVIII Inhibitors -

Preliminary Analysis from the Hemophilia Inhibitor PUP Study (HIPS). Oral presentation, 59th Annual Meeting of the American Society for Hematology, San Diego, USA, December 2016