DR STEVEN PIPE (Orcid ID : 0000-0003-2558-2089)

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Title: New treatment paradigm for hemophilia poses challenges for legacy bioassays

Author: Steven W. Pipe, MD

Departments of Pediatrics and Pathology

University of Michigan

Ann Arbor, MI USA

Correspondence:
Steven W. Pipe, MD
Professor of Pediatrics and Pathology
University of Michigan
ummdswp@med.umich.edu
Phone: (734) 232-9335; Fax: (734) 615-0464

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The one-stage factor VIII (FVIII) clotting assay measures the extent by which a plasma sample corrects the coagulation time of FVIII-deficient plasma in an activated partial thromboplastin time(aPTT)-based assay[1]. The availability of this "surrogate" measure of FVIII activity has been the foundation of *clinical diagnostics* for hemophilia as it can be used to establish a diagnosis of FVIII deficiency, assign severity, and identify the presence of neutralizing inhibitors[2]. Approximately three-quarters of all clinical laboratories globally rely on the one-stage aPTT-based clotting assay to measure FVIII[3]. Other FVIII assays may be required for full diagnostic precision, such as chromogenic FVIII activity assays in order to accurately phenotype patients with non-severe hemophilia A[2].

FVIII activity assays have also become a cornerstone for the *management* of hemophilia A, allowing for dose adjustment of FVIII replacement, monitoring of FVIII levels during treatment and prophylaxis, and optimization of FVIII dosing for pharmacokinetic (PK)-guided prophylaxis[4]. In recent years, the recombinant platform has allowed for the bioengineering of FVIII with improved properties such as extended half-life, reduced immunogenicity, and enhanced affinity for von Willebrand factor (VWF)[5]. The bioengineered FVIII molecules have posed some challenges, as discrepancies have been observed between the one-stage and the chromogenic assays, but these may be overcome with product-specific standards[6, 7]. Where there may still be challenges in the interpretation of one-stage and chromogenic FVIII assays, thrombin generation assays have also been utilized for monitoring FVIII replacement[8]. However, the recent introduction of a novel therapeutic is changing the paradigm of reliance on these surrogate FVIII assays for the management of hemophilia.

Emicizumab-kxwh (Hemlibra, F. Hoffmann–La Roche, Basel, Switzerland) is a humanized bispecific monoclonal antibody which substitutes for missing FVIIIa by bridging FIXa and FX to promote effective hemostasis in persons with hemophilia A[9]. It has no sequence homology to FVIII therefore it does not induce FVIII inhibitors and its function is not impaired in the presence of FVIII inhibitors. Emicizumab has a high bioavailability, allowing for subcutaneous administration, and has a half-life of approximately 30 days[10]. PK profiles demonstrate that at steady-state, trough levels can be maintained sufficient to provide effective bleed control with weekly, 2-weekly and 4-weekly maintenance dosing regimens[11]. As such, dosing regimens can be chosen based on patient and/or physician preference and pharmacokinetic monitoring is not required.

Does this new treatment paradigm mean that laboratory assays are no longer necessary when patients are on emicizumab prophylaxis? Several management issues remain that could be addressed with another "surrogate" assay such as the one-stage FVIII assay has served. There is evidence for interpatient variability of the peak and trough levels at steady state, though the clinical trials have not identified any significant correlation with clinical outcome measures[10, 12]. However, a Japanese study in hemophilia with inhibitors showed a clinical improvement in 4 subjects with dose up-titration[13]. Clinicians have 50 years of experience in

the correlation of bleed control with FVIII levels in most every imaginable clinical scenario (eg. minor and major surgery, trauma, central nervous system bleeding, use of concomitant anticoagulants). There may yet be some value in a similar correlation of bleeding control in such scenarios with emicizumab prophylaxis within the range of inter- and intrapatient variability of their levels. More importantly, we do not have sufficient data on the PK profiles of the youngest infants and how that changes over time. This is an age group that represents a significant treatment gap as they are typically not placed on prophylaxis with FVIII replacement due to the challenges of intravenous infusions, and consequently may be one of the most significant beneficiaries of this therapy. In addition, anti-drug antibodies, though they seem to occur infrequently with the use of emicizumab, can affect the clearance of the drug, or neutralize its function, either of which could impact bleed control[14]. Given the vast clinical experience with FVIII assays to date, it's not surprising that clinicians would be looking for an assay of emicizumab that could provide some estimate of FVIII activity equivalence that could address these remaining management challenges.

The manuscript from Leksa et al. [15] is an excellent evaluation of the potential utility of coagulation assays that have been traditionally used for evaluating FVIII activity, including one-stage clotting assays, chromogenic assays and thrombin generation. They have prepared a sequence-identical version of the commercially available bispecific antibody, emicizumab, as well as a novel bispecific antibody bioengineered for more specificity in its affinity for FIXa and FX. They conclude that the significant differences in the mechanism of action of these bispecific antibodies (bsAbs) leads to a wide range of "FVIII-like" activity readouts such that the utility of these assays to establish FVIII bioequivalence is limited.

This is not unexpected given that emicizumab and FVIII are fundamentally different proteins and are regulated differently[16]: a) FVIIIa has multiple sites of interaction with FIXa, FX and the phospholipid surface whereas emicizumab binds to single sites within FIX(a) and FX(a), b) FVIII needs to be activated (thrombin mediated), emicizumab does not, c) FVIII binds to VWF, emicizumab does not, d) FVIIIa binds to the phospholipid surface, emicizumab does not, and FVIIIa binding limits movement of the FX-activating complex more than emicizumab, e) FVIIIa binds to the surface of activated platelets, emicizumab probably does not, f) FVIIIa has a

much higher binding affinity for enzyme and substrate than emicizumab, g) FVIIIa enhances FXa generation ~10-fold over emicizumab and h) emicizumab can bind both activated and non-activated forms of FIX and FX[17].

The one-stage aPTT-based FVIII clotting assay has particular limitations. Similar to the observations within the preclinical evaluation and clinical trial program with emicizumab, Leksa and colleagues show that the clotting time is maximally shortened by the bsAbs with no evidence for a dose response. The kinetic advantage of the bsAbs leads to this significant overestimation of the FVIII activity. This also has implications for evaluating for FVIII inhibitors while on emicizumab as the Bethesda assay is typically performed with an aPTT-based clotting assay. Given the reliance of most clinical laboratories on these assays, it is likely that patients on emicizumab may still have these assays performed, with misleading results and potential adverse implications for clinical care, even though appropriate guidance is provided in the prescribing information. They next show that a chromogenic FVIII assay that uses human reagents detects the procoagulant effect of the bsAbs. However, the purified reagents include only FIXa, thus this assay does not evaluate any impact of bsAb affinity for unactivated FIX. In addition, the FVIII and bsAb dose-response curves lack parallelism and do not scale proportionately, limiting the ability for this assay to be used to establish FVIII bioequivalence. Finally, they show that the thrombin generation assay has multiple variables that can limit the ability of this global assay to assign FVIII bioequivalence – the triggering reagent, phospholipid concentration, and multiple readout parameters that do not yet have clinical correlates with hemostatic protection from clinical trials. Notably, each of these assays rely on the fact that FVIIIa is the limiting factor, while levels of FIXa are in excess over FVIIIa. In assays with bsAbs, FIXa also becomes a limiting component. FIXa levels can differ strongly from one assay to the other, which may contribute to the challenge of establishing FVIII-equivalence.

The extensive clinical trial program for emicizumab demonstrated that it can be used safely with high efficacy without the need for laboratory monitoring while on prophylactic dosing. Nevertheless, some useful recommendations for the use of laboratory assays have been adopted. A chromogenic-based Bethesda assay that uses bovine reagents has been demonstrated to allow for measurement of FVIII inhibitors[18] and though not widely available,

is now offered nationally to clinicians in the USA through the laboratory at the Centers for Disease Control. Chromogenic FVIII assays that use bovine reagents can also be used to assay the FVIII activity of any additional FVIII concentrate administered or endogenous FVIII. Alternatively, a chromogenic FVIII assay that uses human reagents could be used to follow emicizumab activity for evaluation of PK, to correlate with clinical outcomes, monitor for changes in peak/trough levels over time, or to evaluate any apparent loss of efficacy. However, the widely available conventional aPTT and clot-based FVIII activity assays can still be used in evaluating a case of loss of efficacy. The aPTT should be within the normal range in all persons with hemophilia when obtained during ongoing treatment with emicizumab; therefore, a prolonged aPTT assay and/or a low one-stage FVIII activity in a person treated with emicizumab would be a useful initial evaluation for the presence of a neutralizing anti-drug antibody directed against emicizumab.

The manuscript from Leksa et al.[15], while highlighting the challenges for assigning FVIII bioequivalence with currently available clinical assays, provides important biochemical insights into the mechanism of action of bsAbs and their impact on the kinetics of coagulation. This should help support the development of novel clinical assays, not to derive FVIII bioequivalence, but rather to establish a benchmark "surrogate" activity which can be correlated with clinically relevant outcomes in hemophilia, just as we did for FVIII in the past.

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