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REVIEW ARTICLE

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Laboratory issues in gene therapy and emicizumab

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Abstract

The treatment options for the haemostatic disorders, haemophilia A and haemophilia B, have progressed rapidly over the last decade. The introduction of extended half-life recombinant factor VIII (FVIII) and factor IX (FIX) concentrates to replace these missing clotting factors highlighted discordance between one-stage activated partial thromboplastin time (APTT)-based clotting factor assays and chromogenic factor assays with some products. This raised awareness of the importance of investigation of potential reagent or assay differences by pharmaceutical companies. In 2017, the FVIII mimetic, emicizumab, was approved as a prophylactic treatment for haemophilia A patients with anti-FVIII inhibitors. The mechanism of action of emicizumab causes interference with some commonly used haemostasis tests including the APTT and its associated one-stage APTT-based clotting assays. Chromogenic assays may also be affected but this is dependent on the particular constituents of the reagents. Chromogenic assays containing human factor IXa (FIXa) and factor X (FX) are sensitive to the presence of emicizumab but those containing bovine FIXa and FX are unaffected. Many haemostasis laboratories have been required to evaluate new assays to enable accurate monitoring of emicizumab in patient plasma. A number of gene therapy approaches have been trialled in both haemophilia A and haemophilia B but there are scant data published on the measurement of FVIII and FIX in these patients and whether there are discrepancies between reagents or assay methodologies.

KEYWORDS

chromogenic assay, emicizumab, factor IX, factor VIII, gene therapy, haemophilia

1 | INTRODUCTION

Haemophilia A and haemophilia B (HA and HB) are X-linked haemorrhagic disorders caused by a reduction in clotting factor VIII (FVIII) or factor IX (FIX) activity. For many years, the treatment of haemophilia has been by replacement of the specific missing factor. Advances in novel treatments for haemophilia A and haemophilia B have progressed significantly over the last decade. These include use of a FVIII mimetic drug, emicizumab (HEMLIBRA®, Chugai Pharmaceutical Co), to activate factor X in the tenase complex and generate the thrombin necessary for the formation of a fibrin clot in the absence of FVIII.[1] Gene therapy technologies have also been used to introduce functional FVIII or FIX into human cells with the aim of inducing permanent expression of clotting factor at either low (around 5 per cent of the normal factor level) or near-normal levels.[2] Experience with recombinant FVIII and FIX molecules that have been modified to extend the half-life of the administered protein has highlighted that the assay and reagents chosen to measure these molecules can greatly impact the final result reported by the laboratory.[3-6] The activated partial thromboplastin time (APTT)-based one-stage (OS) clotting assay and the chromogenic (CS) factor activity assay are frequently used in the diagnosis and classification of haemophilia disease severity, as well as the monitoring of haemophilia treatment efficacy. Reagent-dependent discrepancies in the OS factor activity assay, as well as discrepancies between the OS and CS factor activity assays, have been described for a number of the recently approved extended half-life (EHL) HA and HB replacement therapies.[7,8]

2 | GENE THERAPY FOR FVIII AND FIX: WILL THERE BE LABORATORY ASSAY ISSUES?

Gene therapy in haemophilia has evolved over the years since the first human clinical trials for adeno-associated viral (AAV) vector gene transfer of recombinant factor IX (FIX) into skeletal muscle in 2003.[9] The gene therapy approach has been subsequently modified by focusing solely on AAV vector delivery to the liver for both FVIII and FIX.[10] HB gene therapy is currently at a more advanced stage of clinical study than HA due to the smaller size of the gene. As of January 2020, there are 12 clinical trials active or recruiting for FIX and 11 for FVIII.[2,11] All utilize AAV or AAV-like vectors with either wild-type FIX or the Padua point mutation in FIX (p.R338L)[12] or B-domain deleted (BDD) FVIII.[13] The longest running HB clinical trial, the St. Jude Children's research/University College, London, AAV8 FIX wild-type, has recently reported an 8-year follow-up.[14]

Assay discrepancies in gene therapy patients have been reported for some of the AAV vector-mediated liver-directed HA and HB therapies currently in development.[13,15] Unlike conventional factor replacement therapies, which require repeated administration of exogenous plasma-derived or recombinant factor, AAV-based gene therapies rely on the endogenous expression of clotting factor after a single treatment with AAV-based vector containing frequently codon-optimized, bio-engineered transgenes such as BDD FVIII (due to limitations of packaging capacity of the AAV vector) or Padua FIX variant, p.R338L (to achieve higher specific activity).[16] A number of these AAV-based HA and HB gene therapies have provided promising results, achieving sustained factor expression levels close to or within the normal range[13,17] resulting in significant reductions in bleeding episodes and factor consumption. To date, available data for these bio-engineered transgenes on one-stage and chromogenic factor activity assays commonly used to monitor factor replacement therapy in the clinical laboratory are limited.

BioMarin, during their phase I/II study for valoctocogene roxaparvovec (which utilizes a codon-optimized AAV5-hFVIII-SQ transgene), reported a consistent 1.65-fold difference between the OS and CS assays, with higher results being observed in the FVIII OS compared to the CS assay.[13] These results are in contrast to previously published findings for commercially available rFVIII-SQ products (ie ReFacto AF® or Xyntha®), for which lower OS compared to CS FVIII activity has been reported.[18] The nature and biochemistry of these differences are currently still not well understood, and until, such data become available, the question of which of the two FVIII activity results (the OS or the CS) better reflects the haemostatic effect of the gene therapy remains to be determined.

In 2018, factor activity assay data for a FIX gene therapy candidate PF-06838435 (SPK-9001) containing a high specific activity FIX-Padua variant (p.R338L) were presented at the American Society of Haematology Annual Meeting.[15] FIX activity in plasma samples collected from patients receiving PF-06838435 was measured and compared to contrived HB plasma samples spiked with either purified recombinant human FIX-Padua (rHFIXp) or recombinant human FIX (rHFIX, BeneFIX®). Measurements were conducted in four commonly used in vitro diagnostic (IVD)-approved FIX one-stage assay systems: STA®-PTT Automate (PTT-A) and STA®-CK Prest on STA-R Evolution (Diagnostica Stago); Dade Actin® FSL on the BCS® XP (Siemens Healthcare); and HemosIL® Synthasil on the ACL TOP (Instrumentation Laboratories). For comparison, samples were also tested in the ROX FIX chromogenic assay (Rossix AB). A consistent pattern in FIX activity for the PF-06838435 transgene product, as well as for plasma samples spiked with rHFIXp, was observed across all five FIX assay systems. Up to twofold differences in FIX activity were observed in the four IVD OS assay systems. Dade® Actin® FSL provided the lowest FIX activity values, whereas PTT-A resulted in the highest FIX activity levels. In all cases, the ROX FIX CS assay measured the lowest. In contrast, rHFIX (BeneFIX®) recovered within ±25% in all APTT-based FIX activity assays and consistent with previously reported data, modestly under-recovered by 46%-59% of expected target in the ROX FIX CS assay.[15] These results suggest that APTT reagent choice when monitoring FIX activity in patients receiving HB therapy containing a FIX-Padua transgene will be important. Similar to the assay discrepancies observed for the BDD FVIII transgene, the question of which of the assay or reagent systems better correlates with the haemostatic effect of the particular gene therapy remains to be demonstrated. Sangamo recently presented phase 1/2 interim results for SB-525 gene therapy for HA. Laboratory results in 1-4 patients over 28 weeks demonstrated a difference in OS and CS assays with OS results approximately 1.5-fold higher than CS results.[19] Regulators in the United States have already recognized this issue and have requested manufacturers developing FVIII and FIX gene therapies to investigate and address these potential assay discrepancies early during development.[17]

Gene therapy is a constantly evolving field. Genome editing technologies which repair, rather than replace, the affected gene have been trialled in animal models of HB and HA[20,21] CRISPR (clustered regularly interspaced short palindromic repeats) can target FIX using Cas9 (CRISPR-associated protein 9 nuclease).[22] A small piece of guide ribonucleic acid (RNA) binds to both a target in F9, for example exon 2 of murine FIX, and the Cas9 nuclease.[23] Cas9 breaks the double-stranded native deoxyribonucleic acid (DNA) at the target location; then, the replacement FIX RNA, in this example containing FIX-Padua, is inserted into the DNA to repair the affected F9. AAV is still required for delivery of the nuclease.[23] Obviously, these studies are at a very early stage of development but the measurement of FVIII or FIX activities using routine laboratory assays should be considered by the manufacturers.

3 | EMICIZUMAB

Emicizumab is a humanized monoclonal-modified immunoglobulin G4 (IgG4) antibody with a bispecific antibody structure which binds human FX, FIX and activated FIX (FIXa) but does not require

activation to function.[24] Emicizumab prophylaxis improves haemostasis but does not fully correct haemostasis. US Food and Drug Administration (FDA) approval in 2017 and European Medicines Agency (EMA) approval in early 2018 saw the introduction of emicizumab for the prevention and reduction of bleeding in severe HA patients with FVIII inhibitors.[25] Subsequently, FDA and EMA approval from data submitted via the HAVEN 3 clinical trial for the use of emicizumab in HA patients without inhibitors[26] saw a rise in the uptake of emicizumab treatment.

The treatment regimen of emicizumab ensures that a steady-state is achieved; therefore, drug monitoring for dose adjustment is not a requirement. Emicizumab can remain in the patients circulation many months after the last dose so there is the possibility of inter-patient discrepancy due to the inconsistency in the metabolism of emicizumab.[27]

Despite benefits to patients, the functional differences between FVIII and emicizumab in conjunction with a long half-life make this a challenging time for laboratories. Recent publications and helpful manufacturer insight have provided much needed information on how emicizumab can influence haemostasis laboratory tests, the monitoring of emicizumab levels and measurement of FVIII activity level.[28] A summary of laboratory assays to use in the presence of emicizumab is detailed in Table 1.

3.1 | Emicizumab effect on haemostasis tests

In the haemostasis laboratory, emicizumab affects common tests including the APTT and APTT-based OS assays.[29] The most profound effects on haemostasis tests are those affecting the APTT screen or APTT-based assays including single-factor assays, APTT-based activated protein C resistance (APCV) and Bethesda FVIII inhibitor titre assay.[30] The APTT result tends to be shortened, usually below the normal reference range. Even at very low concentrations of drug, an effect can be noted, and thus, this has been seen at sub-therapeutic levels when patients are commencing treatment and in experiments using artificially spiked plasma.[30-32] Initial studies reported that the prothrombin time (PT) and associated extrinsic pathway factor assays are generally not affected by the presence of emicizumab, but its effects are reagent-dependent.[28] As one would expect, other intrinsic pathway factor assays of FIX, FXI and FXII all show a similar falsely elevated activity level and it has been documented that there are dose-dependent relationships of protein C and protein S when measured using APTT-based assays. Again, this can be seen throughout the APCV test results using an APTT clotting-based assay.[28,33]

Published data have suggested that emicizumab will also shorten the APTT in plasma samples which contain unfractionated heparin (UFH) if using the APTT as a screening tool but the chromogenic anti-Xa assay will remain unaffected by emicizumab.[33] Any HA patients treated with emicizumab who require heparin therapy should therefore have a chromogenic anti-Xa assay used as the method of measurement.[28] The summary of product characteristics (SPC) of Hemlibra provides a list of tests which are or are not affected by emicizumab.[34] The UK Haemophilia Doctors' Organisation has also recently published guidance on which laboratory tests to use in the presence of emicizumab.[33] A summary of laboratory assays to use in the presence of emicizumab is provided in Table 1

3.2 | Emicizumab effect on one-stage FVIII assays

The most common type of FVIII activity measurement worldwide is by a OS APTT-based FVIII assay.[35] Emicizumab significantly shortens the APTT, and the FVIII activity levels can be falsely elevated.[30] There is also documented evidence to suggest that the degree of FVIII:C overestimation varies with different APTT reagents.[32] Therefore, this widespread method is unreliable in the presence of emicizumab. It is not recommended for use by the recent United Kingdom Doctors' Haemophilia Organization (UKHCDO) guidelines.[33]

It is possible to quantify emicizumab drug concentration in a modified version of the OS FVIII assay. This involves a higher dilution of plasma than a standard OS FVIII assay and use of Conformité Européenne (CE) marked emicizumab-specific

TABLE 1 Laboratory assays in the presence of emicizumab

Measurement of	Routine assay	Comments
Emicizumab presence	CS assay with human FIXa and FX	This will also measure concurrent rFVIII therapy
Emicizumab drug concentration	Modified OS assay calibrated with emicizumab-specific calibrators	This may also measure concurrent rFVIII therapy
rFVIII in the presence of emicizumab	CS assay with bovine FIXa and human/bovine FX	
Antihuman FVIII antibodies	Bethesda assay with residual FVIII determined by CS assay with bovine FIXa and human/bovine FX	
Anti-emicizumab antibodies	Not routinely available	Prolongation to the APTT may indicate presence of drug neutralizing antibodies

calibrators (R^2 Diagnostics, Enzyme Research Laboratories), to calibrate the assay. There are limited data available describing the use of this assay.[36-38]

3.3 | Emicizumab effect on chromogenic FVIII assays

A number of CS assays are used for FVIII activity monitoring. These vary in source of clotting factors—human or bovine, mechanism of activation of FVIII, incubation time, phospholipid/calcium concentrations and buffer type. Emicizumab binds human FIXa and FX so those CS assays that use both human FIXa and FX components show significant interference in FVIII surrogate activity levels in a concentration-dependent manner.[28] A human-based chromogenic kit may be used to indicate the presence of emicizumab but is not specific just for the drug since the assay will also measure any endogenous or rFVIII present in the test plasma. Local validation should be performed before the use of this assay in patients receiving emicizumab.[33] When the components of the CS assay are of bovine origin, there is no such interference. Emicizumab does not bind bovine FIXa, and no effect is present. There are also hybrid CS kits that incorporate a mixture of human FXa and bovine FIXa proteins and have been described as being able to accurately measure rFVIII in the presence of emicizumab.[39,40]

3.4 | Emicizumab effect on anti-FVIII antibodies

It is recommended that regular measurement of FVIII inhibitor titre be undertaken as part of the routine care of patients with haemophilia A with inhibitors.[41] This will obviously be an ongoing requirement when patients are treated with emicizumab. However, some young haemophilia A patients have only ever been treated with emicizumab so may not require monitoring of anti-human FVIII antibodies. The Nijmegen Bethesda inhibitor assay includes a one-stage FVIII assay to determine the residual FVIII activity which is then used to quantify the inhibitor titre. The interference of emicizumab in the OS assay can cause false negative or much reduced inhibitor titre.[42] Even incorporating a heat inactivation step into the method, which is normally used to inactivate residual FVIII before testing, does not completely remove the emicizumab interference in the assay.[42,43] The HAVEN 1 clinical trial data showed that there are suitable alternative methodologies for FVIII inhibitor measurement including a Bethesda assay which uses CS assay with bovine components to negate the emicizumab interference.[42] To ensure that there is some degree of consistency between inhibitor titre, the FVIII inhibitor assay prior to commencement of emicizumab should also be performed using the same bovine-based CS assay that will be used in the assay during emicizumab therapy.[42] Differences in inhibitor titre between CS and OS Bethesda assays have been reported so this is especially important for patients with pre-existing inhibitors.[43]

3.5 | Measurement of rFVIII in the presence of emicizumab

The HAVEN 3 study which investigated the prophylactic use of emicizumab in patients with HA without inhibitors reported that >90% of participants receiving emicizumab prophylaxis had three or fewer treated bleeding events during the trial duration.[26] Treatment was with undisclosed standard or extended half-life recombinant FVIII concentrates. The recommended treatment for breakthrough bleeding or to cover surgery in patients with FVIII inhibitors on prophylactic emicizumab is the administration of activated recombinant factor VII (rFVIIa).[34,44] National Hemophilia Foundation recommend that the acute bleed management of patients without inhibitors is with standard or extended half-life FVIII concentrates following the same dosing schedule as used prior to emicizumab therapy.[45] To date, there have been no reports of the laboratory monitoring of patients receiving both emicizumab and rFVIII for bleeding or in the peri-operative setting. Data are, however, available for severe HA plasma artificially spiked with emicizumab at 25 or 75 μg/mL and increasing concentrations of either ReFacto AF® or Fandhi where FVIII activity was measured by a hybrid CS FVIII assay containing human FIXa and bovine FX (Technochrom FVIII:C, Technoclone).[40] This hybrid assay was insensitive to therapeutic concentrations of emicizumab and could recover the expected activity of both rFVIII concentrates. A second study of plasma artificially spiked with 10 or 50 μg/mL emicizumab reported the use of a modified OS FVIII assay using higher plasma dilution and r^2 emicizumab calibrators to quantify emicizumab concentration. It is important to note that addition of an unidentified recombinant human FVIII or porcine FVIII (Obizur, Takeda) increased the overall result compared to emicizumab alone indicating that the modified OS FVIII assay is not specific for just emicizumab.[46]

3.6 | Global assays and emicizumab

Global coagulation assays include whole blood thromboelastography or thromboelastometry (TEG, Haemonetics and ROTEM, Werfen) and thrombin generation assays (TGA). TEG and ROTEM are able to measure the viscoelasticity of the whole blood sample through the process of clotting.[47,48] TGA has performed on either platelet-poor or platelet-rich plasma, and it is a quantification of thrombin generation.[49] Global coagulation assays have been used for monitoring the coagulation status of patients while they are receiving emicizumab therapy during pharmaceutical clinical trials; however, these are not often available in routine haemostasis laboratories.[30,50-52] There are also scant data published on the use of a modified clot waveform analysis using APTT and PT parameters to monitor patients on emicizumab therapy.[53]

3.7 | Anti-emicizumab antibodies

As with any drug therapy, there is a small chance of the development of antidrug antibodies. Despite that the mode of action of

emicizumab is different to that of native FVIII such that it does not bind to the phospholipid surface and it is not regulated by the same activation or inactivation mechanisms as FVIII, antidrug antibodies may still develop.[24] Anti-emicizumab antibodies, both non-neutralizing (14/398 patients) and neutralizing (3/398 patients), were reported during the phase III clinical trials.[1] A decline in efficacy of emicizumab was reported in two patients in the HAVEN-1 clinical trial,[25] but no anti-emicizumab antibodies were detected. There are no routine assays currently available for the detection of antidrug antibodies to emicizumab. However, they may be suspected in patients with a sudden increase in unprovoked bleeding and corresponding decrease in measurable emicizumab drug concentration. It has been suggested that an increase to a previous very short APTT may be indicative of the development of antidrug antibodies[54]; however, the APTT in plasma artificially spiked with emicizumab was reported to normalize at sub-therapeutic concentrations of emicizumab[32] so the APTT may not be significantly prolonged until much of the emicizumab has been neutralized.

4 | CONCLUSION

The focus of haemophilia treatment is shifting away from traditional factor replacement therapies towards the repair or alteration to the genome or the overall rebalancing of haemostasis. There may not be specific assays available for the monitoring of the effect on haemostasis of some of these novel therapies. There is no knowing how long the effect of gene therapy FVIII or FIX will last in vivo, and this is likely to be different between individuals; nevertheless, regular monitoring of FVIII or FIX activity will still be required. The rapid regulatory approval and introduction of emicizumab into routine clinical use took many haemostasis laboratories by surprise. The complexity of monitoring those patients receiving emicizumab indicates that this is best performed by experienced laboratories skilled in the establishment of alternative assays.

It cannot be assumed that future therapies with similar pharmacological properties to existing products will exert a similar influence on laboratory tests. It is therefore imperative that pharmaceutical companies conduct thorough laboratory studies prior to introduction of new products into clinical use and that regulatory bodies do not approve new products without ensuring that sufficient laboratory investigations have been undertaken and are available in the literature.

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