# Immunological Aspects of Inhibitor Development in Haemophilia

*a report by*
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The X-chromosome-linked condition of haemophilia is a bleeding disorder that impairs the body's natural control over coagulation. While there is currently no cure for haemophilia, the disorder can be effectively controlled with infusions of the deficient clotting factor, i.e. factor VIII (FVIII) in the case of haemophilia A and factor IX (FIX) in the case of haemophilia B. Technological advances have improved the safety and efficacy of factor replacement products, but patients with haemophilia commonly continue to encounter a major complication in their treatment due to the emergence of inhibitors, or alloantibodies, in response to factor replacement therapy. Haemophilia A is approximately five times more common than the less severe form, haemophilia B, and patients deficient in FVIII are also much more likely to develop inhibitors. Up to 30% of patients with severe (baseline FVIII values <1% of normal) or moderately severe (baseline FVIII values 1–5% of normal) are estimated to develop inhibitors to FVIII at some point in their lives, while fewer than 5% of patients with haemophilia B are affected by inhibitors.

Because FVIII replacement therapy is often initiated in infancy or early childhood, most inhibitors appear in these early stages of life, often within the first 50 days of initiating factor replacement therapy.[1] Although patients with inhibitors do not bleed more frequently than other haemophilia patients, their bleeding exacerbations are much more difficult to manage because their responsiveness to treatment decreases; these inhibitors therefore present serious complications, acting to prevent the clotting activity of the factor concentrates such that the effect of therapy is greatly reduced for patients with higher inhibitor titres, to whom an alternative, less effective, treatment is given. Furthermore, patients with inhibitors may face an increased risk of severe and potentially life-threatening bleeds, as well as hospitalisation, severe pain, immobilisation due to extensive and recurrent joint bleeds and arthropathy. There is currently no unequivocal explanation for why these inhibitors develop in only a fraction of patients, but current research suggests that inhibitor development is a multifactorial immune response. This article will focus on the role of the immune system in haemophilia and the immunological impact of various treatment and disease factors on the management of haemophilia A.

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## The Immunology of Inhibitor Development

A number of diseases are treated using human proteins isolated from biological fluids or produced by recombinant cell lines, but face complications with the development of antibodies against these drugs, which has unfavourable implications for the resulting clinical efficacy (see *Table 1*).[2] The underlying mechanism of antibody generation against protein drugs is poorly understood, but in most cases appears to result from a breakdown of the body's typical immune tolerance to self-antigens.

When an immune response is triggered in response to FVIII or FIX, the protein is endocytosed by antigen-presenting cells (APCs), and the antigenic epitopes are presented on the cell surface in a complex with the major histocompatability complex (MHC) class II molecule. This allows for an interaction between the APCs and T cells, which initiates the production of B-cell antibodies. This interaction requires co-stimulation of molecules on the surfaces of the APCs and T cells, particularly the APC-designated B7 and the T-cell-designated ligand CD28.[3] The antibodies that form in response to exogenous FVIII and FIX in patients with haemophilia A and B, respectively, consist of polyclonal immunoglobulin G (IgG) antibodies, with the majority belonging to the IgG4 subtype. IgG1 and IgG2 subtypes are also present, but unlike IgG4 antibodies do not bind complement. The underlying pathophysiological mechanisms that explain and determine the type of immune response – whether patients are high or low responders – remains unknown, and it is impossible to predict which immune response will be exhibited by individual patients. Oddly enough, the naturally occurring FVIII-specific T cells and FVIII antibodies in healthy subjects with no history of bleeding disorders have been documented in a number of reports, and remain an unexplained phenomenon.[4–7]

Studies have shown that FVIII antibodies localise mainly to the A2, A3 and C2 domains to interfere with the normal function of FVIII (see *Figure 1*). The antibody-binding region of FVIII involves residues Arg484–Ile508 within the A2 domain, with Tyr487 appearing to be of particular importance, and Gln1778–Met1823 within the A3 domain.[8–11] Antibodies that bind to epitopes in acidic areas a1 and a2 flanking the A2 domain and residues Lys1674–Glu1684 and Ser1687–Thr1695 in a3 appear to interfere with the proteolytic cleavage by thrombin to generate activated coagulation factor X (FXa), as well as the normal protective binding of von Willebrand factor (VWF).[12–16] Binding of antibodies to hydrophobic stretches (Glu2181–Val2243 and Val2303–Tyr2332) within the C2 domain are proposed to prevent FVIII from binding to phospholipids and VWF.[17–19]

Some mutations in the A2, C1 and C2 domains of FVIII are closely linked to a genetic predisposition of developing inhibitors in mild/moderate haemophilia A.[20–22] By analysing FVIII produced by

patients with mild/moderate haemophilia A, researchers found that mutations at residues Arg593, Arg2150, Arg2159 or Ala2201 eliminated FVIII epitopes and recognition by monoclonal antibodies.[23–26] Interestingly, there are reports of inhibitory antibodies in patients with mild haemophilia that recognise exogenous but not endogenous FVIII, supposedly because of highly immunogenic mutations.[23,27]

A patient with mild haemophilia carrying an Arg2150His substitution in the C1 domain presented with a high-titre inhibitor towards allogeneic FVIII demonstrated only that the immune system could distinguish between the two types of FVIII at the B-cell level as well as the T-cell level; FVIII-specific T cells could recognise peptides containing Arg2150, but not recombinant FVIII carrying the Arg2150His substitution, suggesting that wild-type FVIII contains T-cell epitopes in the C1 domain that are absent in the mutant FVIII.[28] Furthermore, peptides encompassing Arg2150 were able to interact with multiple human leukocyte antigen (HLA) class II molecules, suggesting that a restricted number of T-cell epitopes that promiscuously interact with multiple HLA class II molecules are involved in initiating immune responses in patients with an Arg2150His substitution.[28] This may explain why some patients with mild/moderate haemophilia A carrying this and other mutations have an increased propensity to develop FVIII inhibitors, as well as the observed lack of association between HLA class II alleles and inhibitor formation.[29]

## An Immunogenetic Predisposition to Inhibitor Development

Studies from patients with autoimmune diseases show that several co-stimulatory and regulatory molecules involved in eliciting an immune response have significant potential in pathophysiology, and may confer susceptibility to antibody-mediated diseases. Indeed, European studies have suggested that certain HLA haplotypes may affect the risk of developing inhibitors.[30–32] Weak correlations were drawn between inhibitor development and HLA class I/II genotypes, where A3, B7, C7, DQA1*0102, DR15 and DRB1*1501 can be designated as risk alleles (relative risk [RR] 1.9–4.0). Conversely, the C2, DQA0103, DQB0602 and DR13 alleles have been associated with a decreased risk (RR 0.1–0.2), occurring less frequently in inhibitor patients than in non-inhibitor patients. However, the Malmö International Brother Study failed to confirm these associations.[33] More recent findings support the initial idea that HLA haplotypes are involved in the development of inhibitors.[34] The study associated HLA-A 34, DRB1*0405 and DRB 1301 with inhibitor development, and HLA-A30, B13, B15, B57, Cw12, DQB1 0303 and DPB1 0201 with some degree of protection against inhibitor development.

Polymorphisms in immunomodulatory genes have also been implicated in influencing the risk of inhibitor development. The tumour necrosis factor alpha (TNF-$\alpha$) locus and the HLA class I/II alleles are closely associated in the MHC complex. The TNF-$\alpha$ cytokine possesses potent pro-inflammatory and immunomodulatory properties, and polymorphisms in this gene have been linked to autoimmune antibody-mediated diseases such as systemic lupus erythymatosus, inflammatory bowel disease and myasthenia gravis.[35–37] The -308 G/A single nucleotide polymorphism (SNP) in the promoter region of the TNF-$\alpha$ gene is associated with increased production and secretion of TNF-$\alpha$, and inhibitors have been reported in 72.7, 39.7 and 46.9% of patients possessing the -308 A/A, G/G and G/A genotypes, respectively (odds ratio

**Table 1: Examples of Antibody Formation Against Commonly Used Protein Therapeutics**

| Recombinant Drug Proteins | Disease/Treatment |
|---|---|
| Insulin | Diabetes |
| Human growth hormone | Pituitary dwarfism |
| Human granulocyte-macrophage colony-stimulating factor | Patients with chemotherapy-resistant solid tumours |
| Interferon alpha | Chronic viral hepatitis |
| | Hairy cell leukaemia |
| | Basal cell carcinoma |
| | Chronic myeloid leukaemia |
| | Cutaneous T-cell lymphoma |
| Erythropoietin | Chronic renal failure |
| | Patients receiving chemotherapy |
| Coagulation factors | Haemophilia |

*Source: Schellekens and Casadevall, 2004.[2]*

[OR] 4.0, 95% confidence interval [CI] 1.4–11.5; p=0.008).[38,39] A microsatellite polymorphism in the promoter region of the interleukin-10 (IL-10) gene (allele 134) has been highly correlated with inhibitor information; inhibitors were present in 72.7% of patients with this specific allele compared with 37.5% of patients lacking the allele (OR 4.4, 95% CI 21–9.5; p<0.001).[33] Allele 134 is associated with increased secretion of IL-10, which promotes the differentiation, proliferation and antibody production of B-lymphocytes. This has been proposed to confer patients with a phenotype that upregulates B-cell activity in response to antigenic stimuli with FVIII infusion, and ultimately inhibitor development. This increase in B-cell clones is considered to be the pathophysiological mechanism of the polymorphism in patients with systemic lupus erythymatosus and myasthenia gravis.[40,41] A significant association has also been found between the -318 SNP C/T SNP in the promoter region of the cytotoxic T-lymphocyte-associated protein-4 (CTL4) gene and the development of inhibitors; the presence of the T allele is proposed to confer a protective effect by enhancing production of the protein and upregulated CTLA-4 activity on activated T-cells to counteract any signals that induce an immune response to infused FVIII. In this regard, patients lacking the T allele were much more likely to develop inhibitors compared with those carrying the allele (57.6 versus 31.2%).[42]

## Inhibitor Development in Haemophilia B

The data on inhibitor development in haemophilia B are extremely limited due to the relatively lower incidence of this form of the disease. The development of inhibitors against FIX is not commonly observed in clinical practice, but often presents concomitantly with or is heralded by an allergic and anaphylactic reaction, the aetiology of which remains unclear.[43] Studies analysing the composition of antibodies in plasma samples of haemophilia B inhibitor patients presenting with an allergic phenotype have suggested that the allergic response may be associated with transient IgG1-subclass antibody production, as these antibodies, though present in plasma procured at the time of allergic episode, were absent when plasma samples were obtained at a later time (four days to over four weeks later).[44]

Few of the risk factors implicated in the development of FVIII inhibitors, such as positive family history of inhibitors,[45,46] African or Latino ethnicity,[47] haemophilia genotype,[48] type of FVIII product used,[49] age at first exposure to FVIII and frequency and intensity of FVIII administration,[50–52] have been as thoroughly explored in the domain of haemophilia B due to the infrequency of FIX inhibitor development.

**Figure 1: Schematic Model Showing the Domain Structure of Factor VIII (FVIII) and the Localisation of the Main Binding Epitopes of FVIII Antibodies**
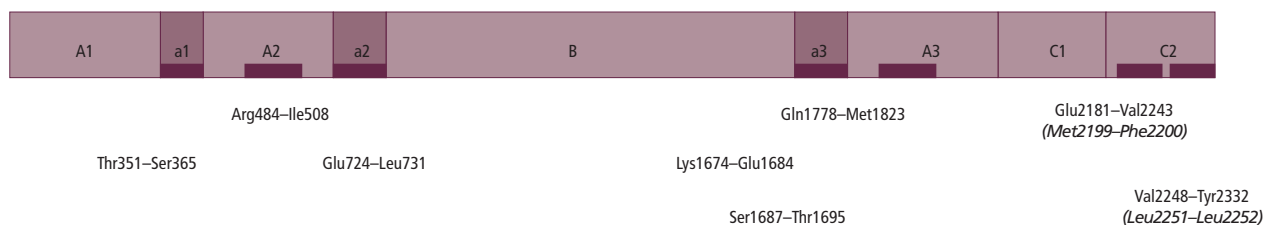


Native FVIII consists of domains designated A1–A2–B–A3–C1–C2, with acidic amino acid regions (a1 and a2) that flank the A2 domain and an acidic region (a3) at the amino terminal end of the A3 domain. The localisation of the main binding epitopes of anti-FVIII antibodies is as shown.
Source: Astermark J, Haemophilia, 2006;12(Suppl. 6):8–13.

**Figure 2: Schematic Model Showing the Domain Structure of Factor IX (FIX) and the Main Binding Area of Inhibitory FIX Antibodies**
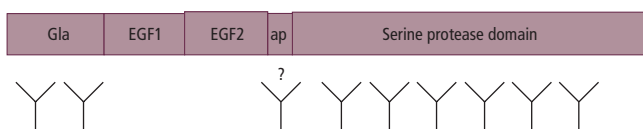


Source: Astermark J, Haemophilia, 2006;12(Suppl. 6):8–13.

However, genotype studies have shown FIX inhibitor development to be associated with deletions, missense mutations and small distinct alterations in the FIX gene.[53] Still, the role of immodulatory genes in inhibitor development has yet to be established for haemophilia B patients. Furthermore, the recognition sites and functional implications are poorly characterised for anti-FIX antibodies. The main binding epitopes recognised by the predominantly IgG1- and IgG4-subclass antibodies include the γ-carboxyglutanic acid (Gla) and serine protease domains in some patients, but not the epidermal growth factor (EGF) domains;[54] it is not yet clear whether antibodies against the activation peptide domain exists (see Figure 2).[55] Functionally, these antibodies against FIX inhibit the activation of FX and interfere with the binding of FIX to phospholipids via the Gla domain, as well as the phospholipid-independent binding of FIX to FVIII. Clearly, there is a need for further studies to confirm the currently available data, and to further explore the immunological and biochemical nature of the immune response against FIX.

**Complications in Haemophilia Treatment**

Various treatment factors can present potential challenges to the immune system and affect the way in which a patient's haemophilia is managed. These treatment factors have been evaluated with respect to the immune response, and the association between the properties of exogenous or modified proteins and inhibitor development has been a very highly debated area of study.

The physical state of the proteins infused into patients is particularly vital in terms of immunogenicity, particularly aggregate formation.[56,57] Although FVIII has a tendency to aggregate, aggregation of FVIII as induced by thermal stress did not enhance the protein's immunogenicity in murine models of haemophilia A.[58] However, O-phospho-l-serine, the head group of phosphatidylserine, prevents aggregation of FVIII under thermal stress, and when complexed with

the C2 domain of FVIII was found to decrease the immunogenicity of FVIII.[59] Notably, this C2 domain contains epitopes with affinity for phospholipids, particularly phosphatidylserine, and several epitopes for CD4+ T cells have been identified within this C2 domain.[4,14,60,61] Similar downmodulation of FVIII immunogenicity has been observed with the use of another phosphatidylserine-orientated chemical modifier,[62] illustrating a correlation between increased phospholipid affinity and a greater frequency of inhibitor development.

While some researchers have suggested that plasma-derived products with high amounts of VWF are less likely to lead to inhibitor development,[63] there are still no conclusive data that favour any one type of factor concentrate over the others. In contrast, other studies have demonstrated no difference in inhibitor development between plasma-derived factor concentrates containing high levels of VWF over recombinant FVIII therapies.[49,64,65] A UK retrospective study of children who had initiated treatment between 1987 and 2003 found that inhibitors developed more frequently in those initially treated with recombinant FVIII compared with plasma-derived FVIII.[51] However, it should be mentioned that there have been great changes in inhibitor assessment over the years, and the study did not account for heterogeneity between initial exposure in children and the classes of factor replacement product. Incidentally, there was no significant difference between the risk of developing inhibitors and the use of either FVIII product when looking at high-titre inhibitors; as high-titre inhibitors may be the clinically relevant inhibitors, this could negate differences in the detection of low-titre transient inhibitors.

Results from the CANAL study found no clinically significant association between switching between recombinant and plasma-derived products and inhibitor development,[49] nor were there any data supporting the use of any FVIII product over the others with respect to preventing inhibitor development. Although there was a comparable risk of developing inhibitors when using recombinant and plasma-derived products, there was a trend towards a lower risk when using plasma-derived FVIII containing low amounts of VWF; however, the validity of this observation is questionable given the low number of patients (n=33) receiving this plasma-derived FVIII with low VWF content.[49]

Pegylation is proposed to reduce immunogenicity by shielding proteins and has demonstrated reduced immunogenicity of bovine proteins, but it is not yet clear whether this reduced capacity to break B-cell tolerance applies in the realm of human proteins.[66] Limited preliminary

data from trials with sucrose-formulated full-length recombinant FVIII reconstituted in an aqueous solution of pegylated liposomes (BAY 79-4980) in treatment-experienced patients have not shown any cases of inhibitor development to date.[67–70] However, none of these studies was designed or powered to look at inhibitors appropriately.

Early prophylaxis has been associated with a significantly lower incidence of inhibitors compared with episodic treatment.[52,71] It has been suggested that FVIII infusions in the absence of immunological danger signals, as in prophylaxis, may inhibit the immune response via peripheral anergy of FVIII-specific T lymphocytes.[72] Other conditions that trigger the immune system and regulate immunomodulatory elements have been proposed to facilitate inhibitor development. Such factors include severe infectious diseases, vaccinations and immunisations that occur in close proximity to the initial infusion of factor concentrates, as well as surgical procedures and traumatic tissue damage that may lead to the exposure of large quantities of potential immunological danger factors. These events can challenge and activate the immune system, modifying the level of cytokines and immune-regulatory molecules, thereby influencing the immune response to the infused protein and promoting the formation of antibodies.

Studies have shown contrasting results regarding this hypothesis; while patients with a low risk of developing inhibitors (e.g. those with mild haemophilia) have developed inhibitors post-operatively in response to FVIII exposure,[73] other studies have shown no association between infections, vaccinations, surgery or central nervous system bleeding and an increased frequency of inhibitors.[50] These results are indicative of a need for further study.

Interestingly enough, inhibitors that develop in haemophilia patients infected with HIV frequently resolve on their own if cellular immunodeficiency is advanced,[74] and until recently the development of new inhibitors against FVIII in severely immunodeficient HIV-infected haemophilia patients had never been reported. One case study examined a 56-year-old male with severe haemophilia A who initiated highly active antiretroviral therapy (HAART) in 1997 and maintained undetectable viral loads between 1997 and 2001; inhibitors were undetected prior to 2001.[75] It is suggested that HAART in severely immunodeficient patients can cause immune reconstitution inflammatory syndrome (IRIS), which can manifest as immune restoration and result in the spontaneous development of antibodies against FVIII. In this particular case, the loss of tolerance against FVIII was not associated with any concurrent opportunistic infection, vaccination, surgery, trauma or switch of FVIII product. The authors of the study recommend that immunodeficient haemophilia A patients receiving HAART undergo regular screening for FVIII inhibitors, as the incidence of novel inhibitors may be underestimated.

The severity of the disease can also influence the management of the disease. Although the incidence of inhibitors is highest in patients with severe haemophilia, the less common development of inhibitors in patients with mild (baseline FVIII level 5–30% of normal) or moderate haemophilia occurs in the second or third decades of life or later; such patients exhibit greater variability in their bleeding phenotype.[76] The bleeding pattern may become similar to that of patients with severe haemophilia, with severe spontaneous joint and muscle bleeds and

FVIII activity dropping below 1%.[77] Some patients can exhibit particularly severe bleeds that are often life-threatening, with large ecchymoses, muscle bleeds and gastrointestinal or urogenital bleeding.[20] Natural history studies of inhibitors in mild or moderate haemophilia have been unable to establish any definitive method of resolving the presence of inhibitors; inhibitors have been found to disappear spontaneously or following immune tolerance induction, while persisting in some patients after a median follow-up of 99

> The effect of treatment intensity on the risk of inhibitor development continues to be debated; the impact of continuous infusion on the immune system is not clear, and more data are needed.

months.[20] The aetiology of these late inhibitors appears to differ from that of inhibitors that arise earlier in life; while the appearance of inhibitors in the initial days of factor replacement therapy suggests primary intolerance to a foreign antigen, late acquisition of inhibitors after hundreds of treatment days suggests a breakdown of tolerance in patients previously tolerant to treatment.[78]

Intensive exposure to FVIII has also been established as a risk factor for the development of inhibitors, where patients who develop an inhibitor following continuous infusion exhibited the typical risk profile for inhibitor formation: severe haemophilia A with a severe gene defect and fewer than 50 factor replacement exposure days.[79] Notably, patients with mild haemophilia are particularly susceptible to inhibitor development in response to intensive treatment; studies suggest that a continuous infusion of FVIII confers a higher risk of inhibitor development than bolus infusions.[73,79] In fact, patients with mild haemophilia may receive more intensive treatment, as the continuous infusion doses are comparable, if not higher, than the commonly used doses in patients with severe haemophilia.[73,80,81] Some centres therefore opt to avoid continuous factor infusion in patients with mild haemophilia.[79]

Uncomplicated venous access is an essential element of factor replacement therapy. Prophylactic infusions are usually administered three times per week in patients with haemophilia A and twice per week in those with haemophilia B. In the ideal scenario, prophylaxis would be initiated at an early age, providing parents with the convenience of administering the factor concentrate in the home setting, but this is often difficult in the peripheral veins of small children. Episodic treatment would also require safe and easy access to veins for the immediate administration of factor concentrate in the case of a bleeding episode.

Central venous access devices (CVADs) facilitate treatment of young children with problematic peripheral venous access, and are commonly used to allow parents to manage home treatment at an early age. However, infection is a frequent complication of CVADs, with rates ranging from 0.14 per 1,000 patient-days to 4.3 per 1,000 patient-days.[82–87] Notably, infection occurs in a greater frequency in patients with inhibitors;[85–87] a review of various studies has found that 50–83% of patients with inhibitors are prone to infection.[88] Possible

explanations for this increased frequency of infection in patients with inhibitors include the occurrence of small haemorrhages around the port following an injection, which can stimulate bacterial growth in subcutaneous tissue, or that patients with inhibitors on an immune tolerance induction programme have high venous access due to their daily, if not twice-daily, required infusions. Use of antibiotics has shown varying outcomes,[89,90] and there is a concern of antibiotic resistance development in response to the general use of antibiotics. Thrombosis has also been a major concern associated with CVADs, and studies suggest that there is an association between a longer duration of catheter placement and a higher risk of developing thrombosis.[91]

### Open Issues

The greatest complication in the treatment of haemophilia is the development of inhibitors, and the immunological mechanisms underlying inhibitor development remain an important area of investigation. Although there is information available on the immune response to factor replacement therapy and immunogenetic- and therapy-related factors affecting inhibitor development, current data regarding the immunology of FVIII inhibitor development remain largely rudimentary, and even less is known about the immunology for FIX inhibitors.

Although not currently possible, the ability to predict inhibitor development with a degree of certainty would prove invaluable, with the possibility of devising treatment regimens to prevent inhibitor development or regimens that induce and maintain tolerance. A potentially useful tool in clinical practice would be the recently developed risk stratification for inhibitor development at first treatment for severe haemophilia A.[92] The authors of the study developed a risk stratification system to predict the risk of inhibitors in previously untreated patients, selecting a risk score of two points for family history of inhibitor, two points for high-risk gene mutations and three points for intensive FVIII treatment at first exposure. Patients scoring no points had a low inhibitor risk (6%), rising to 23% in patients with two points and 57% in patients with three or more points.

Another useful factor in studying the risks of inhibitor development would be the ability to use a genetically well-characterised population of patients in assessing immunogenicity of new replacement factors in clinical trials. Perhaps this goal of unambiguous prediction of inhibitor development can be made more reachable by focusing on the various immunogenetic characteristics associated with increased or decreased inhibitor risk; it is possible that polymorphisms in the immodulatory genes may have more to do with the characterisation of the immune response, while the main determinants of inhibitor development are molecular defects of the coagulation factors and MHC types, but this is an area that can be tested.

The use of episodic treatment, despite haemophilia being a lifelong chronic illness, has complicated attempts to develop evidence-based clinical approaches to therapy, and there exist a limited number of randomised clinical trials in this field. Much remains to be definitively explored in the realm of haemophilia with respect to immunology, and many questions remain unanswered, including: Why do inhibitors develop in only a percentage of patients? What is the impact of specific factors (opportunistic infections, vaccinations, trauma, etc.) in challenging the immune system? What are the crucial risk factors that lead to CVAD-associated complications in small children?

The effect of treatment intensity on the risk of inhibitor development continues to be debated; the impact of continuous infusion on the immune system is not clear, and more data are needed before any recommendations regarding the mode of administration can be made. Furthermore, there are many pathogenic and therapeutic aspects of inhibitor development in mild/moderate haemophilia A that have yet to be elucidated, illustrating a need for large prospective trials to determine which patients are at risk of inhibitor development. Indeed, studies suggest that continuous infusion may be associated with an increased incidence of inhibitor formation; this issue of inhibitors in mild haemophilia A following continuous infusion and other intense treatment regimens can be evaluated in prospective, multicentre studies with genetically well-characterised cohorts. This evidence-based approach could facilitate the management of haemophilia, particularly as these patients age. ■

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