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Comparative analysis of marketed factor VIII products: reply

B. M. REIPERT, J. ANZENGRUBER and F. SCHEIFLINGER
Shire, Vienna, Austria

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Development of factor VIII (FVIII) inhibitors following replacement therapy with FVIII is one of the major challenges faced when treating patients with hemophilia A. Inhibitors develop in 20–32% of previously untreated patients with severe and in 3–13% with moderate or mild hemophilia A [1,2]. The cause of the immunogenicity is not well understood. There is evidence that both genetic and non-genetic factors influence patients' susceptibility to develop these antibodies [3,4]. Novel hypothesis and research approaches are required to obtain more clarity on the molecular basis of FVIII immunogenicity and how FVIII triggers unwanted immune responses in some patients but not in others. The aim of our paper 'Comparative analysis of marketed factor VIII products: recombinant products are not alike vis-a-vis soluble protein aggregates and subvisible particles' [5] was to advance science and help to generate new hypotheses for future research on the molecular basis of FVIII immunogenicity. Our aim was to provide scientific transparency of our rationale and to stay on the level of a scientific debate rather than discussing brands or products. We believe that when it comes to treatment decisions clinical evidence on a robust basis is required. Disclosure of brand names as recently suggested by M Makris and A Farrugia in a letter to the editor of JTH [6] could influence treatment decisions by hypotheses rather than robust clinical evidence.

Data from other protein products suggest that critical quality variables such as soluble protein aggregates (SPAs) and subvisible particles (SVPs) influence the immunogenicity of protein therapeutics [7]. We analyzed SPAs and SVPs concentrations in commercially available recombinant FVIII (rFVIII) products to understand if there are differences between these products after reconstitution. Moreover, we wanted to know if and how levels of SPAs and SVPs change upon exposure of rFVIII products to relevant stress conditions such as agitation and sheer stress. Preexisting SPAs and SVPs may act as seeds that nucleate further protein aggregation upon exposure to stress [8–10].

Our data derived from the analysis of three to six different lots of nine rFVIII products revealed the following.

1 SPAs and SVPs were detected in all lots from all products investigated in varying quantities after reconstitution. SPA concentrations ranged from 0.2% to 11.6%; SVP concentrations ranged from 0.7×10^6/1000 IU to 114.0×10^6/1000 IU. There were lot-to-lot variations in each product.
2 Upon exposure to relevant stress (agitation and sheer stress) the products formed additional SPAs and SVPs to different degrees. Products with the highest concentrations of SPAs or SVPs after reconstitution showed the highest increase in these variables upon relevant stress, indicating that SPAs and SVPs present in the products after reconstitution might act as seeds that nucleate further SPAs and SVPs upon exposure to stress.
3 The size distribution of SVPs in rFVIII products after exposure to relevant stress was similar to that

Correspondence: Birgit M. Reipert, Shire Research and Development, Vienna DC Tower, Donau-City Straße 7, A-1221 Vienna, Austria
Tel.: +43 1 2010 0247 1285
E-mail: birgit.reipert@shire.com

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determined in the products after reconstitution, albeit with a slight increase in larger SVPs (on average 45.8% 0.75–1 μm, 26.4% 1–2 μm, 21.7% 2–4.5 μm and 6.1% 4.5–70 μm in size)

4 The majority (53–99%) of SVPs was protein or contained protein. Individual lots of some products were found to contain increased concentrations of non-protein particles.

5 The use of any single method for assessment of aggregates is not sufficient to provide a robust measure of protein aggregation.

6 No difference was observed in the initial presence or *de novo* formation of SPAs or SVPs that could be attributed to the presence or lack of the B-domain in full-length rFVIII and B-domain-deleted rFVIII products.

The question arises if and how SPAs and SVPs found in the nine recombinant FVIII products influence the immunogenicity of these products. There is experimental evidence that protein aggregates may elicit or enhance immune responses by several mechanisms, including: extensive cross-linking of B-cell receptors, causing efficient B-cell activation [11,12]; enhancing antigen uptake, processing and presentation; and triggering immunostimulatory danger signals [13]. However, other critical information that would be essential to directly correlate the concentrations of SPAs and SVPs with protein immunogenicity in patients is still lacking. In particular, the following information would be required.

1 Types and quantities of SPAs and SVPs needed to generate immune responses for any given therapeutic protein product, in our case for rFVIII products. There is evidence that higher-molecular-weight aggregates and particles are more potent in eliciting immune responses than lower-molecular-weight aggregates [11,12,14]. However, quantitative information about a correlation between the concentration of these higher-molecular-weight aggregates and particles and the immunogenicity of products in patients is missing.

2 The SPAs and SVPs formed and the quantities that efficiently elicit immune responses may differ for different products and in different clinical scenarios. Moreover, the immune system of each patient might have a different sensitivity for immune activation by SPAs and SVPs contained in FVIII products.

In addition to the baseline levels of SPAs and SVPs in each rFVIII product, our data indicate that product mishandling after reconstitution can increase the concentration of SPAs and SVPs. Similar findings for FVIII products were recently published by Tsutomo *et al.* [15]. Thus, it is important to educate end-users about proper product handling to avoid an amplification of potential adverse effects due to increases in SPAs and SVPs induced by mishandling. [15].

In conclusion, we believe the research community needs to pay more attention to the presence of SPAs and SVPs in FVIII products and how these variables influence the immunogenicity of the products in patients. Our current understanding does not yet allow specific conclusions on how levels of SPAs and SVPs in FVIII products translate into product immunogenicity in patients but we believe that thorough assessment of these variables is important.

Addendum

B. M. Reipert wrote the manuscript. J. Anzengruber and F. Scheiflinger revised the manuscript and all authors approved the final version.

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Protease-activated receptor-1 impedes prostate and intestinal tumor progression in mice: comment

C. A. SPEK,* C. TEKIN*†‡ and M. F. BIJLSMA†‡
*Center of Experimental and Molecular Medicine, Amsterdam UMC, University of Amsterdam; †Laboratory for Experimental Oncology and Radiobiology, Amsterdam UMC, University of Amsterdam, Cancer Center Amsterdam; and ‡Oncode Institute, Amsterdam, the Netherlands

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Protease-activated receptor 1 (PAR-1), originally identified as the thrombin receptor on platelets and vascular endothelial cells, is expressed on numerous cells throughout the body. Tumor cells, and cells in the tumor microenvironment such as cancer-associated fibroblasts, macrophages, T cells and endothelial cells, are no exception and PAR-1 expression is abundant in a variety of cancer tissues [1,2]. The potential relevance of PAR-1 expression for tumor growth is underscored by observations that PAR-1 expression levels correlate with cancer progression and overall survival [1,2]. Consistent with such clinical data hinting towards a tumor-promoting effect of PAR-1, experimental studies seem to provide solid evidence for activated PAR-1 as a driver of cancer progression. Indeed, PAR-1 activation induces proliferation, migration and invasion of cancer cells in different *in vitro* experiments (excellently reviewed in references [2]), whereas tumor cell-specific PAR-1 overexpression potentiates tumor growth in preclinical animal models of breast and prostate cancer (Table 1; [3,4]). In line with this, shRNA-mediated inhibition of tumor cell PAR-1, stromal PAR-1 depletion or pharmacological PAR-1 inhibition consistently suppress tumor growth in animal models (Table 1; [3,4]). As a consequence, the current paradigm dictates that PAR-1 promotes cancer progression based on which PAR-1 has been suggested as a promising target for the treatment of cancer [1]. Intriguing recent data are, however, at odds with this paradigm and suggest that PAR-1 could also harbor tumor-suppressive functions.

In a recent issue of the *Journal of Thrombosis and Haemostasis*, Adams and colleagues elegantly addressed the importance of PAR-1 in spontaneously developing tumor models and showed that the genetic elimination of PAR-1 in fact aggravated tumor development [3]. Interbreeding PAR-1-deficient mice with TRAMP (transgenic adenocarcinoma of the mouse prostate) mice that spontaneously develop prostate tumors led to significantly larger tumors with features of aggressive growth. Moreover, PAR-1-deficient adenomatous polyposis coli min ($APC^{min/+}$) mice developed more and larger adenomas as compared with PAR-1 wild-type $APC^{min/+}$ mice. In a concurrently published paper from our own group, we showed that

Correspondence: C. Arnold Spek, Center of Experimental and Molecular Medicine, Amsterdam University Medical Centers, University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, the Netherlands
Tel.: +31 20 566 8750
E-mail: c.a.spek@amc.uva.nl

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