Non-parallelism in the one-stage coagulation factor assay is a phenomenon of lupus anticoagulants and not of individual factor inhibitors

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Dear Sirs,

Individual coagulation factor inhibitors and lupus anticoagulants generally result in prolonged clotting times in coagulation assays. By far the most frequently occurring individual coagulation factor inhibitors are allo-antibodies against factor (F) VIII and FIX. These inhibitors arise in 10–30% of haemophilia A and 4–5% of haemophilia B patients treated with FVIII or FIX concentrates, respectively (1). Acquired FVIII deficiencies due to auto-antibodies rarely occur with an incidence of 1 to 4 per million/year, and mainly in the elderly (2). Most individual coagulation factor inhibitors have fast-acting kinetic properties and completely inhibit the factor concerned except for FVIII inhibitors that are known to be time and temperature dependent (3, 4). Furthermore, two different types of FVIII inhibitors can be recognised, type 1 and type 2. Allo-antibodies are often of type 1 while auto-antibodies generally are type 2. Type 1 antibodies completely inhibit FVIII according to second order kinetics, whereas type 2 antibodies only partially inhibit FVIII and show a non-linear dose-related response.

Lupus anticoagulants are directed to proteins in complex with phospholipids,

like prothrombin and β 2-glycoprotein-1. These antibodies are associated with an increased risk of thrombosis (5) which is in contrast with the prolonged clotting assays in these patients. Furthermore, as both thrombosis and bleeding may occur in patients with lupus anticoagulants and in patients with individual factor inhibitors, both presenting with a prolonged activated partial thromboplastin time (APTT), further laboratory investigation has to confirm the underlying cause of the prolongation as this may determine the choice of the therapeutic strategy (6,7).

In accordance with the prolongation of clotting tests, a further common laboratory finding in patients with lupus anticoagulants and patients with individual factor inhibitors is one or more low-factor activities when assayed with the one-stage coagulation factor assay according to a Parallel Line Bioassay (8–10). The results of these assays are reliable when the curves of reference and patient plasma are parallel (► Fig. 1A). In case of non-parallelism factor activity results should be regarded as incorrect. It is generally accepted that one of the main reasons for non-parallelism is the presence of antibodies like specific coagulation inhibitors, lupus anticoagulants or other non-specific inhibitors.

In case of lupus anticoagulants, mixing increasingly diluted plasma samples with standard volumes of APTT reagent, the lupus activity will be disproportionately neutralised at higher dilutions resulting in increased coagulation factor activity due to non parallelism. For inhibitors against individual coagulation factors like FVIII and FIX inhibitors, non-parallelism in the one-stage factor assay can only be expected when the inhibitor can dissociate from the coagulation factor. However, anti FVIII in-

hibitors have been described to have very low dissociation constants, which indicates that they irreversibly bind to FVIII (11, 12).

The aim of this study was to investigate whether and when lupus anticoagulants and individual FVIII and FIX inhibitors cause non-parallelism in the one-stage factor assay.

One-stage FVIII and FIX assays were performed according to the recommended guidelines with PTT-LA (Stago), APTT-SP (Instrument Laboratory) and ACTIN (Siemens) reagents on a STA-rack evolution (Roche) (8). Multiple dilutions with STA diluent® buffer were analysed from patient plasmas containing: i) high and low-titre lupus anticoagulants (according to STACLOT assay (Stago), ii) high and low-titre type 1 FVIII antibodies (19.5 Nijmegen-Bethesda Units [NBU]/ml and about 2NBU/ml, respectively), iii) high and low-titre type 2 FVIII antibodies (plasmas of patients with acquired haemophilia), 51.5 NBU/ml and about 2 NBU/ml, respectively, and iv) FIX antibodies (Affinity Biologicals, Canada, about 0.5 NBU/ml).

Non-parallelism with the reference plasma in the one-stage FVIII and FIX assay was only detected using lupus sensitive reagents (PTT-LA and APTT-SP) in high-titre lupus anticoagulants containing plasmas (Fig. 1B and C). However, use of a non-sensitive lupus APTT reagent, like ACTIN, or low-titre lupus anticoagulants containing plasmas showed parallelism (results not shown).

The anti-FVIII (both type 1 and type 2) and anti-FIX inhibitor containing samples resulted in curves that were parallel with the reference plasma, identical to samples without an inhibitor (Fig. 1B and C). Results were irrespective of temperature and time of plasma incubation (results not shown) and were seen with both high and low-titre type 1 and 2 FVIII inhibitors (Fig. 1B).

The present study clearly demonstrates that non-parallelism in the one-stage coagulation factor assay can only be detected in the presence of high-titre lupus anticoagulants and by use of lupus sensitive reagent. These results show that in low-titre lupus anticoagulants containing samples, even with the use of lupus sensitive reagent, lupus anticoagulants can be neutralised by

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the amount of phospholipids present in the APTT reagent. However, non-parallelism was never detected in plasmas with high and low FVIII type 1 and type 2 inhibitors and low FIX inhibitors. This indicates that the individual FVIII inhibitors in our patient samples bind FVIII irreversibly, which is in accordance with literature data (11, 12). To our knowledge, binding characteristics of FIX inhibitors are less well known. Our data suggest that they behave like FVIII inhibitors, and irreversibly bind to FIX. Whether our results will be seen in all kinds of individual factor inhibitors is unknown and will mainly depend on their dissociation rate constant.

It is generally recommended to perform factor assays in serial dilutions of plasma in order to exclude the presence of inhibitors (8). The results presented here raise the question whether it is useful to make serial dilutions of every tested plasma in the one-stage coagulation factor. A more practical approach could be to analyse the sample at one appropriate dilution (Fig. 1D). When a factor activity is below the reference value (e.g. FVIII < 50 IU/dl) additional dilutions can be analysed for exclusion of the presence of high-titre lupus anticoagulants when lupus-sensitive APTT reagent is used. Presence of lupus anticoagulants has to be confirmed according to international guidelines for lupus anticoagulants detection (13). In case of parallelism in the one-stage assay individual factor antibodies cannot be excluded and factor activity analysis should be performed in a 1:1 dilution with normal reference pool (in case of FVIII deficiency after incubation for 2

Figure 1: Parallel line bioassay with the one-stage coagulation factor method. The activity of the lowest dilution has been set to 100% for all assays. A) Clotting times of dilutions of reference and patient plasma are plotted against the corresponding log-transformed factor activity. Parallelism of the curves is an indication of correct factor analysis. The horizontal distance between the lines represents the difference in potency. B) Parallel line bioassay of the one-stage factor assay with PTT-LA reagent of FVIII and C) of FIX of patient plasmas containing FVIII and FIX inhibitors or high-titre lupus anticoagulants. D) Flow-chart: follow-up of a deviated one-stage coagulation factor assay.

hours at 37°C). Lack of normalisation of the clotting times indicates the presence of inhibitors and identification and quantification of the inhibitor by the Nijmegen-Bethesda assay should follow (14, 15). Although normalisation of the mixing test indicates the absence of an inhibitor, it can not be excluded that in the case of a relatively high residual coagulation factor, like for type 2 FVIII inhibitors, an inhibitor may still be present.

In conclusion, the recommended procedure, using different dilutions, for performing the one-stage coagulation factor assay is suited, in case of lupus sensitive APTT reagent, to detect the presence of high-titre lupus anticoagulants and lupus like antibodies but not individual FVIII and FIX inhibitors. Screening and quantification of individual coagulation factors inhibitors should be performed in mixing studies with normal pool plasma and specific inhibitor assays.

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Expression of Concern

Concerns have been raised by readers about the accuracy and validity of the data reported in the September 2004 article by Abdelkefi et al., entitled "Prevention of central venous line-related thrombosis by continuous infusion of low-dose unfractionated heparin, in patients with haemato-oncological disease. A

randomized controlled trial" (Abdelkefi A et al. *Thromb Haemost* 2004; 92: 654–661).

Concerns have been raised over the practical aspects of the published protocol.

When asked by *Thrombosis and Haemostasis* to provide evidence that the study was conducted, the authors were unable to provide this

information. Because *Thrombosis and Haemostasis* has no means of establishing the data or demonstrating that research results presented in this article are reproducible, *Thrombosis and Haemostasis* is publishing this Expression of Concern.