Haemostasis is a tightly regulated process that is governed by procoagulant and anticoagulant activities. In the central step of the coagulation cascade, soluble fibrinogen is converted to an insoluble fibrin network by the action of thrombin. However, this primary fibrin network is quite unstable and can not efficiently stop bleeding.

Only after the fibrin strands are crosslinked by the action of the coagulation factor XIIIa (FXIIIa), the clot reaches its final strength and can stop stronger bleedings (1–3).

FXIIIa is generated from its proenzyme factor XIII (FXIII) by the action of thrombin. FXIII belongs to the transglutaminase family of enzymes, and is composed of two A and two B subunits (4). After cleavage of the A subunit by thrombin, the B chains dissociate in the presence of calcium,
whereas the A chain-dimer becomes enzymatically active (5). This process is stimulated by fibrin. The enzymatically active FXIIIa can then crosslink adjacent fibrin molecules by forming isopeptide bonds between $\gamma$ or $\alpha$ chains of fibrin, thereby strengthening the blood clot (3).

Other substrates of FXIIIa are fibronectin or vitronectin, which link the blood clot to the surrounding matrix, or $\alpha$-2 antiplasmin, which inhibits plasminogen-mediated clot lysis when linked by FXIIIa to the clot (3). All these actions of FXIIIa lead to a further stabilization of the blood clot.

Because of its biochemical function, congenital or acquired deficiency in FXIII is associated with a risk for spontaneous bleeding and impaired wound healing (6–8).

Congenital FXIII deficiency is an extremely rare disorder, with an incidence of approximately 1:2000000 (9). It is characterized by e.g.
- intracranial haemorrhage,
- soft tissue hematomas,
- recurrent spontaneous miscarriage and
- abnormal wound healing.

In contrast, acquired FXIII deficiency is more frequent and occurs during a whole variety of clinical conditions that cause a decreased synthesis or increased consumption of FXIII (8, 9), e.g.
- surgical procedures,
- liver diseases,
- inflammatory diseases such as Henoch-Schoenlein purpura or Crohn’s disease,
- disseminated intravascular coagulation (DIC), or
- malignancies.

Most of the bleeding symptoms of congenital or acquired FXIII deficiency were originally reported to occur at FXIII plasma levels of less than 5% (10). However, a questionnaire on congenital factor XIII deficiency in Europe of a Working Party of the European Thrombosis Research Organization (ETRO) on factor XIII identified that bleeding symptoms can also occur at FXIII plasma levels up to 40% (11). Other studies reported an increased bleeding risk after neurosurgery or coronary surgery even at FXIII plasma levels of up to 60% (12) or 70% (13), respectively. Apparently, patients under surgical stress with associated increased FXIII consumption show bleeding symptoms at relatively high FXIII levels (14).

Bleeding symptoms in those patients – as well as in patients that have a congenital FXIII deficiency – are significantly reduced after FXIII substitution therapy (13, 15–17). Substitution of FXIII is only helpful when FXIII plasma levels are below the reference range.

Therefore, measurement of FXIII plasma levels is recommended for the decision of therapeutic intervention with FXIII remedies like Fibrogammin® (13).

Moreover, diagnostic monitoring of plasma FXIII plasma levels during therapy is essential to avoid underdosing for therapeutic as well as overdosing for economic reasons.

Several methods for quantification of either FXIII plasma activity or antigen levels are available for clinical diagnosis (18). Many of them are microtitre plate (MTP) based (19–22). In certain settings that demand highly automated methods for application on routine, random access, high throughput coagulation analyzers or point-of-care instruments other assay formats are desirable. Most automated assays for determination of FXIII on coagulation analyzers are based on a coupled enzymatic reaction that determines FXIII activity (23–26). However, as it is the case with assays for protein S (27) or anti-phospholipid antibodies (28), some laboratories may prefer an automated immunoassay for quantification of FXIII rather than an activity test due to its ease of use, broader measuring range and higher sensitivity. Thus, we set out to develop an automated, latex–enhanced, turbidimetric immunoassay for the quantitative determination of FXIII, and to assess its preliminary performance characteristics. The prototype assay is based on a monoclonal antibody (mAb) that specifically binds FXIII A chain. The mAbs are coupled to latex particles. FXIII in a plasma specimen causes agglutination of the latex particles, which can be quantified turbidimetrically.

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**Methods**

**Material**

Affinity-purified FXIII from human plasma and FXIII-deficient plasma were from Innovative Research (Novi, MI). FXIII A and B chains were kindly provided by Dr. Hubert Metzner (CSL Behring, Marburg, Germany). Standard Human Plasma (SHP), Control Plasma Normal (CPN, plasma with a FXIII concentration in the normal range) or Pathologic (CPP, plasma with a FXIII concentration in pathological low range), enzyme-linked immunoabsorbent assay (ELISA) reagents and materials, Berichrom® factor XIII reagent as well as 280 nm latex particles were from Siemens Healthcare Diagnostics Products (Marburg, Germany). Cell culture material and media were obtained from Nunc (Langenselbold, Germany). Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum were from PAN Biotech (Aidenbach, Germany). HAT (hypoxanthine, aminopterine, thymidine) medium was purchased from CC Pro (Oberdorla, Germany). All fine chemicals as well as goat and rabbit anti-mouse polyclonal antibodies were purchased from Sigma (St. Louis, MO), Polyethylene glycol 4000 was from Merck (Darmstadt, Germany). Immune EasyMouse Adjuvans was from Qiagen (Hilden, Germany). Chromatography material as well as Bicore sensorchips, kits and buffers were purchased from GE Healthcare (Munich, Germany). A sheep anti-human FXIII polyclonal antibody conjugated to horseradish peroxidase (HRP) was obtained from Affinity Biologicals (Ancaster, Canada).

**Hybridoma generation**

A Keyhole Limpet Hemocyanine (KLH) conjugate of purified human FXIII was prepared as described by Harlow and Lane (29). BALB/c mice were immunized and boosted twice intraperitoneally with 20 μg KLH conjugate diluted in Immune EasyMouse adjuvants, respectively. A final booster was administered intravenously three days prior fusion. Hybridoma were produced according to a modification of the method from Kohler and Milstein.
After splenectomy, the suspension of spleen cells from immunized mice was mixed with a suspension of myeloma cells Sp2/0 in DMEM in a cell ratio of 1 : 5. The cells were fused in a solution of 50% polyethylene glycol 4000 in DMEM. Hybridomas were grown in a HAT medium containing 10% fetal calf serum. After two weeks, the specificities of the hybridoma were evaluated by ELISA. Positive hybridomas were cloned using a micromanipulator. For antibody mass production, cells were cultured in roller bottles, and supernatants were purified over Protein A Sepharose Fast Flow columns.

Antibody screening

All reactions were performed at room temperature. 96-well MTPs pre-coated with a goat anti-mouse IgG antibody were incubated with hybridoma supernatants or purified monoclonal antibodies to human FXIII diluted in Tris-buffered saline (TBS) for 1 h. After three washing steps with Enzygnost® washing buffer (Siemens Healthcare Diagnostics Products, Marburg, Germany), human FXIII, FXIII A chain or FXIII B chain were added to the wells. After 1 h incubation the plates were washed three times with Enzygnost washing buffer. Subsequently, HRP-labeled anti-human FXIII polyclonal antibody diluted in TBS was added and incubated for 30–60 min. After washing three times with Enzygnost washing buffer 100 μl tetramethylbenzidine (TMB)-substrate was added for 15 min and the reaction was stopped after 15–30 min with 100 μl Enzygnost stopping solution POD. Absorption was measured at 450 and 650 nm. For determinations of kinetic and affinity constants, selected antibodies were immobilized on Biacore CM5 biosensorchips according to the manufacturer’s instructions on a Biacore 2000 instrument. FXIII diluted in HBS-P buffer was injected over the chip surface at different concentrations. K_a, K_d and K_D values were calculated from the resulting sensograms using the BiaEvaluation software.

Turbidimetric immunoassay

Coupling of monoclonal antibodies to latex particles and immunoassays were performed as described (31). Preliminary prototype assay methods were adapted to the BCS XP (Siemens Healthcare Diagnostics) and Sysmex CA-1500 instruments. For the BCS XP prototype assay, 10 μl sample and 80 μl of a buffer containing salt, detergent and protein stabilizers were combined and incubated for 105 s. Subsequently, 80 μl latex reagent were added, and the change in absorption was determined at 570 nm for 180 s. For the CA-1500 prototype assay, 10 μl sample and 58 μl of a buffer containing salt, detergent and protein stabilizers were combined and incubated for 140 s. Subsequently, 80 μl latex reagent were added, and the change in absorption was determined at 800 nm for 180 s.

Results

In order to obtain mAbs that recognize both FXIII and FXIIIa, we first immunized mice with human FXIII conjugated to KLH, and screened resulting hybridoma for specific binding to FXIII A chain. MAbs directed against FXIII A chain were coated on 280 nm latex particles, and their agglutination potential was characterized by tur-
Tab. 1 Kinetic and affinity constants of the mAb used in the prototype assay. The mAb is directed to human factor XIII A chain. FXIII was injected at different concentrations over a Biacore chip surface coated with the mAb2. $K_D$, $k_a$, and $k_d$ values were calculated from the resulting sensograms using the BiaEvaluation software.

<table>
<thead>
<tr>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_a$</td>
</tr>
<tr>
<td>$k_d$</td>
</tr>
<tr>
<td>$K_D$</td>
</tr>
<tr>
<td>$1.36 \times 10^5$</td>
</tr>
<tr>
<td>$7.96 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5.85 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

Tab. 2 Imprecision of the FXIII antigen prototype test on BCS XP and Sysmex CA-1500 instruments. CPN CPP were measured eightfold per run on five consecutive days on each instrument. Variance components were subsequently determined by ANOVA analysis.

<table>
<thead>
<tr>
<th>Test System</th>
<th>CPN</th>
<th>CPP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCS XP</strong></td>
<td>102.4</td>
<td>109.2</td>
</tr>
<tr>
<td><strong>Sysmex CA-1500</strong></td>
<td>102.4</td>
<td>109.2</td>
</tr>
<tr>
<td><strong>Repeatability (% CV)</strong></td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Between run imprecision (% CV)</strong></td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Within-lab imprecision (% CV)</strong></td>
<td>3.4</td>
<td>3.0</td>
</tr>
</tbody>
</table>

In FXIII-deficient plasma. The FXIII level of every dilution was determined and plotted against the theoretical FXIII amount calculated based on the determined FXIII level of the undiluted pool (Fig. 1c). Linearity, i.e. recoveries within 84–116%, was given over the entire range tested (12–127% FXIII) for both platforms. The mean recovery was 97% FXIII on BCS XP ($r = 0.999$) and 99% FXIII on Sysmex CA-1500 ($r = 0.997$).

In order to determine the analytical sensitivity, an analyte-free medium (0.9% NaCl solution), lowest calibrator (10% FXIII), and its 1:2 dilution (5% FXIII) were measured 20-fold on both instruments. Linearity below 10% FXIII was verified and analytical sensitivity was determined as being the mean test result of the analyte-free medium plus two standard deviations of test results. The lowest amount of FXIII that can accurately be measured with this prototype assay is 0.51% FXIII on BCS XP and 0.44% FXIII on Sysmex CA-1500.

Comparison of results from 169 patient samples, which were evaluated using the BCS XP adaptations of the FXIII prototype assay (Y) and the Berichrom factor XIII activity assay from Siemens Healthcare Diagnostics (X) showed good agreement by Passing-Bablok regression analysis (Fig. 2). Further analysis of these patient samples with the Sysmex CA-1500 adaption of the FXIII prototype assay confirmed the good correlation of test results of the FXIII antigen prototype assay and the Berichrom factor XIII activity assay ($r = 0.94$, slope = 1.18, intercept = −12.93), and showed an excellent system consistency ($r = 0.98$, slope = 1.22, intercept = −4.45) between FXIII prototype adaptations on Sysmex CA-1500 and BCS XP (data not shown).
Tab. 3  Interference testing: SHP was spiked with interfering substances and tested on BCS XP and Sysmex CA-1500. The bias of the test results obtained with the respective samples from the test results obtained with an unspiked sample is provided.

<table>
<thead>
<tr>
<th>substance</th>
<th>final concentration (mg/dl)</th>
<th>bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bilirubin</td>
<td>60</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>haemoglobin</td>
<td>400</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>cholesterol</td>
<td>300</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>triglycerides</td>
<td>3000</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

Discussion

Accurate determination of FXIII is necessary for a respective substitution therapy. Emerging and existing substitution therapies for FXIII-deficiency with recombinant FXIII or factor concentrates would benefit from a widespread availability of adequate FXIII assays (11–17). Tests for FXIII antigen provide a good alternative to currently used activity assays due to their ease of use, broad measuring range and high sensitivity.

However, genetic polymorphisms that influence FXIII activity are quite common in the human population. Despite the good agreement between FXIII activity and FXIII antigen levels in general, the correlation between determinations FXIII antigen levels and FXIII alloenzyme activities as well as the clinical relevance remains to be determined (2). Hence, further studies are needed to demonstrate the clinical utility of a FXIII antigen assay in comparison to FXIII activity assays with respect to detection of FXIII deficiency.

Up to now, an ELISA for FXIII antigen determination has been published (22). However, ELISAs can be difficult to integrate into the workflow of the coagulation section of a central laboratory (34). In particular in perioperative settings were a short turnaround time is needed faster assay formats are necessary. An automated assay for FXIII antigen quantification has been reported in a meeting abstract (35). The reported assay is based on polyclonal antibodies directed against FXIII. As polyclonal antibodies typically exhibit a higher lot-to-lot variation when compared to monoclonal antibodies, we set out to develop a fully monoclonal, automated immunoassay prototype for quantitative determination of FXIII on a broad set of coagulation analyzers. The prototype assay exhibits the precision, linearity, sensitivity and insusceptibility to interfering substances required for accurate determination of FXIII antigen levels. Moreover, both the BCS XP and Sysmex CA-1500 adaptations compare well to the Berichrom factor XIII method that quantifies FXIII activity.

Conclusion

The preliminary research assay prototype exhibits excellent analytical sensitivity, precision, and dynamic range suitable to measure reliably FXIII antigen levels in human plasma.

Acknowledgements

We would like to thank Dr. Hubert Metzner (CSL Behring, Marburg, Germany) for kindly providing human FXIII A and B chains.

Conflict of interest

All authors are employees of Siemens Healthcare Diagnostics Products GmbH. They declared that there are no conflicts of interest regarding the publication of this article.

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