

Fibrinogen and Factor XIII

Chairs: M. de Maat (The Netherlands) and R. Seitz (Germany)

Co-Chairs: R. Ariens (UK), P. Bishop (USA), A. Ichinose (Japan), H. Kohler (Switzerland), J. Koopman (The Netherlands), M. Maurer (USA), L. Medved (USA), N. Weinstock (Germany), J. Weisel (USA)

This was the first meeting of the combined Fibrinogen & Factor XIII subcommittee in a single session of 4 hours and some experiences were shared: A common concern is that the time for the meeting is short, and that several aspects could not be discussed with sufficient detail (especially the nomenclature and the laboratory aspects).

PART 1: Fibrinogen

In the first presentation, dr. W. Koenig showed us that the biological variation of fibrinogen levels in plasma affects the relationship between plasma fibrinogen levels and risk of cardiovascular disease. The reliability index of fibrinogen measurements is around 0.5-0.7, which means an 30-50% underestimation of the risk estimate in epidemiological studies. Multiple measurements (2-3 samples collected at least 2 weeks apart) and exclusion of samples collected during inflammatory conditions improves the risk estimation.

In the next presentation, dr. L. Medved and dr. J. Weisel gave an update on the nomenclature of the fibrinogen molecule and on the nomenclature of the fibrin formation. A major point to consider is the numbering of the amino acids, since it is common use for fibrinogen to use a numbering system, based on the mature protein, while the recommendations of the Human Genome Variation Society use the transcription initiation site as +1. This approach is now also being discussed for other (hemostasis) proteins. Since everybody is very much used to the old numbering system, it has been suggested to use double numbering to avoid confusion. A report is being prepared which is expected to be finalized in 2008.

The next presentation was also on nomenclature, now of the fibrinogen variants, and dr. M. de Maat and dr. J. Koopman prepared and presented a suggestion for a clearer nomenclature. A report is now being prepared that will be circulated among the fibrinogen investigators and will also be finalized in 2008.

Since it was noticed last time that it is difficult to compare different publications on the characteristics of fibrinogen mutations, Dr. M. Neerman-Arbez presented a minimum set of assays that should always be performed. Of course, different types of mutations (resulting in afibrinogenemia or dysfibrinogenemia) require different approaches. This list will be included in a SSC report and several investigators that were present agreed to participate in the discussions.

The next two presentations focused on the effects of measuring fibrinogen with different assays in the association with cardiovascular risk. First dr. A. Silveira showed that in the recent meta-analysis of the Fibrinogen Research Collaboration, there is no indication that the association with risk is different for the different types of assays. Also, in a recent QTL analysis, they showed that the association between genetic markers and fibrinogen levels is similar for the Clauss assay and

a nephelometric assay. In the next presentation dr. D. Peetz presented first results of the Gutenberg Heart Study, in which 5 different fibrinogen assays were used, and here the correlation between the assays varied between 0.5 and 0.9. A common concern is that fibrinogen assays need to be further standardised.

PART 2: FXIII

Session I. International Standardization and Registry Issues.

A. Ichinose gave a report about the FXIII SWP business meeting in Dresden in February 2007:

A) Political/Social issues: The current chair of the FXIII subcommittee explained that he initially could not accept the chair of the merged subcommittee, but was able to accept a joint chairmanship. The FXIII Standardization Working Party (SWP) decided to continue to work under the FXIII subcommittee of SSC/ISTH, and to centralize to the FXIIISWP all activities regarding the standardization of Factor XIII to ensure formal and consistent practices. B) Credit issues: On petition of the chair of the FXIIISWP, ISTH/SSC headquarters opened a homepage in their website in order to publicize/announce the major contributions of the FXIIISWP for the establishment of the 1st International standard for plasma (ISP) of FXIII. In addition, one FXIIISWP member was asked to complete a paper on the 1st ISP of FXIII. In order to avoid the recurrence of complications, the FXIIISWP decided to make written agreements for confirmation in advance and when needed. C) Financial issues: Financial difficulties were reported individually, and possibilities to raise funds for the standardization activities were discussed. The FXIIISWP decided to estimate the amounts of money required for direct research costs and travel expenses to attend the FXIIISWP meetings, and agreed to seek possible financial supporters. D) Scientific & Technical Issues: Difficulties in reproducible measurements of XIIIa were considered carefully. The FXIIISWP decided to perform a pilot study to explore possible solutions to this problem. The requirements for good FXIII-deficient plasma and antibodies against various forms of FXIII were also confirmed. E) Clinical issues: the FXIIISWP addressed the necessity of standards for plasma FXIII concentrates and rec. FXIII preparations, for clinical management of FXIII deficiency.

A Report on standardisation of FXIII Concentrate on behalf of the FXIII SWP was presented by Sanj Raut. Following ISTH/SSC FXIII & Fibrinogen Subcommittee & SWP meetings where the need for a FXIII concentrate reference standard was established, it was proposed to carry out a pilot study (activity & antigen) on all available FXIII concentrate materials. Aims of the study was to evaluate candidate materials for the establishment of the 1st IS for FXIII concentrate, to investigate the relationship between measurement of FXIII in the concentrate vs plasma and to investigate the relationship between measurement of FXIII activity and FXIII antigen levels. Trial fills & accelerated degradation stability studies of candidate materials have been completed and samples & assay kits have been shipped out to the SWP participating laboratories together with protocol and assay design. Study is currently on hold whilst a number of issues are being resolved but it is envisaged that the study will resume later in 2007. 5 materials were provided: (X) FXIII Concentrate (02/170), activity potency ~ 40 IU/amp; (Y) WHO 1st IS FXIII Plasma (02/206): activity potency 0.91 IU/amp; antigen potency 0.93 IU/amp; (Q) rFXIII Concentrate (06/021PM), activity potency ~ 40 IU/amp; (R) rFXIII Concentrate (06/022PM), activity potency ~ 40 IU/amp; (J) FXIII Concentrate J, potency ~ 40 IU/vial. Laboratories were asked to

use routine and/or provided FXIII activity and antigen (A2B2-pdFXIII; A2 subunit-rFXIII) assays. They were requested to carry out 4 independent assays on each sample (on 2 separate days) using preparation Y as standard and following assay instructions/design as described in the protocol. They were to pre-dilute in FXIII deficient plasma and submit all raw data for analysis (by Nov 2007). It is envisaged that results will be analysed and presented at the SSC in June 2008. Based on this, selection of materials for definitive fills will be carried out and a full international collaborative study would be initiated. The objective is to submit study report to WHO/ECBS for establishment in Oct 2009.

V. Ivaskevicius focused on the progress of the international FXIII registry. Recently the data summarizing the former FXIII Registry of ETRO Working Party was published in *Throm Haemost* (2007, 97;6:914-21). A new on-line Questionnaire for patients affected by FXIII deficiency was presented. This questionnaire is available on the www.f13-database.de website. Further, Standardization of Genetic Terms were discussed in relation to FXIII genes and of clinical issues. Advantages and disadvantages were shown of new nomenclature supported by Human Genome Variation Society and old traditional nomenclature. Proposals were provided regarding classification of degree of severity depending from FXIII activity and bleeding symptoms.

Session II: Scientific and Clinical Issues:

W. Korte showed data confirming previous publications that a seemingly moderate intraoperative decrease of FXIII to levels below ca. 60% is associated with subsequent bleeding. A prospective study on intraoperative FXIII substitution was terminated early after 22 patients already, since a significant reduction of bleeding was observed. Further studies on the postoperative setting will follow.

Concerning diagnosis of FXIII deficiency, five cases were presented by H.P. Kohler, where FXIII levels had been overestimated by the most widely used Berichrom assay finding up to 15% FXIII activity despite non-detectable FXIII antigen; a problem already described in recent literature. Notably, it has been shown that overestimation can be amended by the subtraction of the blank; however manufacturer and user do not appear to be aware. L. Muszbek presented an algorithm for the laboratory diagnosis and classification of FXIII deficiencies: 1/ Screening test: A quick functional assay for the determination of plasma FXIII activity; 2/ Mixing study for the detection of neutralizing antibody; 3/ If FXIII activity is <5%, further functional test for the precise assessment of FXIII activity in the low activity range (amine incorporation assay, evaluation of fibrin cross-linking by SDS PAGE); 4/ Determination of FXIII A2B2 complex (R-ELISA); 5/ If the concentration of the complex decreased determination of individual FXIII subunits in the plasma; 6/ Determination of platelet FXIII activity and FXIII-A concentration; 7/ Detection of non-neutralizing antibodies against FXIII subunits by binding assays; 8/ Molecular genetic investigations. Guidelines on diagnosis and monitoring of FXIII therapy are missing, and it is proposed to publish an SSC position paper. This proposal was endorsed by the attendees.

Dr. Kitano discussed genetic and molecular bases of phenotypes of the B subunit of Factor XIII. Three major protein phenotypes of FXIII-B (FXIIIB*1, FXIIIB*2, and FXIIIB*3) are determined by isoelectric focusing and immunoblotting. FXIIIB*1 is the most common

phenotype among Europeans, while FXIIIB*2 and FXIIIB*3 are common in Africans and Asians, respectively. FXIIIB*4 is a rare phenotype. Recently, we determined amino acid residues responsible for each phenotype by nucleotide sequencing analysis using genomic DNAs. Assuming that FXIIIB*1 would be a basic phenotype, FXIIIB*2 had an amino acid substitution of codon 95 in exon III from His to Arg, and FXIIIB*4 had an exchange of codon 368 in exon VII from Glu to Val. For FXIIIB*3, we discovered a C-to-G change in intron K. This nucleotide substitution would create a better splicing acceptor AG dinucleotide, result in differential splicing of intron K, and produce a totally new exon. The presence of this message was confirmed by RT-PCR using hepatic mRNA. As a result, FXIIIB*3 has a 15 residues-longer carboxy-terminal than other phenotypes as well as two additional basic and one extra acidic amino acid residues. Accordingly, all four phenotypes contain variable numbers of charged residues, which ultimately contribute to their differential isoelectric points.

N.T.P. Bakker spoke about the hypothesis that chronic changes in blood flow and blood pressure induce an adaptation of vascular calibre, and that this remodelling depends on the cross-linking enzyme tissue-type transglutaminase (tTG). Blood pressure-dependent and flow-dependent remodelling was studied in wild-type (WT) and tTG-null mice using a surgically imposed change in blood flow in small mesenteric arteries. WT mice showed inward remodelling after 2 days of low blood flow, which was absent in arteries from tTG-null mice. Yet, after continued low blood flow for 7 days, inward remodelling was similar in arteries from WT and tTG-null mice. Studying the alternative pathways of remodelling, we identified monocytes/macrophages as a source of factor XIII and backup mechanism in tTG null mice.

R. Ariens addressed in his presentation the role of gamma dimer formation in determining clot elasticity and lysis rates is discussed. Cross-linking of fibrin by FXIIIa occurs between gamma-gamma and alpha-alpha chains. The relative contribution of gamma-gamma chain cross-linking is poorly understood. We made mutations in the gamma chain cross-linking sites and investigated fibrin structure and function. We found that gamma-dimer formation contributes significantly to clot rigidity. Gamma-alpha hybrid cross-links did not effectively increase clot rigidity. Differences in fibrin degradation products were observed in fibrinogen cross-linking mutants. However, no differences in fibrinolysis rates were observed when gamma-gamma cross-linking was eliminated. We conclude that gamma chain cross-linking plays a major role in determining clot rigidity but not lysis.

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3 July 2008

Vienna, Austria

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DRAFT

Factor XIII

Standardisation work

The report of the 1st Business Meeting of FXIII Standardization Working Party in 2008. Prof. A. Ichinose, L. Muszbek, R. Seitz, R. Ariens, S. Raut, and H. Kohler. Prof. Ichinose reported the summary of the 1st Business Meeting of FXIII Standardization Working Party (former FXIII Standardization Working Group) in 2008, chaired by Prof. Akitada Ichinose (A. Ichinose, L. Muszbek, R. Seitz, R. Ariens, S. Raut, and H. Kohler), held in Wiesbaden 4:00 PM-5:30 PM, Feb. 20, 2008, after the Factor XIII Symposium at the GTH meetings. Major agenda were; 1) Merging Issue of two subcommittees, Factor XIII and Fibrinogen (already merged, likely to be a single chair), 2) Chairmanship of the merged subcommittee (a candidate from the Factor XIII side; Prof. Hans Kohler, because of the current co-chair), 3) Standardization Working Party (must continue), 4) Negotiation with the SSC HQ (financial support for SWP, single or double chair?), 5) Negotiation with the Fibrinogen Subcommittee (about joint chair, rotation?). The SWP meeting also confirmed the current condition: 1) Members' situations; funding, possible conflict of interests, 2) Needed Costs (requested from 2 labs.); ~7000 USD (experiments), ~4000 USD (travel), 3) the SSC policies of 2004 and of 2006-8, 4) Possible supportive companies; A (plasma-derived Concentrates), B (rec XIII-A), etc., 5) Possible other organizations to co-operate; IFCC, (not JSTH, JCT), 6) The choices; dissolution, pause, or continue. Decision to be made in Vienna in July.

L. Muszbek (Hungary) gave an update on methodological problems concerning the measurement of FXIII activity and antigen in FXIII concentrates. When calibrated against company standards, FXIII activity estimates showed wide differences, which diminished with calibration against 1st IS. Antigen values were ca. 9 to 13% higher than activity, though both were calibrated against 1st IS; this might be due to some loss of activity during virus inactivation (pasteurisation). If concentrate was diluted with buffer, the Berichrome test grossly underestimated activity; addition of extra thrombin did not help. In other tests, concentrate FXIII activity was ca. 16% higher with buffer or buffer + fibrinogen than with deficient plasma as diluent, suggesting the possibility of inhibitory constituents in plasma. The prudent choice of the diluent is crucial, dilution with plasma might reflect more closely the physiologic situation, while it may to some extent underestimate the catalytic activity in the concentrate. A concentrate standards appears useful, and needs to be calibrated against the IS. In the discussion, R. Seitz noted that the experience in other cases (e.g. FVIII) showed that dilution with deficient plasma did not avoid the need to have a concentrate standard.

S. Raut (UK) presented the current concepts and available materials necessary for further experiments needed, before the actual standardisation can proceed. For the measurement of recombinant FXIII, particular aspects have to be considered; this preparation is of higher purity than the present plasma products, and contains only subunit A. Thus, the presence of subunit B in the system (e.g. dilution with deficient plasma) is an important point. So far, it is open, whether one common or separate plasma derived and recombinant concentrate standards are needed; candidate materials of both types need to be included in the experiments.

Clinical minisymposium

Diagnosis and Management of patients with transient/acquired factor XIII (FXIII) deficiency; Re-revisit.

By Ichinose A1, Souri M1, Sasabe M2, Kotani N2, Mizobe T3, Tanaka A3, Tsukada J3, Ishida F4, Ito T4, Sugita K5, Aki K6, Sawada A7, Higasa S7, Nishikawa T8, Eura R8, Kawakami K8, Matsuura Y9, Tamai Y10. [1Yamagata Univ., 2Matsuyama Red Cross Hosp., 3Occ. Env. Health Coll., 4Shinsyu Univ., 5Dokkyo Med. Coll., 6Nippon Med. Coll., 7Hyogo Med. Coll., and 8Kagoshima Mun. Hosp., 9Narita Red Cross Hosp., 10Hiroasaki Univ. (Japan)] Prof. Ichinose discussed the increasing bleeding disorder due to transient/acquired factor XIII (FXIII) deficiency. He summarized 13 cases of this disease state, among which about a half turned into having inhibitors against FXIII, and the remaining half being idiopathic. He presented several representative cases including 1) a life-threatening cervical bleeding case of 3 y.o. boy, who was saved by the replacement therapy using FXIII concentrates, 2) a case of type I complete inhibitor of FXIII, 3) a case of type II incomplete inhibitor of FXIII, 4) a case with a possible inhibitor against the fibrin cross-linking (ligation) reaction, and 5) a case with an inhibitor against the activation cleavage step by thrombin. He reviewed the current status of 'Acquired Haemophilia' mainly due to antibodies against Factor VIII, and proposed to call acquired FXIII deficiency as 'Acquired Haemophilia XIII'. He also reviewed 'Hemorrhagic Disorders of Fibrin-stabilization' published in 1977, and 'Guidelines of Testing Modalities for Differential Diagnosis of Bleeding Disorders of Fibrin-stabilization' published in 1994 by Prof. L. Lorand, and pointed out the necessity of rejuvenation, because of emerging additional factors as well as new medicines. Finally, he proposed 1) to conduct a large-scale survey of Acquired Haemophilia XIII, 2) to establish reliable definition for inhibitor and mild FXIII deficiency, 3) to make new recommendation for/guideline on global screening tests of Acquired Haemophilia XIII, 4) to make some recommendation for/guideline on diagnosis & management of Acquired Haemophilia XIII including.

H.P.Kohler (Switzerland) talked about „mild“ reduction of FXIII: between bleeding and thrombosis. Acquired non-immunological reduction of FXIII antigen levels is mainly explained by consumption due to various medical conditions. Such a consumption can lead to a significant decrease in FXIII antigen levels far below the lower normal range limit. However, smaller reduction of FXIII antigen levels within the “normal range” can also be associated with severity of disease or even clinical outcome. Examples from patients with stroke, pulmonary embolism, sepsis and from subjects undergoing surgery give evidence that the so called “normal range” of FXIII antigen levels has possibly to be reconsidered.

Z. Berczky (Hungary) presented the results of a case control study assessing risk factors in more than 900 patients admitted to the hospital for coronary angiography. Subgroups were formed according to the presence of significant (>50%) stenosis of coronary vessels and/or Acute myocardial infarction. Elevated FXIII level turned out to be an independent risk factor in women. Similar results were found also in patients with peripheral arterial disease. Thus, elevated FXIII levels appear to be a gender specific risk factor of atherothrombotic diseases.

É. Katona (Hungary) showed measurements of Factor XIII in body fluids other than plasma. In bronchoalveolar lavage fluid FXIII activity can be found mainly as free subunit A, probably stemming from macrophages as demonstrated by FACS analysis. FXIII levels are elevated in inflammatory bronchoalveolar disorders. In normal cerebrospinal fluid, obtained during lumbar anaesthesia, low FXIII levels are present. Also in tears FXIII can be found. Stimulation of tear flow with intranasal alcohol can strongly increase the volume of tears, but not FXIII concentration.

Fibrinogen

There has been some concerns regarding the availability, stability and validation of the high fibrinogen standard. In addition, this standard was not approved by the WHO. Therefore, Sanj Raut of the NIBSC explained to us the procedures that the NIBSC used for the standards and he started the discussion on the need for a ± 10 g/l standard. The audience did not see a need for such a standard, they were happy with the currently available standards. It was agreed that at the Fibrinogen Workshop (In Venice in July) Moniek de Maat will ask the participants for their opinion, and decide after that whether a new high fibrinogen standard is required (added 13.07: also at the Fibrinogen Workshop there was no interest expressed in a high fibrinogen standard).

There are a number of fibrinogen assays available and Ann Rumley presented a comparison of three assays (Clauss, PT-derived and nephelometric method) in prediction of cardiovascular risk using the Monica study. Although the nephelometric method showed a weak correlation with the two functional assays ($R \pm 0.6$), the

relationship of the three assays with risk was comparable. Combination of different assays did not improve the risk prediction.

Leonid Medved and John Weisel present the final version of the fibrinogen/fibrin nomenclature that has been developed over the last years. The manuscript has been prepared and approval of the SSC was asked. The only question that was raised was about the genetic nomenclature: it was decided that both the numbering, based on the mature protein, and the new numbering, based on the advise of the Standardisation Committee Nomenclature Working Group and the HUGO Gene Nomenclature Committee (HGNC), would be used in the manuscript. The manuscript has been circulated to fibrinogen experts and approved by them.

The Fibrinogen Variants Database is growing and Michel Hanss told us that at the moment 454 molecular abnormalities are included, of which 248 in the A α -chain (82 mutations A α 16), 66 in the B β -chain and 137 in the γ -chain. It appeared that a large number of mutations associated with bleeding problems were found in the N-terminus of the A α -chain, those with amyloidosis were in the C-terminus of the A α -chain, and those associated with thrombosis were often in the C-terminus of the γ -chain, at positions 14 of the B β -chain and 554 of the A α -chain. Although interesting, these data are only observations depending of the way cases have been collected and described. Several modifications of the data presentation have been proposed, including progressive retranscription of the abnormalities on a different reference sequence, and the translation of mature protein location to native protein location. Direct submission of cases should allow maintaining an accurate representation of variant occurrence.

A new item for standardization is the description of the fibrin clot structure. More and more researchers are measuring the fibrin clot structure characteristics, but there is no consensus on how this is done. Marlien Pieters gave a very complete overview of the methods that are being used at the moment and of their advantages and disadvantages. It was agreed that fibrin clot structure is potentially an interesting variable and that it would be good to have a minimum set of standardized variables available.

Submitted by M. de Maat and R. Seitz