1996 MINUTES
FIBRINOLYSIS SUBCOMMITTEE

Sunday, 23 June, 1996, 8.00 - 12.00
Room Rossini, Fira Palace Hotel
Barcelona, Spain

Chair: Cornelis Kluft, The Netherlands
Co-Chairs: Bernd R. Binder, Austria; Jorgen Gram, Denmark;
Dusan Keber, Slowenia; D. Strickland, USA (unable to attend).

Attendance was approximately 80.

Dr. Cornelis Kluft summarized the activities of the last two years to establish a pilot project group for coordinated standardization of materials and methods (PGM), and the actions of this PGM to define its procedures. In brief, for each selected analytical target, a working group will be formed to define criteria for specificity of method(s) to suggest how this specificity in practice can be verified and to suggest the required reference material. Further in the session this procedure was considered for a number of analytical targets.

Urokinase Antigen and Scu-PA

Dr. Bernd Binder and K. Benraad reported on the status of standardization of assays for urokinase-type plasminogen activator antigen and the single-chain form of u-PA. Assay of both plasma and tissue extracts of tumors was considered, and it was felt to be important to combine standardization in these different areas of research. It was concluded that for u-PA antigen residual, problems needed to be solved first. These problems included differences between tumor-derived and recombinant u-PA preparations in some assays; differences in quantity and composition of the extract of tissues depending upon the extraction procedure; large inter-assay variability of some methods in some laboratories; and the presence of a significant portion of u-PA antigenic material in plasma that is unidentified.

For scu-PA assay, Dr. Bernd Binder concluded that a fair agreement exists between methods about its plasma concentration, while initial problems with an interfering component in 10% of patients with coronary heart disease were practically solved. Furthermore, evidence was accumulating that assay of scu-PA is relevant for coronary heart disease.

Dr. Patrick Gaffney summarized the work carried out in 1993 to create a standard for scu-PA used for thrombolytic treatment and to formulate an activation procedure essential for measuring its biological activity. It was concluded that Drs. Binder, Benraad and Gaffney should join forces and act as a working group within the PGM frame work to establish multiple standards in relation to specific assay methods. A call for further participants was issued (respondents thus far are Drs. V. Gurewich and W. A. Gunzler).

Standards for Thrombolytic Agents

Dr. Patrick Gaffney reported that the WHO (1995 meeting ECBS-WHO) is recognizing that mutant proteins may be significantly different from the wild-type original protein and have independent properties; thus requiring a separate reference material. Reteplase (93/726) is presently under study and might be proposed this year. Also staphylocoagulase (94/718) is under consideration. Dr. Patrick Gaffney issued a call for participants of a collaborative study to assign a value to the new DAI-I activity standard.

Antiplasmin

Dr. Bart Hennis, on behalf of Mr. Piet Meijer who could not be present, reported problems with the specificity of antiplasmin assays were particularly noted in automated versions of this method. A major determinant of the problem was identified and appeared to be the use of too high concentrations of plasmin. It was concluded that based on this work, a working group of the PGM should be formed. A call for participants was issued (respondent thus far is Dr. M. Hanss).

Histidine-Rich Glycoprotein

Dr. Bart Hennis reported on the occurrence of two molecular forms of HRG determined by the presence of a polymorphism in the population. This polymorphism results in variable glycosilation of HRG. He reported that two polyclonal antisera used in radial immunodiffusion showed a different reactivity towards both forms (ratio 1.4). It was concluded that better assays should be developed that are either insensitive to the difference or specific to the forms.

Tissue-type Plasminogen Activator

Dr. Cornelis Kluft reported on the impact of heterogeneity in the Actilyse preparation on assays. The heterogeneity concerns glycosilation in kringle 2 of t-PA which is absent in a part of the molecules. It could be shown that this heterogeneity has no impact on two commercially available antigen assays of t-PA, but a check of other methods remains necessary. It could be shown (and was known from literature) that in measurement of activity, the two forms showed a large difference. It was concluded that for t-PA activity more work is required before standardization can be successful. For t-PA antigen,it was decided to form a working group within the frame of the PGM. A call for participants was issued (respondents thus far are Drs. M. Stegnar and D.C. Rijken).

Future Activities

Next to the working groups for scu-PA, antiplasmin activity and t-PA antigen, the subcommittee members suggested the following activities: (a) to have the canceled discussion on receptors, (b) to discuss the D-dimer standardization also in this subcommittee, (c) to discuss genetic methods and nomenclature and summarize the situation about fibrinolysis components, and (d) to discuss the practical aspects of anticoagulation for fibrinolysis; notably the problem of requirement for multiple anticoagulants.

Dr. J. Gram reported on a collaborative study to evaluate suitability of the secondary coagulation standard of the SSC for fibrinolysis quantities. The conclusion was that the matrix standard is

suitable for t-PA antigen, PAI-I antigen, antiplasmin and plasminogen activity assays. The next step to assign value to the standard is considered difficult and will be attempted in collaboration with Dr. Patrick Gaffney.

1997 MINUTES

FIBRINOLYSIS SUBCOMMITTEE

Saturday, 7 June, 1997, 8:00-12:00
Tiziano, Fortezza da Basso
Florence, Italy

Chair: C. Kluft, The Netherlands

Attendance fluctuated between 80-120 persons.

Blood collection and handling procedures (Moderator: Dr. I. Walker)

Dr. I. Walker summarized the items that were rather well established and focused on (a) residual items that should be considered and were not well documented, (b) a future report aiming at defining minimal requirements, taking into account haemostasis assays as broad as possible (fibrinolysis, coagulation, platelets). She questioned whether the SSC should take over the recommendation of ICSH to use uniformly one citrate concentration being 0,109 mol/l. Dr. J Conard introduced the issue of influence of menstrual cycle. Dr. B. Polack summarized critical items of quality control of the increasingly used CTAD tubes and identified the absence of external quality control on such specialized tubes. Mr. P. Meijer showed data that the low pH in Stabilyte tubes might interfere with the assay system by lowering the pH. Dr. M. Stegnar identified the nearly complete absence of data on stability of analytes upon storage of plasma and showed some preliminary data suggesting the importance of this item.

It was decided that Dr. Walker should proceed to prepare a proposal for minimal requirements, as above, which should be circulated among other subcommittees to provide a broad basis for discussion of the proposal next year. In addition, a report form accompanying blood collection will be drafted for discussion (Drs. Walker and Kluft).

In addition, subgroups will work on filling in gaps in knowledge that are considered of importance. Dr. Conard with the assistance of presently known volunteers, Drs. Stegnar, Kluft, Jespersen, van de Ende and Douglas, will produce a report on fluctuations of haemostasis factors during the menstrual cycle. A practical approach of recording the time of the last menses will be included in the aforementioned report form.

Dr. Stegnar, with the assistance of Drs. Gaffney, Douglas and Kluft, will further evaluate how studies on stability of stored samples should be organized. Dr. Kluft will submit a request to selected scientists, both within and outside the fibrinolysis field, to ask for data they might have on stability of stored samples. This matter was considered urgent in view of the use of the SSC secondary standard, and Dr. Jespersen suggested that specific attention be paid in this respect to lyophilization and other potential matrix problems.

SSC Secondary Standard: Report on assignment of a value for t-PA and PAI-1 antigen (reported by Drs. Gram and de Maat)

Using the NIBSC standard as a calibrator for the methods, values were assigned to the SSC secondary standard. Five companies with commercially available kits reacted very positively and provided free reagents. Unfortunately, the results between the methods used disagreed strongly by a factor of three to five for t-PA antigen and by a factor of two for PAI-1 antigen. No value could therefore be assigned. It was decided that the assistance of the company scientists would be requested to find the reason for the discrepancies and a renewed discussion about establishment of a reference method might be needed.

The assignment for plasminogen and antiplasmin was delayed to await the introduction of a new NIBSC batch of the required standards and is expected to be available next year. Dr. Gaffney reported to have ampouled a new plasmin standard (97/536) to succeed 77/588 and also a new glu plasminogen standard to replace the British standard 78/646. It was decided to seek WHO approval for the glu-plasminogen standard, as well.

Assignment of a value to the t-PA antigen plasma standard (reported by Dr. Gaffney)

The subcommittee agreed with the proposal of the assigned value on the new plasma t-PA antigen standard (94/730). Dr. Gaffney will propose this to the WHO. It was further agreed that information on the variation in detected t-PA antigen in the assignment study and information about the added amount of t-PA should be accessible for users.

Assignment of an antigen value to the PAI-1 standard for activity (reported by Dr. Gaffney)

The activity standard 92/564 established by the WHO showed in a collaborative study a variation in results too wide to be acceptable. The subcommittee agreed with Dr. Gaffney's suggestion not to proceed to WHO.

Update on the scu-PA standards and methods of assay (reported by Dr. Gaffney)

New batches of non-glycosilated (NG)(95/564) and glycosilated (G) (95/668) standards for scu-PA show excellent stability at six months (20¼ and 37¼ C). Measurement (after activation) directly on chromogenic substrate shows virtually identical contents of both preparations in agreement with amino-acid analysis. Expression in SI units (katal) is now possible. It was decided that this was now the preferred method for comparison with other preparations. The previous discrepancy with tests of *in vitro* biological activity was strongly reduced by introduction of a clot lysis assay using exogenous lysis instead of endogenous lysis. The warning was issued not to confuse *in vitro* resemblance and standardization of NG and G with thrombolytic efficacy which might depend on other factors as well.

Criteria for specificity of methods and methods of testing: Plasmin inhibitor and t-PA antigen

Mr. Meijer assisted by Drs. Hanns, Christensen, Wiman and Kluft had prepared a report and a set of criteria for specificity and proposed methods of testing. Dr. Booth, assisted by Drs. Chandler, Ersdal, Rijken, Kinnby and Stegnar, did so for t-PA antigen. The subcommittee added suggestions and finally approved the criteria and methods for both analytes. Both groups will

prepare a full report for publication, which will be reviewed by the PGM before further processing within SSC.

The working group on Scu-PA (Drs. Binder, Dooijewaard, Gunzler, Benraad) issued an interim report and will report next year.

In view of the assignment of the SSC secondary standard, it was decided to form a new working group for plasminogen which will report next year (chairman and participants to be invited).

Nomenclature of genetic polymorphisms

Dr. M. de Maat, on behalf of a working group (plus Drs. F. Green and N. Sala) on genetic methodology formed within the frame-work of ETRO (Population genetics of hemostatic risk factors for arterial vascular disease), summarized the nomenclature that should be used for intron and exon mutations in genes of interest for vascular diseases when the recommendations of Beaudet and Tsue (*Human Mutation* 1993; 2: 245-8) were followed. It was felt it would be of great help to publish a table of old versus new "names" and to explain the principles. It was decided to submit this approach as a proposal to the SSC before a report was made and ask for more general support and approval of this effort

Quality control of measurements of genetic polymorphisms

Dr. M. de Maat, on behalf of the working group mentioned above, summarized measures that should be taken to assure quality of genetic methods. Measures are different for different methods. Publication of quality control techniques that are known to expert laboratories might be useful to support the dissemination of genetic methods to many new laboratories. It was decided that Dr. de Maat would arrange a collaborative study, including distribution of samples among interested laboratories, to document the magnitude of the problem. This will be reported next year.

Items for 1998

In addition to what has been mentioned above, it was suggested to pay attention to standardization of D-dimer and to explore the developments around TAFI.

1998 MINUTES
FIBRINOLYSIS SUBCOMMITTEE
Sunday, 21 June, 1998, 13:00-17:00
Cankarjev Dom
Ljubljana, Slovenia
Chair: Nuala Booth, UK
Co-chairs: P. Declerck, Belgium; C. Kluft, The Netherlands;
Osamu Matsuo, Japan

The meeting was attended by 50-90 members.

Carboxypeptidases, including TAFI

This was a new topic for the subcommittee. Dr. Dirk Hendriks summarized the current state of knowledge on these enzymes, including specificity and physiological location. He discussed assay methods and the inhibitors that were available to identify which enzymes are active in a particular situation. He recommended that the standard nomenclature be adopted, including the recently agreed EC number. Dr. Lazslo Bajzar then addressed the specific topic of TAFI (also known as carboxypeptidase U) and gave information on a functional assay and on a novel ELISA. He presented data on a small group of normal individuals. It was agreed that this topic was of growing interest and that it should be on the program for 1999.

Measurements of proteins of the fibrinolytic system in animal models
Dr. Paul Declerck presented an overview of methods suitable for non-human samples, both functional and immunological. Additional information on assays and availability of antibodies was presented from the floor (Dr. HR Lijnen). It was agreed that a written summary of available antibodies should be made available and Paul Declerck offered to prepare this with a view to publication in *Thromb. Haemost.*

Standards for proteins of the fibrinolytic system
Dr. Patrick Gaffney made the case for a new standard for plasmin. He presented data on assignment of a value of 5.3 IU/ml to the new standard. It was agreed that this material should be recommended to the WHO Expert Committee on Biological Standardization. He and Dr. Booth, as Chairman, would discuss its presentation to the Committee with the SSC's presentative to the WHO.
Dr. Gaffney's second topic was on single-chain uPA. A collaborative group had assayed two preparations of this material, one produced in *E coli* and one in CHO cells. The agreement between all five centres was excellent, but there was a discrepancy in the activity of the CHO product in chromogenic versus clot lysis assays. It was agreed that, in the light of this, it was too early to propose either of these materials as a standard. It was agreed that Dr. Gaffney should offer these two preparations as NIBSC reagents, giving the full data on site of production and activities in both assays. The average data from the collaborative group would be included in the information given.

Blood collection for assays of fibrinolysis
Dr. I Walker was unable to be present so Dr. C Kluft summarized a document she had prepared. Since this was of relevance to other subcommittees, he undertook to consult with other interested parties and to bring together a final version of the report to be made available to any member of the subcommittee for eventual publication.

Fluctuations in fibrinolysis throughout the menstrual cycle
Dr. C Kluft presented the results of a literature review of this topic. There were strong indications of changes in some components of the system over the menstrual cycle in some studies but no clear changes emerged. Dr. Kluft intends to pursue this topic in further studies but there was little support for making this a subcommittee initiative at this stage, in view of the difficulty of carrying out a sufficiently wide-scale study to achieve a clear outcome.
Reports for PGM working group
Dr. C Kluft chaired this session, reporting on the activities of the Project Group on Methods, consisting of Drs. J Gram, J Jespersen, TW Barrowcliffe, PJ Declerck and CW Francis, with Dr. Kluft as chair. He explained that this group was part of general SSC activities that had initially concentrated on fibrinolysis. It aims to examine the methods used to measure particular analytes and to produce reports on the criteria necessary to achieve reproducibility across laboratories. Two such reports, on tPA antigen and plasmin inhibitor, are in preparation.
Dr. B Binder presented a review of methods to measure scuPA. His conclusion was that there was no single analyte measurement of which there was good experience at this stage. It was therefore decided to put the issue of standardization aside until this became available.

Dr. J Sidelaman presented data on plasminogen measurements, showing the wide fluctuations in data achieved in a WHO EQAS initiative. It was decided that several collaborators were required to assay a number of samples and to report back to the subcommittee.

The introduction of the IFCC/SSC working party
Dr. C Jackson explained the background to the formation of The Joint Committee on Standardization of Coagulation Effects, comprising Drs. J Jespersen, J Rosing, P Esnouf, G White, TW Barrowcliffe, C Kluft and J Lenahan with Dr. Jackson as Chair. This initiative will impact on the Fibrinolysis Subcommittee in that it will cover some of the issues covered up to now by the PGM.
Plans for 1999

It was agreed that topics for the SSC meeting in Washington should include

- an update on carboxypeptidases
- local fibrinolysis on the surface of platelets and other cells
- D-dimer

Further suggestions should be sent to Dr. Booth (n.a.booth@aberdeen.ac.uk), Dr. Declerck (Paul.Declerck@farm.kuleuven.ac.be), Dr. Kluft (Kluft@euronet.nl) or Dr. Matsuo (kr9o-mto@asani-net.or.jp).

The meeting finished at 5:15.

1999 MINUTES

FIBRINOLYSIS

Saturday, 14 August 1999

1:00 to 5:00 PM

Room 20-22

Washington Convention Center

Washington, DC

Chair: N. Booth, UK

Co-chairs: P.J. Declerk, Belgium; C. Kluft, The Netherlands;

O. Matsuo, Japan

The meeting was attended by 80-120 people.

Procarboxypeptidase U / TAFI

This topic was first discussed by the subcommittee in 1998, since then there has been considerable progress both in functional and antigen assays. The recent work of five groups was presented, allowing a reasonable consensus to emerge. Dr. Dirk Hendriks summarised a functional assay in which all the procarboxypeptidase U (PCU) is activated, after which it can be measured by the release of hippurate by HPLC. This correlated well with a measure of total PCU by ELISA. There was a strong correlation between plasma PCU and time for t-PA-mediated plasma clot lysis. Dr. Laurant Mosnier then presented his studies, using very similar approaches, this time with released hippurate being measured colorimetrically. Dr. Anthony Chan then made a presentation on behalf of Dr. Lazslo Bajzar, whose group has developed ELISA methods, based on either polyclonal or monoclonal antibodies. The assay is sensitive to native TAFI and sees it less well after activation. They too examined the relationship between plasma TAFI and endogenous fibrinolytic activity, this time measuring euglobulin lysis time, and found no correlation. Dr. Kees Kluft presented data on TAFI, measured by ELISA, in normal individuals. The most notable findings were the wide variation in normal concentrations and correlation with markers of inflammation. Dr. Irene Juhan-Vague presented ELISA data on normal individuals and in a group of patients. Correlations were observed between TAFI and some known risk factors for cardiovascular disease. General discussion of all these findings included suggestions that both activity and antigen needs to be measured, that care should be taken to make sure that there is no interference in the assays from other plasma factors, and that the wide normal variation might reflect genetic polymorphisms. It was suggested that the speakers and any others interested should form a working group to summarise assays that are readily available, and to compare data before the meeting in Maastricht in 2000. Exchange of samples in order to compare different assays may also be possible.

Standards for proteins of the fibrinolytic system

Dr. Patrick Gaffney, in his final presentation before he retires, summarised the standards and reference materials that are available and discussed their use in different studies. The t-PA standard was discussed in detail by Dr. Colin Longstaff. A new standard for t-PA is now required and a collaborative study was recently completed, in which the new putative standard was compared to the existing t-PA standard. The report on this study was sent last month to a panel of members. All responses to date supported the acceptance of the new standard and several commented favourably on the study. The potency is 10000 IU based on 12 laboratories using fibrin-based assays. A minority of contributors to the study felt that the value of 10500 IU should be adopted; this was based on 14 laboratories, the additional 2 using a different assay. It should be noted that previous standard was measured only in fibrin-based assays. Both these issues, the acceptance of the new standard and the actual value, were put to a vote. There were no votes against the acceptance of the standard. The value of 10000 IU was accepted by 12 votes to 1. It was noted that there were a very large number of abstentions, especially in the second vote, most of those present not having a strong view. This t-PA preparation will now be recommended as a new standard, with a value of 10000 IU, to the WHO Expert Committee on Biological Standardisation.

Physiological fibrinolysis by u-PA

Dr. Victor Gurewich reviewed the data that suggest that u-PA has a role in fibrinolysis independent of the receptor, u-PAR. This included data from animal models, including knockout mice, and from studies on human blood. His own work shows that clots of platelet-rich plasma were lysed more readily by single-chain u-PA (also called proUK) than were platelet-free clots; the opposite is true of lysis by t-PA. He concluded that physiological fibrinolysis is critically dependent on the strong binding by platelets of u-PA and encouraged others to assess its role.

D-Dimer

This was a new topic for this subcommittee, formerly having been discussed by the fibrinogen committee. Dr. Dempfle reviewed different assays for D-Dimer and concluded that some of them are equally sensitive to soluble fibrin and D-Dimer, some of the assays being related to plasmin degradation and some not. Dr. Piet Meijer presented the ECAT experience of measurement of D-Dimer. It became clear that there was a problem of using different assays and different plasma samples in many studies, such that a solid basis for comparison is lacking. Dr. Willem Nieuwenhuizen summarised his work on measurement of D-Dimer. He had hoped to make available a set of plasma samples, suitable for comparisons between different laboratories; he now intends to provide these. This was warmly welcomed and it is intended that studies on these samples should be presented to future meetings of the subcommittee and to liaise with the fibrinogen committee.

Secondary SSC standard

Dr. Kees Kluft discussed the measurement of the SSC standard for proteins of the fibrinolytic system, which had been hampered by discrepancies between assays. The problems of assaying t-

PA and PAI-1 have now been resolved. Dr. J Sidelaman, who last year presented data on the wide fluctuations in plasminogen measurements in a WHO EQAS initiative, reported on the conclusions of a small working party on measurement of plasminogen. It recommends the use of streptokinase activation and functional measurement, essentially as in the ECAT procedure.

Plans for 2000

It was agreed that topics for the SSC meeting in Maastricht should include

PCU / TAFI

D-Dimer

Results of current work on standards

Further suggestions should be sent to Dr. Booth (n.a.booth@aberdeen.ac.uk), Dr. Declerck, (Paul.Declerck@farm.kuleuven.ac.be), Dr. Kluft (Kluft@euronet.nl) or Dr. Matsuo (kr9o-mto@asani-net.or.jp).

The meeting finished shortly before 5 p.m.

FIBRINOLYSIS

15 June 2000
13:30 to 17:30
Room 0.4
Maastricht Meeting and Convention Center

Chairman: N. A. Booth--UK
Co-chairmen: P.J. Declerck--Belgium; C.-E. Dempfle--Germany; O. MatsuoJapan

D-Dimer

This topic was first discussed by the Subcommittee in 1999, when it was clear that there were important issues on measurements in different assays. Four speakers presented data. Dr M Nesheim discussed his studies on an *in vitro* model of fibrin degradation, which showed the presence of essentially the same products over the time course of degradation, and stressed their large size, consisting of variable numbers of repeating units. Dr Dempfle presented the results of his fibrin assay comparison trial (FACT), in which all manufacturers of kits for the measurement of D-Dimer had measured the same set of 86 samples, consisting of modified plasma samples containing various fibrin derivatives, and plasma samples from patients with DVT or DIC. The variations in response could be normalized by an approach previously used by Dr W Nieuwenhuizen. Concordance of assay results could be improved by using fibrin derivatives comparable to those found in clinical plasma samples for calibration instead of low molecular weight fibrin degradation products. The FACT trial will continue with the aim of developing calibrators usable for all D-Dimer assay systems. He now proposed to build on this work, with more detailed analysis of a limited number of samples. Dr Nesheim offered to provide well-characterized fibrin derivatives for inclusion in this exercise, and it was agreed that this would strengthen the study. The clinical utility of different assays was reviewed by Dr J J Michiels, who discussed the diagnosis of DVT, and the variation in these assays was discussed by Dr P Meijer on behalf of the ECAT group. These presentations underscored the differences between assays and the large inter-assay and intra-assay variations in data, further encouraging Dr Dempfleís efforts in defining methods. It was agreed that these continuing studies should be presented at the next meeting of the Subcommittee.

Standards for proteins of the fibrinolytic system

Dr C Longstaff presented information on three issues about standards. The first arose from the report on the replacement standard for plasminogen activator, tissue type (commonly called tPA), presented and accepted at the meeting in Washington, 1999. The recommendation of the Subcommittee was that the new standard should be adopted, with an agreed potency of 10000 IU per ampoule. The standard was approved by ECBS/WHO in 1999, which decided that it should be called the 1st standard, reflecting the change to recombinant tPA (melanoma tPA was used in the 2nd international standard); they also included Alteplase in the title.

There was concern that this name might cause confusion among users, possibly causing them to view this standard as appropriate only for the measurement of recombinant material. These issues were discussed at a small meeting in May, as a result of which members of the Subcommittee were asked by Dr Booth for their views. All 15 responses agreed that the continuity of the standards was a more important issue than the source of protein, and supported the request that the standard be named the 3rd international standard. 14/15 favoured or were neutral on the inclusion of the word recombinant. The same large majority (14/15) felt that inclusion of the word Alteplase was undesirable. These responses were presented to the meeting by Dr N A Booth. The meeting approved the recommendation that the name of the standard be amended to "3rd International Standard (Recombinant) for Plasminogen Activator, Tissue Type (Enzymatic Procedure)". This wording is based on the nomenclature adopted by ISTH/SSC (*Thromb Haemost* 71: 375-384, 1995) but could be shortened for convenience to "3rd International Standard (Recombinant) for tPA". Information should be included with the standard on: the source of tPA in this standard and in the 2nd international standard it replaces; information on the assay used for the standardization (fibrin clot lysis); suitability of the assay for measuring all natural sources of tPA as well as Alteplase.

Dr Longstaff went on to explain that supplies of the current international standard for streptokinase were running low. The same is true for the current British standard for plasminogen, but there is an international standard for plasmin, adopted by the Subcommittee in 1998. Members were asked to inform him of any requirement for replacement standards. His final topic was the stability of standards. He showed data on stability on standards for tPA and plasminogen activator, urokinase type (uPA) and indicated his desire to hear from any user who had information on stability or problems with any standard (clongstaff@nibsc.ac.uk).

PAI-1 in animal models

Dr P J Declerck presented data on the plasma and platelet pools of PAI-1 in several species, most of which were broadly comparable with human, the concentrations in mice and rats being lower in both plasma and platelets. Rabbit platelets contained no detectable PAI-1; this has important implications for studies using animal models.

Procarboxypeptidase U / TAFI

This protein has been a topic for discussion by the Subcommittee since 1998, Dr D Hendriks playing the major role in organising the discussions. Nomenclature was discussed by Dr Hendriks, who made the case for its name being in line with those of other carboxypeptidases. The discussion that followed accepted the need for a common name but there was a reluctance to drop the name TAFI, for reasons including bibliographical searches. It was agreed that all authors should include both names at first mention, including also the EC number. Data on current antigen and activity assays were presented by Dr L Mosnier and Dr D Hendriks. Considerable progress has been made over the last year and there was a lively discussion. There was agreement that normal plasma contains no detectable active enzyme. There was good correlation between antigen and total activity generated by thrombin. The data on normal plasma concentrations differed between groups, and it was agreed that groups should exchange standards to achieve harmonization of values.

Secondary SSC standard

Dr Booth explained that assay methods for measuring functional plasminogen (Dr J Sidelman) and plasmin inhibitor ($\propto_2$-antiplasmin) (Dr P Meijer) were now in place. Laboratories ready to participate in measuring this material were asked to make contact with her (n.a.booth@aberdeen.ac.uk).

Abbreviations for plasminogen activators

Dr Booth had explored the question of whether the abbreviations for the plasminogen activators should be hyphenated or not, and had sought the views of members of the Subcommittee. There was a perception that the hyphen was the official abbreviation, based on discussions in 1984. The responses suggested a majority (9/14) in favor of tPA rather than t-PA, but some people strongly preferred t-PA, and there were some concerns about confusion in bibliographic searches with the phorbol ester and with tissue polypeptide antigen. The nomenclature adopted by ISTH/SSC (*Thromb Haemost* 71: 375-384, 1995) does not recommend any abbreviations, so it appears that authors may use whichever version they prefer. Dr Booth recommended that at first use in any manuscript, the correct name should be given, followed by the chosen abbreviation. This would make it unnecessary to get agreement (which in any case appeared unlikely) for an official abbreviation.

Plans for 2001

Suggestions for topics were requested but none were made; attendees were encouraged to send suggestions to Dr. Booth (n.a.booth@aberdeen.ac.uk), Dr. Declerck (Paul.Declerck@farm.kuleuven.ac.be), Dr. Dempfle (dempfle@verw.ma.uni-heidelberg.de) or Dr. Matsuo (kr9o-mto@asahi-net.or.jp). It was agreed that discussion at the meeting in Paris should include the topics of PCU / TAFI, D-Dimer and Standards.

The meeting was attended by about 120 people, including all the co-chairs. Dr Booth expressed her thanks to them and to all participants and closed the meeting at 5.20.

FIBRINOLYSIS

6 July 2001
13:00 to 17:00
Room 251
Palais des Congrès

Chairman: P.J. Declerck--Belgium
Co-chairmen: N. Booth--UK; C.-E. Dempfle--Germany; O. Matsuo--Japan; M. Nesheim--Canada

Procarboxypeptidase U / TAFI
Dr. D. Hendriks provided an overview on the nomenclature, on the current assays and on the function of this enzyme in the fibrinolytic system. This was followed by the presentation of a newly developed chromogenic substrate based activity assay that, in contrast to existing functional assays, might allow to analyse large numbers of samples. Dr. Hendriks briefly mentioned that at a recent wet workshop in Leiden, three commercial assays (one for activity, two for antigen) had been evaluated. Since only a limited number of people (often unexperienced) participated and only a limited number of samples were included, no firm conclusion could be drawn. Dr. Mosnier reported the results of their study on the measurement of TAFI levels in 25 normal plasma samples using 5 different assays (3 antigen and 2 activity assays). As presented at the 2000 Maastricht SSC meeting, the TAFI levels as measured by the 5 assays, showed good correlation. However, additional analysis of the results indicated that there might be a systematic variation in the TAFI levels (related to the use of the commercially available sheep anti-TAFI polyclonal antibody) that needs further investigation. The meeting agreed with the organisation of a collaborative interlaboratory study on available proCPU/TAFI assays. Laboratories interested in participating were requested to sign up at the end of the session.

Standards for proteins of the fibrinolytic system
Dr. Longstaff reported that at the WHO/ECBS meeting in October 2000 it was agreed that the recommendations of the SSC be adopted to change the name of the new IS for tPA (98/714) FROM: *Alteplase (recombinant tissue plasminogen activator), 1st IS*, TO: *Tissue plasminogen activator, human, recombinant, 3rd IS*.

Dr. Longstaff presented the results of a collaborative study that was organised in 2000/2001, involving 16 labs, to replace the 2nd IS for Streptokinase. The study included 4 coded Streptokinase samples and participants were asked to perform one or more of the 2 methods provided, or their own in-house method. At the end of the study there was universal agreement by participants with the two proposals arising from the study, 1) on potency and identity of the new standard; and 2) on methodology. 1) It was agreed that preparation 00/464 should be recommended to the WHO as the 3rd IS for Streptokinase with a potency of 1030 IU/ampoule. 2) It was agreed that a chromogenic assay without fibrin (one of the methods provided in the study) would make a suitable reference method for Streptokinase activity determinations. It is likely that this method will be adopted as the reference method of the European Pharmacopoeia. Prior to the SSC meeting the report of this study had also been sent to various members of the

subcommittee for comments. Eleven responded and all approved the report. The report was subsequently approved by the meeting.

D-dimer

Dr. Nesheim briefly discussed the structure of fibrinogen, the polymerization of fibrin momomers, as well as the subsequent cleaveage of fibrin by plasmin to form a family of fibrin degradation products. This was followed by a brief presentation of results wherein the products released from a Factor XIIIa cross-linked clot perfused with dilute plasmin were characterized with respect to their average molecular weight, their molecular weight distribution, and their chain composition. Because the fragments are soluble and well characterized with respect to absolute concentration, chain composition, molecular weight and D-dimer content, they would be excellent candidates for D-dimer standards by which to calibrate and compare various assays.

Dr. C.-E. Dempfle presented the results of the FACT-3 trial (Fibrin Assay Comparison Trial) in which 23 samples (including normal samples as well as samples from different pathologies) were subjected to analysis by 22 commercially available assays. A wide range of values was observed when comparing the results obtained with the various kits; however, a good correlation between the methods was obtained. The latter allowed the generation of conversion factors by which the data from each respective assay could be normalized, resulting in a harmonization of the data obtained from all DVT and most of the DIC samples. For some particular assays this procedure appeared not to be applicable for the data obtained with normal samples. Larger studies will be needed to confirm the general applicability of these conversion factors. The study also demonstrated that a harmonization of the data obtained with the different assays could also be achieved by using a common calibrator consisting of a high-molecular weight, partially plasmin degraded, cross-linked fibrin preparation.

Dr. P. Meijer presented data of the "D-dimer comparison trial," a joint project of the ECAT Foundation and INSTAND performed within the framework of their external quality assessment programmes. The scope of this project was to evaluate the analytical performance of quantitative D-Dimer assays in daily laboratory routine and was focussed at the inter-laboratory variability per assay and the difference in outcome between different methods. A set of 7 samples generated by mixing pooled normal plasma with pooled patient plasma containing elevated D-dimer levels, at different ratios to about 450 laboratories. Preliminary data analysis on the results of about 200 participants from the ECAT Foundation was discussed. The mean inter-laboratory CV ranged from 5 ó 80%. The ratio between the lowest and highest mean value of different methods on different D-Dimer levels ranged from 3 ó 14. Dr. P. Meijer concluded that, with respect to harmonisation of D-Dimer methods, not only the standardisation of the calibration but also the difference in sensitivity should be taken into account.

Dr. Kitchen presented data of recent studies (September 1999 and November 2000) in which two test samples were distributed to more than 300 centres. Latex agglutination was used by 165-215 centres. During this period the use of quantitative (automated and ELISA) methods increased from 40 to 110. Each sample was a lyophilised pool of plasmas from hospitalised patients with elevated D-dimer. Results were grouped by technique and the median D-dimer with different latex agglutination methods varied form 400 to 2000 ng/ml. This was similar for both samples. For one automated assay the median result was 359 ng/ml (range 250-430) with a CV of 15%,

for another method the median was 3030 ng/ml (range 1800-3200). For one automated method the CV of results was 54%. For one method the median test result was only 20% above the upper limit of normality. For others the median test result was more than 6-fold higher than the upper limit of normal. It was concluded that D-dimer assays in routine use vary widely in the results obtained, in the discrimination between normal and abnormal, and in their precision . Improved standardization is required.

Matrix Metalloproteinases

The matrix metalloproteases (MMPs) form a family of over twenty closely related zinc dependent proteases. MMPs are involved in the degradation of many components of the extracellular matrix. MMPs are believed to be involved in various (disease) processes involving matrix remodelling like rheumatoid arthritis, scar formation and invasion and metastasis of tumor cells. Increasing evidence for a role of MMPs in fibrinolysis and cardiovascular disease is accumulating, i.e. MMPs can be activated by plasmin as well as by thrombin and activated MMPs may induce activation of platelets. Dr. J. Verheijen gave an overview on assays for quantitation of MMP activity and antigen. Quantitation methods include: zymography, degradation of matrix components, immunological methods like ELISA and fluorogenic or chromogenic methods involving synthetic peptide or protein substrates. The specificity of the methods is often poorly documented. In biological samples MMPs occur in multiple forms, i.e., free active enzyme, inactive pro-enzyme, active or inactive complexes with protein inhibitors and membrane- or matrix-bound forms. The extensive structural and functional similarity between the various MMPs, the occurrence of multiple forms and the lack of standard preparations and reference methods complicate a reliable quantitation of these enzymes and make interlaboratory comparison of results very difficult. In vitro and in vivo methods for the investigation of MMPs were subsequently discussed by Dr. Z. Galis. Her studies, involving a variety of experimental approaches (in vitro, in vivo, including particular knock-out animal models), clearly demonstrated the role of upregulation of gene expression of various MMPs in atherosclerotic disease, vascular remodeling and angiogenesis. It could also be concluded that MMPs play an important role in the degradation of fibrin matrices during angiogenesis.

Topics for 2002

Attendees were encouraged to communicate with the co-chairpersons. At the 2002 meeting progress on proCPU/TAFI measurements and D-dimer standardization should be included as well as the topic "Standards" .

The meeting was attended by 130-140 people including all the co-chairs. The meeting was closed at 4.55 pm.

Fibrinolysis

July 19, 2002
9:00 to 13:00
Plaza Room
Boston Park Plaza Hotel

Chair: P. Declerck, Belgium
Co-chairs: N. Booth, UK; C. Dempfle, Germany; Dirk Hendriks, Belgium; O. Matsuo, Japan; M. Nesheim, Canada

D-dimer

Dr. Dempfle commented on the issue of novel assay technologies for measurement of D-dimer antigen, including latex-enhanced photometric immunoassays (LPIA) and quantitative point of care (POC) assays. Rapid quantitative D-dimer assay results are needed in clinical practice for exclusion of venous thromboembolic disease, diagnosis and monitoring of DIC, and diagnosis or exclusion of heparin-induced thrombocytopenia type 2. This cannot be achieved with microtiter plate ELISA systems, nor with qualitative D-dimer assays. Clinical studies have shown equivalence of LPIA to ELISA and a high analytical standard of the novel whole blood POC assay technology. The majority of assays currently available are LPIA. LPIA display variable reactivity with different size crosslinked fibrin derivatives, as well as variable influence of fibrinogen degradation products on assay results. In whole blood assays, there may be additional influence of RBC content, hemolysis, and platelets. Apart from the analytical optimization of assays, common standardization is required, especially when integrating POC assays into the diagnostic array of a hospital. For preparation of a D-dimer standard, reproducible procedures for preparation and standardization are needed. The standard should contain a 'physiological' variety of fibrin derivatives rather than simply purified fibrin fragment D-dimer. Specific antigen reactivity profiles of the assays should be taken into consideration when interpreting results. Due to differences in monoclonal antibody reactivity and influences of assay technology on assay response, clinical studies are needed for validation of each individual assay and assay-instrument combination.

Dr. Walker discussed the heterogeneity of non-covalently associated products derived from the double-stranded fibrin protofibrils subsequent to plasmin-mediated fibrin degradation. Using a perfused clot system employing purified components, he characterized these FDPs with respect to their weight-averaged molar mass, chain composition, mass distribution and tPA / DSPAa1 cofactor activity. A comparison of two different paired monoclonal-based D-dimer assays showed marked differences in sensitivity toward D-dimer and the heterogeneous FDPs, underscoring the need for defined D-dimer standards. The methods employed for FDP production and analysis may prove useful in defining heterogeneous FDPs as standards for D-dimer assays.

Dr. Meijer presented the results of the external quality assessment programmes of INSTAND and ECAT Foundation, the so-called D-Dimer Comparison Trial, performed in 2001. The aim of

this trial was to investigate the performance of quantitative D-Dimer assays in daily laboratory practice. A set of 7 lyophilised plasma samples was distributed to about 500 laboratories. Samples were prepared by the addition of different amounts of a patient pool with a high D-Dimer level to normal pooled plasma. The results of 423 laboratories were included in the final evaluation.

Besides differences in the absolute D-Dimer concentration measured by the different methods in the plasma samples, differences in the analytical performance (within- and between-laboratory variation) were also observed. This is the first study which shows the daily performance of D-Dimer assays in clinical laboratories on a large scale. The results were used to set-up a harmonisation model based on linear regression between the mean value of the different samples and the amount of patient plasma added to the sample. This was performed for each method individually. Linear regression is possible due to the number of samples used in this trial. A consensus line was constructed by correlation of the mean value of all included methods for the different samples to the amount of patient plasma added to the sample. The difference between a method related regression line and the consensus regression line has been estimated. This was used to recalculate the values for each method of the different plasma samples. With this procedure the between-laboratory variation for the methods was reduced from 70 – 80% to about 20%. The model presented seems to be a feasible method to harmonise the different D-Dimer methods currently used in the clinical laboratories.

Dr. Dempfle proposed the generation of an inventory of D-dimer assay reactivity in animal models. D-dimer assays are needed for animal models of thrombotic disease, as well as intravascular coagulation and related conditions. Studies observing the lysis of clots prepared from human plasma in animal models require D-dimer assays reactive with human D-dimer antigen, but without cross-reactivity with animal fibrin and fibrinogen.

No valid information is currently available on the reactivity of available D-dimer assays with animal fibrin. The proposal involves the preparation of individual pools from mouse, rat, rabbit, pig, and dog plasma and the preparation of standardized crosslinked fibrin preparations similar to those used in the FACT trial. These fibrin preparations will be diluted in plasma from the same species. In addition, similar fibrin preparations from human plasma will be diluted in the same animal plasmas for comparison of reactivity profiles with the D-dimer assays. Comparison of different sub-species and strains, using the D-dimer assays reactive with the specific species samples, will be performed at a later stage of the trial. Audience and readers are encouraged to contribute animal plasma for preparation of the samples.

Standardization and Methodology

Dr. Longstaff discussed the significance of methodology in the establishment and use of WHO international standards based upon the experiences with tPA and molecular variants thereof. International Standards (IS) prepared by NIBSC and established by WHO have traditionally been calibrated in International Units (IU), determined in collaborative studies using a range of methods. Thus IU may be arbitrary and many have obscure origins. An alternative approach argues that primary reference materials should be calibrated as far as possible in SI units and using only clearly defined Reference Methods. The current status of Fibrinolysis IS was

discussed, including some history of the origin of the IU for different IS. The theoretical and practical merits and problems of the different approaches to calibrating primary reference materials were also discussed. It was concluded that, obviously, appropriate standards are useful but need to be considered with sufficient flexibility. In view of the increasing number of recombinant, molecular, variants of particular factors (e.g. thrombolytic agents) it should be evaluated how useful the currently available standards are for these other variants.

Procarboxypeptidase U / TAFI

Dr. Nesheim discussed the development and characteristics of a functional assay for activated TAFI (plasma carboxypeptidase B, CPU). It is based on the TAFI-mediated reduction of the bat saliva plasminogen activator (bPA)cofactor activity of high molecular weight purified fibrin degradation products (FDPs). The plasma sample with TAFIa in it is incubated in a microtitre plate with the FDPs for three hours in the presence of hirudin and aprotinin. A recombinant variant of plasminogen (S741C), labelled covalently at C741 with fluorescein is then added along with bPA. The conversion of the fluorescent plasminogen to inactive plasmin is accompanied by a 50 percent decrease in fluorescence intensity. The time course of cleavage of the fluorescent plasminogen is determined in a fluorescence plate reader. Initial rates are calibrated with TAFIa at known concentrations in TAFI deficient plasma. The assay is sensitive to TAFIa over the concentration range of 20 to 200 picomolar. No interference from the constitutive plasma carboxypeptidase, CPN, is evident in the assay. The assay has been developed for the analysis of reseach and clinical specimens in order to obtain a better understanding of the biochemistry, physiology and pathophysiology of the TAFI pathway.

Dr. Declerck gave an overview on the problems associated with the measurement of proCPU/TAFI activity and antigen. A procedure for the start of a collaborative study (coordination by Dr. Hendriks and Dr. Declerck) was proposed and discussed. In an initial phase 150 samples will be analysed by two or three different laboratories using all available methods. Also pooled (and depleted and supplemented) plasma will be included. Particular attention will be focussed on the differential recognition of various polymorphic forms in the different assays. After presentation of the data at the 2003 meeting, a larger study, involving a smaller number of samples (but more defined) and a larger number of participating laboratories will be started.

Dr. Booth presented her group's recent work on the effect of physiological inhibitors of fibrinolysis on lysis of thrombi. This included the use of model thrombi to assess the contributions of leukocytes and analysis of activity in situ. The effect of TAFIa has been assessed in these systems and the inhibition of lysis (induced by tPA, uPA or scuPA) by TAFI, present in the plasma in which model thrombi were bathed, was highlighted.

Topics for 2003

Upon request of the SSC Working Group on Coagulation Standards, the Fibrinolysis Subcommittee agreed upon evaluation of the "SSC-ISTH Secondary Coagulation Standard, lot 2" for t-PA and PAI-1 antigen and activity levels. Dr. Kluft raised the problem of large variations between the different methods. Dr. Declerck and Dr. Longstaf will work out a procedure (including previously evaluated NIBSC preparations and involving various laboratories). The

data should be presented at the 2003 meeting. The meeting also agreed to include a topic on assays for measurement of global fibrinolytic activity in blood in various disease states. It was also suggested to consider discussions on assays for fibrinolytic therapeutics exerting their effect through a direct (i.e. non-plasmin mediatied) degradation of fibrin.

Progress on proCPU/TAFI measurements and D-dimer standardization should be included.

The meeting was attended by 35-45 people including all but one co-chair. The meeting was adjourned at 12.10 pm.

Fibrinolysis

July 13, 2003
08:00 to 12:00
Hall 9
The International Convention Center, Birmingham

Chair: P. Declerck, Belgium
Co-chairs: N. Booth, UK; C. Dempfle, Germany; Dirk Hendriks, Belgium;
O. Matsuo, Japan; M. Nesheim, Canada

Procarboxypeptidase U / TAFI

Dr. Nesheim discussed the development and characteristics of a functional assay for activated TAFI (TAFIa) . It is based on the down regulation by TAFIa of the cofactor activity of high molecular weight, soluble fibrin degradation products in the activation of a fluorescent plasminogen derivative by vampire bat plasminogen activator. The standard curve of the assay spans the concentration range from 0 to 200 picomolar; thus the assay is very sensitive. The basal level of TAFIa was measured in a group of healthy young volunteers and was found on average to be 11 picomolar. TAFIa levels as high as 2000 picomolar were measured in the plasmas of baboons infused with thrombin at low levels. With the cessation of thrombin infusion, TAFIa levels decayed with a half life of about 20 minutes. A corresponding prolongaton of in vitro clot lysis time was observed. These baboon experiments show that TAFI can be activated in vivo in a primate to levels that substantially effect fibrinolysis.

Two polymorphisms i.e. Ala/Thr at position 147 and Thr/Ile at position 325 in the coding region of TAFI have been reported. Dr. Gils described the development and characteristics of two monoclonal antibody based ELISAs for measurement of TAFI/proCPU antigen in plasma. One (MA-T12D11/MA-T28G6-HRP) appeared to be genotype independent whereas the other (MA-T32F6/MA-T9G12-HRP) revealed to be highly genotype 325 dependent, i.e. it does not recognize the Ile(325) variant. A polyclonal based commercial assay appeared to be partially genotype dependent (i.e. a decreased sensitivity towards the Ile(325) variant). It is concluded that the interpretation of currently available data on TAFI antigen should be interpreted with caution and should be done in conjunction with the genotyping. Dr. Alessi presented additional data using these assays in the analysis of 1000 patient samples confirming the genotype dependency.

Dr. Guimaraes reported the determination of TAFI levels in plasma from 92 healthy individuals making use of 3 distinct assays, namely, a commercial chromogenic assay – Actichrome® TAFI Activity assay (American Diagnostica), an in-house developed rocket immunoelectrophoresis – (electroimmunoassay) using rabbit polyclonal antibodies (van Tilburg et al. 2000) and an ELISA using commercial sheep polyclonal antibodies against TAFI (Affinity Biologicals Inc.). Each individual was also genotyped for the – 438 A/G and 1040 C/T polymorphisms. The association between TAFI levels and genotype in each assay was evaluated and a linear regression and Bland-Altman agreement analysis in the whole sample group and in genotype sub-groups was performed. The results demonstrate that artefacts may arise when measuring TAFI antigen

levels by ELISA with a lower response for the Ile-325 variant (T allele). No artefacts were found with the Actichrome“ TAFI assay and with the electroimmunoassay. The latter two assays support, however, a genotype-related variation of TAFI concentration.

The overall problems on genotype dependency of TAFI assays was further discussed. It was concluded that this raises problems in interpretation of data and that there is a need to further explore these observations. It was concluded that in an intial phase approximately 100 genotyped plasma samples should be analysed by various expert laboratories using various methods (coordination Paul Declerck). The outcome of these data will be reported at the 2004 meeting.

D-dimer

Dr. Dempfle showed data on a new method for standardization of D-dimer antigen assays, which is not based on consensus values or pooled plasma samples. He discussed the possibility of using a well-characterized purified DD-domain containing model peptide, generated from fibrin by using a novel endoproteinase, in the calibration process. The model peptide would be used to calibrate existing standards with regard to their content of this dimerized D-domain. The meeting agreed that expressing all calibrators in ‘dimerized D-domain’ may be a first step in harmonization of D-dimer levels as measured by different assays. Therefore all interested attendants were asked to contact Dr. Dempfle and to provide him with their currently used calibrators for analysis. These data and the progress in the harmonization will be reported at the 2004 meeting.
Dr. Dempfle also reported on his study of the measurement of D-dimer in animal samples. It could be concluded that very few of the available commercial assays tested recognized to a certain extent D-dimer from animal origin (mainly horse, rat and bovine). Again interested scientists are urged to contact Dr. Dempfle in order to look for assays/reagents that may recognize D-dimer in rabbit, sheep, pig, cat or dog and to provide material (plasma) to generate calibrators for D-dimer from rat, horse and bovine origin.

Dr. Wada reported on an assay that reacts preferentially with granulocyte elastase-digested XDP (GE-XDP). Increased levels of GE-XDP were observed in severe trauma, burn and infections, which were not associated with disseminated intravascular coagulation (DIC). There were good correlations between the levels of D-dimer and plasmin plasmin inhibitor complex (PPIC) in patents with DIC, indicating that the plasmin mediated fibrinolysis predominantly functioned in these patients. On the contrary, no good correlations were found between GE-XDP and PPIC in patients with severe infections, trauma and burn without DIC. Since GE-XDP is a distinct criteria from the D-dimer (or p-XDP), measurement of GE-XDP may provide a new tool for the analysis of intravascular fibrin degradation.

Standardization and Methodology

Standardisation of bioassay methods is a means of reducing interlaboratory variability when measuring biological parameters. Dr. Longstaff discussed the development of a method for measuring potencies of plasminogen activators which has many advantages over current methods and would make a good reference method. The method is precise and accurate (it has been field

tested in 2 international collaborative studies with excellent results). The assay system includes fibrin and can be compared to the physiological situation during thrombolysis. However, the inclusion of a chromogenic substrate means that good quality data can be collected and results can be expressed in SI units (molar plasmin concentration generated per second). This allows comparison of different plasminogen activators, which is a particular advantage in fibrinolytic standardisation due to the proliferation of molecular variants and the history of arbitrary units. The meeting agrees that this method may well suit the requirements of a reference method. Before making a final decision this method will be evaluated by different labs. Dr. Longstaff will coordinate this study and report the findings at the 2004 meeting.

Dr. Babu discussed current methods of plasminogen-based fibrinolysis methods for the quantitation of plasminogen activators. He reported the discovery of a bacterial enzyme with a direct fibrinolytic action and with potential clinical application. He raised the problem of expressing the activity of such compounds in defined units.

Dr. Antovic presented data on an assay for the evaluation of overall fibrinolytic potential (OFP) derived from the overall hemostatic potential (OHP). The meeting expressed its concerns on the overall applicability of such a test since, dependent on the reagents used to perform this test and dependent on the possible presence, in (patient) plasma, of drugs that affect coagulation as well as fibrinolysis, the outcome may not necessarily reflect the real physiological fibrinolytic potential. More detailed characterization of this assay would be needed.

General Discussion and topics for 2004

The meeting agreed that its activities on the methods for measurement of proCPU/TAFI and D-dimer should be continued. The progress on the planned proCPU/TAFI study and on the D-dimer standardization should be included. In addition an update on the evaluation by various laboratories, of the method described by Dr. Longstaff will be presented. Any of the (co-)chairs can be contacted for further suggestions concerning the meeting in 2004.

The meeting was attended by 100-130 people including all (co-)chairs. The meeting was closed at 11.30 pm.

Fibrinolysis

June 18, 2004
8:30 to 12:30
Barbantini
Fondazione Giorgio Cini

Chair: O. Matsuo, Japan
Co-chairs: P. DeClerk, Belgium; C. Dempfle, Germany; D. Hendriks, Belgium; C. Longstaff, UK; M. Nesheim, Canada

At the start of the session, the chairman gave an overview of the ongoing activities of the Fibrinolysis Subcommittee. Then the various presenters provided a summary of the achievements of the past year.

I. **TAFI session:**

Three speakers presented the recent assay methods and their results. **Dr. Nesheim** developed the assay method for TAFIa in plasma (The development and use of an assay for plasma TAFIa). The mean concentration of TAFIa in plasma was 11.7+/- 3.6 pM. When thrombin was injected in the baboon, plasma TAFIa increased transiently at the peak value of around 2000 pM at 100 min after thrombin injection. After injecting E. coli to baboon, TAFIa also increased transiently. In human samples, TAFIa in patients with pre-eclampsia increased at 0-6 hour after post partum, and then decreased gradually at 12-24hr. In the factor VIII deficient plasma, it is well known that the clotting time is prolonged. In this process of the clotting, TAFIa in the factor VIII deficient plasma was not increased as in the normal plasma.

Dr. Declerck presented new data on different monoclonal antibody-based ELISAs that exhibited different reactivities towards different forms of (pro)CPU/TAFI(a) (Development of ELISAs exclusively reacting with either the released activation peptide from TAFI (proCPU) or with TAFIa (CPU). One assay recognized exclusively the zymogen. Another assay reacted exclusively with the released activation peptide whereas the third assay reacted only after activation of proCPU/TAFI. The availability of these different assays may facilitate the evaluation of proCPU or its activated forms as putative risk markers in cardiovascular diseases. However, the evaluation of these assays in a variety of clinical samples will need to provide the ultimate proof.

Dr. Frere on behalf of MC Alessi presented data about the influence of the TAFI gene polymorphisms on TAFI levels in plasma. Different TAFI assays were presented with variable sensitivity towards the Thr325Ile polymorphism. Independently of the Thr325Ile polymorphism which can alter the value of TAFI in some ELISA's, two TAFI gene polymorphisms are strongly associated with TAFI levels : G-1102T as well as T+1538A (Polymorphisms in the TAFI gene contribute to TAFI levels regardless of the Thr325Ile polymorphism).

In this TAFI session, **Dr. Declerck** also announced that, as discussed at the previous meeting, his laboratory (Dr. A. Gils) together with the laboratory of Prof. I. Juhan-Vague has been collecting plasma samples for evaluation of the currently used assays. It is anticipated that by the end of

2004 or early 2005 these samples will be sent for analysis to participating laboratories. The results of these analyses will be presented at the SSC-fibrinolysis meeting in 2005.

II. **Standardization session**

Dr. Longstaff summarized the whole activity for the standardization in previous period in SSC as well as NIBSC. He introduced the present protocol to measure the plasminogen activator activity in the microtiter plate (Standardizing methodology in Fibrinolysis assays). He delivered the standard PAs (tPA, UK, SK), standard plasmin, standard fibrinogen, plasminogen, thrombin, and chromogenic substrate. Some of the raw data is very excellent, but there were some problems. A large amount of variability in results was seen in the overall results. These findings highlight the difficulties of measuring absolute reaction rates in different laboratories as opposed to relative rates against a standard reference preparation. He is waiting for all data coming from all member of working group, and then he can analyze carefully and may present the model assay format in the next SSC committee.

III. **III D-dimer**

As the first speaker in this session, **Dr. Dempfle** talked about the definition of D-dimer, and compared various assay kits available from the commercial source at present moment (Definition of D-dimer antigen). Further, the effect of FgDP, or fibrinogen, on the assay of D-dimer was examined and he pointed out the problem of the calibrators. Dr. Meijer introduced the harmonization procedures of D-dimer antigen assay. The method for harmonization is different from the standardization. Although several important issues (e.g. how to establish an independent harmonization equation) regarding the implementation of the harmonization procedures need to be clarified, the diagnostic companies are willing to participate into the process of harmonization. Dr. Meijer organized the D-dimer harmonization study-project group, and will present the results at next meeting. The hot discussion was followed on the implementation of harmonization.

IV. **General discussion and future activities**

Currently used (pro)CPU/TAFI(a) assays will be evaluated by various laboratories (coordination Dr. A. Gils) and the results will be presented at the 2005-meeting.
Consequent to discussions with the Working Group on Secondary Standards and preliminary evaluation by some members of the SSC-fibrinolysis subcommittee the meeting agreed to calibrate the newly prepared SSC Plasma standard lot 3 for t-PA antigen and for PAI-1 antigen and activity. This will be coordinated by Dr. Longstaf and the results will be presented at the 2005-meeting.

D-dimer as well as the standardization, which are further handled for the next SSC subcommittee. In addition, as the new issue in this SSC subcommittee, we will work on plasmin and streptokinase preparation, the detail of which is announced later. Any of the (co-)chairs can be contacted for further suggestions concerning the meeting in 2005.

The meeting was attended by around 60 – 70 people including all (co-)chairs. The meeting was closed at 12:00.

Fibrinolysis

6 August 2005
11:00 to 14:30
Harbourside Meeting Room 4
Sydney Convention and Exhibition Centre

Chair: O. Matsuo, Japan
Co-chairs: C. Dempfle, Germany; D. Hendriks, Belgium; C. Longstaff, UK; M. Nesheim, Canada

TAFI/proCPU

Dr Willemse presented a new kinetic assay for measuring proCPU/TAFI levels in plasma. He first gave an introduction about currently available assays and pointed out the major pitfalls of antigen and activity-based assays. The novel assay he presented is based on the quantification of arginine-cleaved from hippuryl-L-arginine by CPU using 3 coupling enzymes (arginine kinase, pyruvate kinase and lactate dehydrogenase) finally leading to the consumption of NADH which can be followed continuously at 340 nm. The assay shows excellent correlation with the HPLA-assisted reference assay and has a high precision. Compared with HPLC, the assay is much easier to perform and it allows a much faster determination of proCPU concentrations. This, combined with the broad linear range of proCPU determination (100-2400U/L) makes it a useful tool for sensitive screening of clinical samples. Because the assay measures the cleaved arginine it can be used with all kinds of C-terminal arginine-containing substrates and can be used as a useful tool for screening different synthetic and physiological CPU substrates.

In discussion the problem of availability of one of the enzymes needed for the coupled assay was highlighted since it must be purified and is not commercially available.

Standardization of fibrinolytic factors

Calibration of SSC Plasmas

Dr. Longstaff reported on the calibration of SSC plasma #2 and #3 to measure t-PA antigen, PAI-1 antigen and activity. Eight labs joined in the collaboration study to assess the feasibility of calibration. GCV results for t-PA antigen in SSC #2 and #3 were variable, and a little less so for 94/730. However, there was some improvement in variability after the removal of outlier and 94/730 was close to the expected value of 25 ng/ml. Normalisation of SSC2 and 3 using 94/730 as calibrator improved the spread of data and suggests the preparation will be useful as a standard for tPA antigen assays.

Table 1 Summary of potencies
t-PA Antigen (ng/ml) [Expected plasma quoted values <10, or 1 – 12, or 95% <9 ng/ml; 94/730 25 ng/ml]

Sample	geo mean pot	95% lower	95 % upper	%gcv

SSC2	3.38	1.87	6.12	103.4
SSC3	3.67	1.89	7.11	120.9
*94/730	24.45	19.47	30.7	27.9

*One statistical outlier removed

With the same aim, PAI-1 antigen was compared with 92/651, SSC #2 and #3. The mean value in 92/654 was 73.5 ng/ml, though the expected value was 250 ng/ml. SSC #2 and #3 gave consistent and reasonable results. Labs 1 and 6, using the same kit, gave consistently low results for all samples (see table 2). Results from this small study do not allow us to assign a potency for PAI-1 antigen in ng/ml in plasma with confidence.

Table 2 Summary of results
Summary of PAI-1 Antigen after removing labs 1 and 6 [Normal: 4 -43 ng/ml]

	Assay	geo mean pot	95% lower	95 % upper	% gcv
SSC2	25	13.16	8.75	19.8	39.0
SSC3	25	13.94	9.98	19.46	30.8
92/654	25	107.9	70.61	165.0	40.7

In the study to calibrate the SSC plasmas for PAI-1 activity, laboratories split into 2 groups reporting activity in ng/ml or inhibitor units/ml. These results could not be easily compared. Both groups showed large variability, especially the ng/ml group. Further work is needed before standardization of PAI-1 activity can be attempted.

During the discussion with the audience it was generally agreed that some problems with 92/654 may be due to the recombinant nature of the PAI-1 used to spike the plasma in this International Standard. It was suggested that a new standard of plasma containing a high level of (native) PAI-1 would be useful, especially for the diagnosis of elevated PAI-1 in patient samples.

Standardization of methodology for plasminogen activator activity.

C Longstaff gave a final report on the methodology study for determination of thrombolytic potency of plasminogen activators. The aim of the study was to investigate the feasibility of a proposed assay for the determination of absolute enzyme activity is SI units (pM/s plasmin production), as an alternative to International Units (IU). This approach would fulfill some recommendations for assay methods and allows different thrombolytics to be compared, which is not the case currently as IU are different for current plasminogen activator IS. A very detailed assay protocol was agreed before the study and all participants were provided with all critical reagents to measure streptokinase, tPA and uPA activity. In spite of this there was a wide spread of final results returned for absolute enzyme activity. Means of all assay results gave satisfactory dose response curves for all activators. Some improvement in variability (expressed as %GCV) was observed if streptokinase was used as a standard for the other plasminogen activators, but variability was still quite large, around 35%. The conclusions of the study were that absolute determination of enzyme activity is very difficult. It is also problematic to provide a new method

to laboratories and expect them to perform the method well without training. The traditional method of calibrating International Standards by recruiting as many labs as possible and allowing them to use familiar methods is a pragmatic approach that will be difficult to change in favor of a specific assay if this involves much complexity.

Standardization problems with recombinant streptokinase

Colin Longstaff reported on observations with potency determinations of native and recombinant streptokinase using the current 3 rd International Standard (IS) for Streptokinase. The international standards for streptokinase have been used successfully for more than 40 years and international collaborative studies show excellent agreement between consecutive standards. Many companies around the world manufacture streptokinase to treat the global epidemic of cardiovascular disease seen in developing countries and in Eastern Europe. Some of these products are recombinant (rec) and some of these rec products do not behave well against the 3 rd IS for streptokinase in different assay formats. For example, using two standardized methods, (1) without fibrin and (2) with fibrin the potency of two recombinant streptokinase products available in India was measured relative to the 3 rd IS for Streptokinase. The ratio of potency for normal, native streptokinase is 1.0 comparing these assay methods, but for one rec streptokinase the ratio was 0.3 and for a second rec streptokinase was 1.5. Thus the inclusion of fibrin in the assay can dramatically affect the potency of the product and the dose given to patients. This is especially important since different pharmacopeias recommend assay methods, without fibrin (eg European and British Pharmacopoeia) or with fibrin (Indian Pharmacopoeia). Changing the assay format could result in lethal doses of streptokinase being given to patients. Further work is needed to determine the cause and possible solutions to these problems, which may include provision of additional standards or pharmacopoeial methods.

D-dimer

Assay of D-dimer in multicenter trial I. Jennings

The measurement of D-dimer is used for diagnosis of DIC, monitoring the treatment of DIC, diagnosis of DVT/PE and the prediction of recurrence The multicenter trial for the assay of D-dimer was performed (431 centers participated). For this trial, 4 D-dimer samples were prepared; high D-dimer level (pool 1: ~1000ng/ml), low D-dimer level (pool 2: ~300 ng/ml), mixture of equal quantities of pool 1 and pool 2 (pool 3) and pool of plasma with ~300 ng/ml of D-dimmer (pool 4).

This trail demonstrated the large variation in results between reagent groups. The comparison of findings in different centers was difficult. The calibration curve was made by plotting overall median D-dimer level versus individual laboratory D-dimer levels for low, mix and high sample. Although the calibration curves could be constructed, comparison of results only valid where linearity was good and data was not extrapolated. Difference may exist between different samples that preclude valid comparison between methods.

Harmonization of D-Dimer assays: Results of the FACT4 study C. Dempfle

At first, Dr. Dempfle presented the consensus of D-Dimer antigen. Monoclonal antibodies used in D-dimer antigen assays should display minimal cross-reactivity with fibrinogen, (monomeric) fragment D from fibrinogen, non-crosslinked fibrin, other proteolytic fragments of fibrinogen or non-crosslinked fibrin.

In clinical plasma samples, fibrin fragment D-dimer represents only a portion of the total D-dimer antigen. A major portion of D-dimer antigen in clinical plasma sample has a higher molecular weight than fibrin fragment D-dimer. Based on these findings, fibrin fragment D-dimer is not a primary candidate for a calibrator. Distribution of fibrin compounds and matrix should closely match with clinical plasma samples. Since D-dimer antigen is not a homogeneous and monoclonal antibodies against D-dimer antigen react with different antigenic sites of the D-dimer antigen structure, a primary reference standard cannot be formulated. Therefore, pooled patient plasma samples could be used for harmonization of D-dimer antigen assays. The pools should contain a variety of clinical plasma samples including the target groups DVT, PE and DIC. Different responses of D-dimer antigen assasys in different concentration ranges preclude the use of simple conversion factors.

For D-dimer assay, a common calibrator should be need. Fibrin fragment D-dimer is not suitable for D-dimer assay. One standard is sufficient if the standard is a pooled plasma with a large number of donors. It is necessary to evaluate assays individually for determination of the cut off for DVT exclusion. Dr. Dempfle proposed the preparation and validation of a lyophilized reference preparation based on pooled human plasma. Dr. Dempfle also recommended the definition and validation of the procedure for calibration of the reference preparation, and the clinical evaluation of the calibrator.

Problems with the D-Dimer Assay J. Olson

In reporting the D-Dimer level, two units are usually used. One is D-Dimer Units (D-DU), another one is fibrinogen equivalent unit (FEU). Surprisingly, 1 ng/ml (D-DU) of D-Dimer equals to 2 ng/ml (FEU) of D-Dimer. Data from a study in the U.S.A. indicated nine commercial kits for D-Dimer are available, six recommends reporting FEU and three recommends reporting D-DU. Among all methods for reporting the quantitative D-Dimer, there is wide variation in the type and magnitude of units reported.

Nearly 40% of laboratories are converting the analyzed units and reporting in unit other than those recommended by the manufacturer. Many laboratories are unclear about which type of units they are reporting. This is the major problem with the D-dimer assay performance in the U.S.A.

General discussion and activity for next term

Since we have still several questions and issues to be clarified, we would like to keep the studies on TAFI, Standard of fibrinolytic factors and D-Dimer. Requests to the audience were made to suggest new issues that might be appropriate for the committee.

Fibrinolysis

Chair: O. Matsuo, Japan
Co-chairs: C. Dempfle, Germany; D. Hendriks, Belgium; C. Longstaff, UK; M. Nesheim, Canada

Section I TAFI /CPU

Dr Nesheim reviewed the structure and function of TAFI and discussed the mechanism of the prolongation of lysis time resulting from TAFI activity. Functional methods for measuring active TAFI (TAFIa) were reviewed. Three assays were described: 1. A colorimetric assay was described using the substrate anisoylazoformyl arginine (AAFR); 2. A functional assay based on FDP cofactor activity with a fluorescent plasminogen substrate and bat t-PA (DSPA); and 3. A functional assay based on the direct binding of fluorescent plasminogen to FDPs. The colorimetric assay involving AAFR is simple and shows a good dose response but is not so sensitive. In the functional assay with DSPA the normal background level of TAFIa from 6 subjects gave a mean of 11.7 ± 3.6 pM, which is only 0.2% of the circulating zymogen concentration. Thus the method is sensitive, specific and accurate, however it is technically demanding and requires specialized reagents not commercially available. Dr Nesheim described the functional assay based on the binding of fluoresecent plasminogen to FDP which was also sensitive, accurate and precise and is a one step, quick and easy method. This method should be suitable to use on plasma samples and should be free of interference from t-PA and fibrinolytic inhibitors. Dr Nesheim expressed a wish to collaborate with other workers on these assays using clinical and experimental specimens and invited them to contact him.

Drs Willemse and Hendrick introduced activity based proCPU/TAFI assays with regard to the importance of polymorphism and substrate specificity. The principle of this assay is the cleavage of hippuryl-L-rginine to hippuric acid and arginine. It was shown that the threonine 325 isoleucine polymorphism has a significant effect on CPU stability such that the thr325thr variant has a half-life of 8 minutes versus 15 min for the ile325ile variant, although there was no difference in the activation kinetics. Intrinsic activity of proCPU was analysed using Bz-AA-Arg where AA is substituted by a range of amino acids. Ala and Met were the residues showing highest intrinsic activity of proCPU. Conditions to optimize proCPU activation kinetics were explored that minimized genotype dependent artifacts. Recommendations included activation at room temperature rather than 37ºC and the use of high substrate concentrations.

Drs Gils and Declerk demonstrated ELISA methods using monoclonal antibodies for the detection of TAFI and TAFIa and isoforms. An ELISA with MA-T32F6/MAT9G12-HRP was used to measure TAFI and showed no difference between a control group and patient groups. Different combinations of antibodies were sensitive to the presence of different isoforms of TAFI. Isoform of The325 Ile was not recognized by some antibodies leading to underestimates of TAFI levels in plasma. Surprisingly some polyclonal antibodies were also sensitive to different TAFI isoforms. Monoclonal antibodies in 144 combinations were screened to detect different forms of TAFI including intact zymogen, activation peptides and active TAFIa. Thus selecting different combinations of antibodies could be used to monitor the process of TAFI

activation and decay of activity. The clinical application of these assays can be used to shed light on the role of TAFI in vivo.

Section II Standardization of fibrinolytic factors

Dr Longstaff presented a summary of a recent study to measure t-PA antigen in plasma. This was a follow-up of a smaller study presented last year on t-PA antigen and PAI-1 antigen and activity. The aims of the present study were to assign an agreed value of tPA antigen to in the SSC plasma lot 3 and in a spiked plasma preparation 94/730. The previous 2 nd IS for t-PA activity was also included as this was apparently used in the past by kit manufacturers to calibrate their standards. Results were presented from 14 groups comprising 8 different methods (in-house and commercial kits). As in the previous study there was significant variability in values for SSC lot 2 and lot 3 with a mean value close to 3 ng/ml (normal value less than 10 ng/ml) and %gcv around 70%. A mean value of 25.26 ng/ml, in line with expectations, was calculated for 94/730 (%gcv 21.0 for 12 labs after removal of 2 statistical outliers). A mean value of 1.5 µg/ ml was derived for 86/670 also in line with the expectations. Recalculation using 94/730 as a common standard of t-PA antigen in SSC plasma lot 2 and 3 resulted in no change in t-PA antigen concentration and improved %gcv marginally by around 10%. There were clear differences between methods for all samples, however, results could be harmonized using a method-specific correction factor. In this way lot 2 could be used to determine the correction factor to apply to the results for lot 3 and vice versa. After this procedure the value of t-PA antigen in lot 3 was still very close to 3 ng/ml but the %gcv fell to 18.2. Results from earlier studies were also presented which showed t-PA antigen in plasma were close to 3ng/ml and in 94/730 was close to 25 ng/ml. Dr Longstaff asked if there were objections to proceeding with proposals that SSC plasma lot 3 could be assigned a t-PA antigen level of 3 ng/ml and 94/730 could proceed as a proposed international standard with a t-PA antigen of 25 ng/ml. No objections were raised.

In a second talk, Dr Longtaff summarized the results from the earlier studies including PAI-1 and highlighted the difficulties found. Recent results from NIBSC on the 1 st IS for PAI-1 suggested that this preparation was stable, after low values for antigen were determined in the earlier studies. Proposals were presented on future studies aimed at calibrating SSC plasmas for PAI-1 which could be performed using a range of plasma samples with different PAI-1 activities as a means of harmonizing different methods. These studies were planned for 2006/7.

Dr Longstaff presented an update on work involving recombinant streptokinase which generates discrepant results in different assay formats relative to the 3 rd IS for Streptokinase. These observations have significant implications for the assignment of potency values to streptokinase therapeutic products and correct dosing for treatment of myocardial infarction. Data were presented from collaborative work with an Indian Biotchnology company (Biocon) on recombinant streptokinase suggesting that an N-terminal methionine, in place of the expected isoleucine, present as a result incomplete processing of the protein in ***E. coli*** . was the source of the problems. A modified recombinant variant without the N-terminal methionine was demonstrated to show no evidence of discrepancies in assays with or without fibrin present. In light of other results where the N-terminal sequence has been modified in recombinant streptokinase it is not unexpected that changes in activity might be seen in the presence of fibrin.

It was concluded that manufacturers of recombinant streptokinase should ensure that the N-terminal sequence of their protein is correct if they are to correctly assign a potency using the 3 rd IS for Streptokinase

Section III D-dimer assays

Dr. Carl-Erik Dempfle on behalf of the FACT study group presented first the review and then possible proposal. Based on the work of Patrick Gaffney on the structure of fibrin degradation products, assays based on monoclonal antibodies generated by immunization with fibrin fragment D-dimer have been available since 1983. A variety of monoclonal antibodies have been developed, and assay technology has shifted from manual latex agglutination assays and microtiter plate ELISAs to quantitative latex particle assays with photometric detection and rapid fluorometric immunoassays.

In clinical plasma samples, D-dimer assays mainly detect high molecular weight crosslinked fibrin complexes in addition to fibrin degradation products. The consensus statement from the preceding ISTH SSC meeting concerning the definition of D-dimer antigen is as follows: 'D-dimer antigen indicates antigenic material detected by use of monoclonal antibodies generated by immunization with fibrin fragment D-dimer and related compounds. The minimal structure detected is fibrin fragment D-dimer, but larger compounds containing dimerized D-domains are detected as well.'

Various calibrators are being used for D-dimer assays, including fibrin fragment D-dimer, terminal plasmin digests of crosslinked fibrin clots, plasmin digests of crosslinked fibrin clots with digestion stopped before the terminal stange, and plasma pools from patients with high levels of D-dimer antigen. Although fibrin fragment D-dimer and terminal digests of fibrin clots work well with some assays, others display a totally different reactivity with this material than with clinical plasma samples, resulting in over- as well as underestimation of D-dimer antigen levels in the clinical plasma samples. Therefore, the calibrator should contain a physiological array of crosslinked fibrin derivatives. This can be achieved either by using pooled plasma from patients with high levels of D-dimer antigen, or in vitro-preparations containing fibrin derivatives with similar composition of fibrin derivatives. According to the results of the Fibrin Assay Comparison Trial (FACT) part 4, a pooled plasma from patients with disseminated intravascular coagulation (DIC) shows identical performance with all 28 D-dimer assays tested both using serial dilutions with plasma from healthy blood donors, and buffer. Therefore, only a single calibrator pool plasma is needed, which may be diluted with assay-specific diluents.

Since patient plasma pools, as well as in vitro fibrin preparations may be heterogeneous, a reliable procedure for assigning D-dimer concentration values is needed. The FACT working group suggests the following approach: Aliquots of the plasma pool or fibrin preparation are incubated with a high concentration of plasmin in presence of calcium and a thrombin inhibitor. Both fibrin, and fibrinogen in the sample are degraded, resulting fibrin fragment D-dimer/E complex as main terminal breakdown product of the crosslinked fibrin. Proteolysis of the fibrinogen, in contrast, yields fibrinogen fragments D and E. After ensuring that proteolysis is complete by SDS-polyacrylamide gel electrophoresis and immunoblotting, using polyclonal anti-fibrinogen antiserum for detection, concentration of fibrin fragment D-dimer is measured. For

measurement of fibrin fragment D-dimer, several D-dimer assays are used which are not influenced by presence of fibrinogen degradation product D (FDP-D) and show good reactivity with fibrin fragment D-dimer. These assays are calibrated with purified fibrin fragment D-dimer. The resulting concentration levels of fibrin fragment D-dimer reflect the total concentration of D-dimer antigen (dimerized D-domains) in the original plasma sample.

By means of plasmin proteolysis, the D-dimer antigen is 'homogenized' and concentration measurment is made independent of the molecular size and composition of the fibrin compounds containing the dimerized D-domains. This allows calibration with fibrin fragment D-dimer/E complex as a well-defined primary reference material.

The measured D-dimer value is then assigned to the pooled plasma, which is then used for calibration of the D-dimer assays.

The following round of FACT (FACT-5) will require calibration of the participating assays with the common calibrator pool plasma and will include a set of clinical plasma samples from patients with DIC, DVT and pulmonary embolism. The study will evaluate the effect of common calibration of D-dimer assays on the conformity of the D-dimer assay results.

Section IV General discussion

For the activity in the following term, the participants are requested to contact with speakers to collaborate in each item. The number of the participants is estimated about 150 at the end of the subcommittee.

D-dimer session with Fibrinolysis, DIC and Hemostasis and Malignancy was held after Fibrinolysis subcommittee, and this was successful as the first trail.

Fibrinolysis

Chair: C. Longstaff (UK)
Co-Chairs: C. Dempfle (Germany), D. Hendriks (Belgium), O. Matsuo (Japan), M. Nesheim (Canada)

TAFI/CPU Chaired by D Hendriks

M Nesheim presented work on a new assay for TAFIa in plasma based on modifed plasminogen with a fluorescently labelled active site and a chemically-derivatized FDP containing a quencher molecule. Removal of C-terminal lysines by TAFIa is measured by increasing fluorescence. The assay was sensitive down to 10-15 pM TAFIa with good results for intra- and inter assay variabliltiy of 6.3 and 8.3%. There was no interference from normal plasma plasminogen in the normal physioligical range or the TAFI polymorphism at position 325. Several normal plasma samples from volunteers were tested and an average of 20 pM TAFIa determined (range 4-32). Data were presented from animal experiments following injection of procoagulant FXa and PCPS demonstrating a dramatic, transient increase in TAFIa levels with the expected half-life. Sub-lethal doses of *E.coli* injected into a baboon as a model of sepsis were able to activate up to 30% of available TAFI zymogen. Conditions suitable for collection of plasma samples to optimise TAFIa stability were discussed.

J Willemse discussed approaches for the measurement of proCPU and CPU using specific small chemical substrates with the structure Bz-Xaa-Arg (where Xaa represents a naturally occurring amino acid). These assays are complicated by the presence of constitutively active CPN, the activity of which must be subtracted to determine CPU activity. A total of 15 synthetic substrates were screened with CPU and CPN to determine which amino acids optimised the ratio of activity (expressed as kcat/Km) for CPU/CPN. Aromatic residues or chemically modified aromatic residues were optimum for maximising the ratio of CPU/CPN activity. A summary of results from a pilot study measuring CPU levels generated during thrombolytic therapy for ischaemic stroke was presented demonstrating significant generation of CPU activity. Conditions for the minimisation of ex-vivo proCPU activation were discussed.

A Gils discussed the application of 3 different ELISA approaches to investigate the extent of TAFI activation in clinical studies. It was hypothesized that not the total amount of TAFI protein but the amount of activated TAFI may play a critical role in the interference with fibrinolysis. Therefore, two ELISAs were developed measuring either the activation peptide or activated TAFI (TAFIa). Intact TAFI and TAFI fragments were determined in three different groups of patients i.e. patients with hyperlipidemia, patients with stroke and patients with sepsis.

From the data obtained it was concluded that the ELISAs that measure the extent of TAFI activation are more sensitive markers in studies on the relationships between TAFI and cardiovascular diseases, but assessment of all possible markers may be needed and should be assessed on a case by case basis. Standardisation of the assay was discussed and it was confirmed that recombinant active peptide did not react in the same way as native peptide found in plasma and could not be used as a standard, however it may be possible to express results in pM of peptide with further work.

T Lisman discussed 3 epidemiological studies on venous and arterial thrombosis in which thrombosis risk associated with hypofibrinolysis was investigated. Hypofibrinolysis was assessed with a plasma-based global fibrinolysis assay where plasma is clotted with tissue factor in the presence of calcium and phospholipid vesicles and fibrinolytic potential estimated from clot lysis curves. Three studies were discussed. LETS (leiden thrombophilia study), a case/control study on venous thrombosis (421 pts/469 controls) where hypofibrinolysis was associated with a 2-fold increased risk for a first venous thrombosis. MEGA (multiple genetic and environmental assessment of venous thrombosis), a case/control study on venous thrombosis (2913 pts/2129 controls), where increased thrombosis risk was associated with hypofibrinolysis, and showed an interaction of hypofibrinolysis with factor V Leiden. SMILE (study of myocardial infarction leiden), a case/control study on myocardial infarction in men (426 pts/646 controls), where hypofibrinolysis was associated with an increased risk of MI only in men below the age of 50. The SMILE study also showed elevated TAFI levels (measured by activity assay, Pentapharm) to be *protective* against MI.

During the general discussion D Hendriks proposed that a number of common samples should be compared using the different approaches described for measuring active CPU/TAFIa.

D-Dimer Chaired by C-E Demple

I Jennings reported on two separate UK NEQAS for Blood Coagulation exercises to explore the degree of precision amongst laboratory D-Dimer measurements, and the degree by which inter-method agreement could be improved using a calibration curve model. The first exercise demonstrated generally good within-centre precision, with 82% centres reporting results for two identical but differently coded samples within 10% of each other. However, 6 centres reported results which would have excluded DVT for one sample but failed to exclude DVT for the other, identical sample. In the second exercise, overall between-method precision of D-Dimer results for two samples was shown to improve markedly when a calibration model was applied, using the consensus median values obtained by all participants for three “calibration plasmas” to recalculate D-Dimer values. For centres reporting results in fibrinogen equivalent units (FEUs), between-centre coefficients of variation (CVs) fell from 25.9% to 11.6% and 22.4% to 7.7% respectively for the two samples. For centres reporting in ng/ml, CVs fell from 45.3-21.6% and 40.8-11.6% respectively. Improved harmonisation of D-Dimer results by different methods may be achieved by a calibration model and common calibrant plasmas.

P Meijer reported results from the latest ECAT study involving 600 participants and covering 25 methods, although 8 methods account for 90% results. More participants report results in FEU rather than ng, in contrast to UK NEQAS studies. Issues of repeatability and the importance of repeat testing for results around the cut-off level were discussed as an approach to minimise false positives and negatives. Sources of variability in assay results were discussed which include the usual reasons of differences between antibodies and reference standards used in different methods but also additional problems from different lots of reagents in the same kits.

Both NEQAS and ECAT quality control studies highlight the large discrepancies of numerical D-dimer values reported, which add to the complications surrounding interpretation of assay results in the clinical context of VTE exclusion. The main cause of discrepancy is the different

calibrations used by the manufacturers of the assays, but problems for clinical decisions arise from choice of cutoff values. These may be taken from package inserts but may be in-house evaluations. Even for individual assays, the cutoff values used may vary considerably between laboratories and VTE exclusion cutoff values have been validated in appropriate clinical trials only for a minority of D-dimer assays. Assay results in the high concentration range are also highly discrepant, which makes it difficult to use D-dimer assays in scoring systems, such as the ISTH-DIC score, or establishing cutoff values for other indications apart from VTE exclusions.

P Meijer also discussed harmonisation of immuno-assay in general with particular relevance to possible approaches to measurement of plasma tPA and PAI-1 antigen. Ideally a standardisation hierarchy should be adopted with a SI units and a primary reference methods at the head, which also requires clear definition of the entity to be measured. This is often not possible for complex biological mixtures ("soups") and approaches to harmonisation may propose the use of consensus values, but it should be recognised that this approach is "unstable". A number of proposals were made to improve standardisation and harmonisation of immuno-assays including the organisation of collaborative studies with samples having a range of values to be measured, inclusion of all available methods and assessment of clinical samples where possible and clear understanding of the entity being measured.

C-E Demplfe discussed applications of D-dimer assays for purposes other than VTE exclusion including DIC, aortic aneurysm exclusion, monitoring of anticoagulant therapy (including detection of heparin-induced thrombocytopenia), and monitoring of intensive care patients and risk stratification after stopping anticoagulant therapy. Reporting on a common scale is particularly important in these cases as is the ability to measure a wide range of D-dimer values.

A summary of a consensus statement issued at a meeting on D-dimer hosted by the ECAT was presented which included the following points:

- Fibrin fragment D-dimer is a terminal product of plasmin proteolysis of fibrin containing crosslinked C-terminal gamma-chains.
- D-dimer antigen indicates antigenic material detected by use of monoclonal antibodies generated by immunization with fibrin fragment D-dimer or related compounds.
- The minimal structure detected by D-dimer antigen-specific monoclonal antibodies is fibrin fragment D-dimer. Larger compounds containing dimerized D-domains are detected as well.
- In clinical plasma samples, fibrin fragment D-dimer represents only a portion of the total D-dimer antigen. A major portion of D-dimer antigen in clinical plasma samples has a higher molecular weight than fibrin fragment D-dimer. Based on these findings, fibrin fragment D-dimer is not a primary candidate for a calibrator.
- Since D-dimer antigen is not a homogeneous entity and monoclonal antibodies against D-dimer antigen react with different antigenic sites of the D-dimer antigen structure, a primary reference standard cannot be formulated.

It was suggested, that pooled plasma from patients with high D-dimer antigen concentration be used for harmonization, applying a series of dilutions of the pooled plasma as used in NEQAS and ECAT quality control

C-E Dempfle showed results from the Fibrin Assay Comparison Trials (FACT) parts 4 and 5. FACT4 compared assay reactivity of fibrin fragment D-dimer and pooled plasma from patients with DIC, using serial dilutions in plasma from healthy blood donors, and in buffer. Some D-dimer assays displayed identical dose-response with fibrin fragment D-dimer and the pooled plasma samples, some assay reacted considerably better with the low molecular weight fibrin degradation product than with the predominantly higher molecular weight fibrin contained in the plasma, and other assays responded poorly to fibrin fragment D-dimer. For the pooled plasma, assay reactivity was similar for dilutions with plasma and buffer. It was concluded that a single pooled plasma with high concentration of D-dimer antigen would be sufficient as common calibrator, with no need to prepare sets of dilutions, or pooled plasma with different levels of D-dimer antigen.

In the recently completed FACT5 trial, reference laboratories of assay manufacturers received a pooled plasma with high concentration of D-dimer antigen for preparation of serial dilutions with assay-specific diluents, and a set of 50 pooled plasma samples with different levels of D-dimer antigen. The correlation of assay results for all 30 assays included was excellent, with a mean regression coefficient of 0.946±0.054 (range 0.703 – 0.999). Common calibration with the pooled plasma reduced the coefficients of variation from nearly 60% to approximately 20%.

Since the procedure for harmonization based on consensus values produced variable results depending on the set of assays included, a new procedure was suggested by C-E Dempfle to generate D-dimer antigen values on a common scale for all D-dimer assays. This procedure is based on the distribution of a pooled reference plasma with high concentration of D-dimer and an assigned D-dimer level to the assay manufacturers. The D-dimer concentration is assigned by a procedure involving ‘homogenization’ of the D-dimer antigen by extensive plasmin digestion of the reference plasma and quantitation of the amount of D-dimer generated with a calibrator consisting of terminal plasmin digest of a cross linked fibrin clot prepared from a known amount of fibrinogen. By the plasmin digestion, all D-dimer antigen present in higher molecular weight form is transformed to fibrin fragment D-dimer, which is a homogeneous analyte. This allows the preparation of successive plasma pools with constant levels of D-dimer antigen.

Future activities will include quality control issues of the procedure used for assigning D-dimer concentration values, and investigations on the effect of lyophilization of the plasma on the results as part of the process of making long term stable standards. The effect of common calibration on future quality control exercises, and the performance of diagnostic algorithms involving D-dimer antigen will be other topics.

Plasminogen Activators and Plasmin chaired by O Matsuo

C Longstaff reported the conclusions of a collaborative study to determine tPA antigen in 4 samples: (1) SSC/ISTH secondary coagulation standard lot 2; (2) SSC/ISTH secondary coagulation standard lot 3; (3) NIBSC Preparation 94/730; (4) NIBSC Preparation 86/670. In total 14 sets of results comprising 48 independent assays were analysed using 8 different methods: 6 commercial kits and 2 in-house methods. Results for the 2 SSC/ISTH plasma samples were similar and within the expected range at 2.9 and 3.0 ng/ml for lot 2 and 3, respectively. The overall mean antigen value for 94/730 was close to 25 ng/ml, the expected

value based on the formulation of this preparation and on past studies. Data were also analyzed using local standards and a common standard for all assays: 94/730 with an assigned value of 25 ng/ml. In this analysis the mean antigen values for the SSC plasmas were not changed from the analysis using local standards but there was a modest reduction of up to 7 % in inter-laboratory gcv. Analysing data according to method, grouping different methods or kits, highlighted significant differences between methods. However, it was possible to correct for these differences and harmonise results for normal plasma pools using data from the SSC plasma samples which produced significant reductions in % gcv, and left the antigen values unchanged. Sample 94/730 (recombinant tPA in plasma) would make a satisfactory reference preparation for tPA antigen determinations in plasma with a consensus value of 25 ng/ml. It was recommended that 94/730 be proposed as the WHO 1 st International Standard for tPA antigen in plasma. SSC coagulation plasma lot 3 can be assigned a consensus value of 3.0 ng/ml tPA antigen. The process of approval developed by SSC/ISTH was followed to prepare a report for the WHO Expert Committee on Biological Standards to recommend establishment of 94/730 as an International Standard for tPA Antigen. Approval statistics and comments from the collaborative study participants (10/13), a panel of experts with a background in fibrinolysis standardisation issues (9/13) and Fibrinolysis Subcommittee co-chairs were summarised. Among the responders no one disagreed with the proposals that 94/730 should be established as the WHO 1 st IS for tPA antigen in plasma with a value of 25 ng/ampoule or the ISTH/SSC coagulation plasma lot 3 should be calibrated at 3.0 ng/vial. The only comments received suggested possible improvements to the calibration process which would include the use of expert laboratories rather than collaborative studies and consensus values Another respondent pointed out a possible source of variability in the data obtained not identified in the report which could be due to matrix effects and “cryptic” tPA. No further comments or objections were received from the meeting.

C Longstaff reported results from a collaborative study to measure PAI-1 antigen in plasma recently completed with an aim to investigate the possibility of harmonising results obtained for PAI-1 measurements using different methods. Participants were provided with 5 different samples comprising 3 freshly collected frozen small plasma pools containing Low (L), Medium (M) and High (H) levels of PAI-1 and 2 lyophilised plasma preparations from pools of donors, which were the SSC/ISTH secondary coagulation standard lot 3 and a sample prepared at NIBSC coded 06/053. Twelve sets of data were returned comprising 7 methods, designated A-G. As expected, results for the 5 samples were highly variable between methods. The 5 samples were assigned a consensus value for PAI-1 antigen content as the arithmetic mean value from the 12 sets of data. A regression equation was then calculated from plots of each laboratory’s results for the 5 samples versus the consensus value. Conversion factors for slope and intercept were calculated for each method to make it possible to convert results from each method into values on the consensus scale. The harmonisation process worked well for most methods except one, which determined a different ranking for the PAI-1 content of the 5 samples from all other methods. The harmonisation procedure allows expression of results on a common scale so results can be compared. However, the consensus approach does not allow determination of PAI-1 antigen content in absolute units of real ng/ml and further work is required to achieve this. The results obtained raised questions about the normal range of circulating PAI-1 antigen in plasma.

C Thelwell reported on the possible usefulness of a standard of 4-nitrophenol for the standardising active site titrations using NPGB. Traditionally IS have been calibrated IU

following an international collaborative study using laboratory's own in-house methods. There has been a recent movement towards introducing SI units for standard preparations. Such a standard might be useful in conjunction with active-site titrants available for a range of proteases including plasmin, thrombin, urokinase and factor Xa, and associated inhibitors. NPGB, a suitable active-site titrant for trypsin, and an example of a collaborative study to establish the 1st IS for Alpha-1 antitrypsin (proteinase inhibitor) in units of moles of active inhibitor was brieflydescribed. Trypsin cleaves NPGB to release 4-nitrophenol, which can be measured by absorbance and converted into moles of active enzyme based on a 4-nitrophenol calibration curve. This approach relies on the accuracy of generating the calibration curve. This accuracy could be improved if a standard for 4-nitrophenol was available to eliminate variation introduced in the preparation of stock solutions. Fluorimetric active-site titrants also exist, such as MUGB, which offer greater sensitivity. A standard for 4-methylumbelliferone could be used to calibrate titrations with MUGB. This approach would allow new and replacement protease (and associated inhibitor) standards to be calibrated in molar concentrations as well as assigning SI units to existing IS. This was recommended as a possible approach for standardising plasmin as it will soon be necessary to work on replacing the existing 3 rd IS.

P Vandeberg presented work on the use of active site titration for the value assignment of a reference standard for plasmin, used during development of therapeutic plasmin which is being investigated as a direct acting thrombolytic. This standardization was done for the purpose of maintaining consistent dosing. The method used is essentially the same as what was published by Chase and Shaw (Biochem Biophys Res Commun. 1967 Nov 30: 29(4): 508-14.), but has been adapted for use in 96 well plates for manual or automated execution. Work involving crossover testing with the International Reference Preparation for Plasmin (97/536) using an amidolytic assay (chromogenic substrate S-2403) was also covered, although concerns were raised over the long term stability of the WHO IS for Plasmin which mitigated against over-reliance on this preparation.

Fibrinlysis Subcommittee Announcements by C Longstaff

C Longstaff closed the meeting with several short announcements related to Subcommittee activities. These included a request from the European Pharmacopoeia for a standard for plasmin inhibitor (alpha-2-antiplasmin) which is needed to measure the remaining inhibitor activity in virus inactivated human plasma used therapeutically. The feasibility of making such a standard will be explored and a request was made for groups interested in taking part in a collaborative study should contact C Longstaff.

An update on the modification of the Instructions for Use accompanying the 3 rd IS for Streptokinase 00/464 was provided. It is proposed to recommend that the IS only be used for native streptokinase or for recombinant streptokinase that has been checked for suitable activity in fibrin and non-fibrin-based assays. The presence/absence of fibrin can lead to discrepant results using the 3 rd IS for Streptokinase with some recombinant products and this is potentially dangerous. Where discrepant results are obtained there is currently no way of assigning a potency with the 3 rd IS for Streptokinase.

An update was provided on the status of the WHO 1 st IS for Streptodornase. This IS will now likely need to be replaced following earlier consideration that it might be discontinued.

There was one Fibrinolysis SSC publication in the past year briefly reporting the outcome of a study on fibrinolysis methods for potency determinations of streptokinase, tPA and urokinase, C Longstaff et al, J Thromb Haemost 5(2) 412-4: 2007.

Fibrinolysis

4 July 2008
Vienna, Austria

Chair: *Colin Longstaff, UK*
Co-Chairs: *Carl-Erik H. Dempfle, Germany; Ann Gils, Belgium; Dirk Hendriks, Belgium; Osamu Matsuo, Japan; Michael E. Nesheim, Canada*

DRAFT

TAFI/CPU Chaired by D Hendriks.

Update on a sensitive assay for functional TAFIa (CPU) in plasma

M Nesheim presented an update on a sensitive assay for functional TAFIa (CPU) in plasma. The assay is based on the ability of TAFIa to release bound fluorescent plasminogen from soluble high molecular weight fibrin degradation products that have covalently attached QSY moieties which quench the fluorescence of the bound plasminogen. When the plasminogen is released the fluorescence intensity increases and the rate of this can be used to measure the level of TAFIa in the sample. The level of TAFIa in a small group of normals was shown to be 20 pM with a range of around 5 to 30 pM. The assay was applied to chimp samples in experiments in which coagulation *in vivo* was stimulated with a combination of factor Xa and PCPS vesicles infused as a bolus. TAFIa levels at levels as high as 6000 pM were observed within 10 minutes of the infusion. Very high levels (35,000 pM) were found *in vivo* in lethal E. coli induced sepsis in a baboon model. A modest (2-fold) increase was found in samples of unstable, but not stable, angina in humans. In whole blood *in vitro* with clotting induced with tissue factor in the presence of and intrinsic pathway inhibitor, TAFIa was found to increase to levels of the order of 2500 pM after clotting, with a time course that tracked very closely to the thrombin-antithrombin complex time course. The extent of TAFIa activation was measured in the same way with 14 hemophiliac samples. The results ranged from values less than one tenth that of normal to near normal. One protein C deficient sample showed supra normal TAFIa generation. These results indicate that readily measured TAFIa levels appear in plasma samples under a variety of conditions and this assay is sensitive enough to measure TAFIa levels down to resting levels which are low

A new substrate allowing sensitive and specific determination of proCPU/TAFI and CPU/TAFIa in plasma samples

E Heylen presented work on a new sensitive assay for CPU (TAFIa) in plasma samples. There is a need for new sensitive assays to determine the importance of CPU levels in the circulation for population studies and during treatments such as thrombolysis for stroke. The assay is based on new sensitive small substrates for CPU which have improved specificity (kcat/Km) over CPN which has a constitutive activity in plasma and complicates the activity determination of CPU. The zymogen form, proCPU (TAFI) also has some intrinsic activity on small synthetic substrates which requires correction. The assay method was described and sensitivity discussed in relation to the expected ranges of CPU in circulation. Further validation of the assay is underway.

Comparison of different methods for measuring human TAFI

Pierre Morange presented an overview and data comparing different methods for measuring human TAFI. Assays may be complicated by the different forms present in circulation, including zymogen TAFI (which may also be complexed with plasminogen), active TAFIa and the conformationally inactive form, TAFIai. There are a number of commercial assays based on activity measurements of TAFI following activation with Thrombin/Thrombomodulin and immunoassays. Some of the immunoassays have different sensitivities for isoforms of TAFI with Ile/Thr at position 325, which in the past has led to overestimation of the genotype effect of this polymorphism. Other polymorphisms have been identified in the gene promoter region that can affect circulating TAFI levels. A number of studies have been performed to investigate correlation of circulating TAFI

or TAFIa + TAFIai levels with coronary artery disease and stroke. The prospective PRIME Study evaluated the association between TAFI plasma levels and the risk of coronary disease. in 10,000 healthy men recruited in France and Northern Ireland, TAFI plasma levels were similar in individuals with and without cardiovascular events after 5 years of follow up. However, it important to note that this assay is poorly sensitive to TAFIa and measure mostly the zymogen. Recent data have shown that the amount of activated TAFI (TAFIa) plays a more crucial role than that of TAFI in retarding fibrinolysis. This has led to the development of new TAFI assays measuring either exclusively TAFIa, or associated with TAFIai (TAFIa/TAFIai) or the released TAFI activating peptide (TAFI-AP). It has been recently shown that plasma levels of TAFI-AP (measured by a specific ELISA) were associated with the 4 major types of ischemic stroke in the retrospective SAHLSIS Study. The association between TAFI levels (using 4 TAFI measurements: two evaluating the zymogen, one measuring the TAFIa/TAFIai and the last one the TAFI-AP) and the risk of cardiovascular events have been assessed in the Atherogene Study. This study has included 1668 individuals from Germany with a significant coronary disease. These individuals were followed up for 3 years. TAFIa/TAFIai plasma levels measured at baseline were associated with an increased risk of cardiovascular death. This association was not observed with the test measuring the zymogen or with the ELISA measuring TAFI-AP.

Discussion

D Hendriks presented a short summary and chaired a discussion of the current problems and questions surrounding measurement of TAFI/CPU both zymogen and activated species. Circulating levels of the active enzyme may be of special interest but are very low and few assays have the necessary sensitivity. It is also not clear how to compare results from an assay such as that described by M Nesheim with immuno assays measuring activation peptide, for example. There are also a number of questions and problems surrounding the collection and processing of samples used to measure TAFIa/CPU due to the well known thermal instability at temperatures above zero deg. C. At this stage it was proposed that a collaborative study comparing different assay methods on a common set of samples to measure TAFI/proCPU would be useful and achievable. There was a discussion on the design of such a study including what samples and the number of samples should be used. It would be useful to include samples with different polymorphisms at position 325. Consideration was also given to the preparation of clearly defined samples formed by adding back purified (native or recombinant TAFI/proCPU) to depleted plasma. Further discussion will take place over the coming months to finalise the details of a study before samples can be shipped via NIBSC. The audience were requested to contact the chair and co-chairs of the subcommittee to express an interest in taking part and in making further suggestions.

D-dimer Chaired by C-E Dempfle

Introduction

C-E Dempfle made a short additional presentation to summarise the current situation regarding harmonization approaches for the large number of d-dimer methods currently in use. C-E Dempfle showed results from the Fibrin Assay Comparison Trials (FACT) parts 4 and 5. FACT4 compared assay reactivity of fibrin fragment D-dimer and pooled plasma from patients with DIC, using serial dilutions in plasma from healthy blood donors, and in buffer. A procedure for homogenisation of D-dimer samples was presented which allows quantitation of d-dimer in plasma following immunoblotting. This method has been found to be reproducible but needs testing in more laboratories. A request was put forward for laboratories to contact C-E Dempfle who would provide the method and further help where needed. Data were presented using the harmonisation procedure proposed on 50 plasma samples and a number of assays These results appeared to validate the use of 0.5 ug/ml cut off used in many assays.

Update on the NEQAS EQA for d-dimer

Ian Jennings covered the experience of the UK NEQAS studies on d-dimer determinations and showed how harmonisation procedures could be used successfully to improve inter-laboratory variability. However, between laboratory agreement remains poor in the absence of a suitable international reference preparation for d-dimer in plasma. Further problems were also identified including the variety of units reported and lack of local determination of cut-off values when d-dimer is used for the exclusion of DVT.

Report on the d-dimer harmonisation standardisation project-3 of JSLH for 13 d-dimer tests from 11 companies worldwide.

Optimising the harmonisation strategy to maximise the benefit for clinical practice.

K Fukutake presented results from the Standardization Committee of The Japanese Society of Laboratory Hematology, The Japanese Society of Laboratory Medicine and The Japanese Society of Thrombosis and Hemostasis on Standardization of FDP/D Dimer. These studies have been progressing as a joint project since 2002 in Japan. Project-3 was conducted for optimising the harmonization strategy to maximise the benefit for clinical practice in early 2008 and included 13 dimer tests from 11 companies. Plasmas from 80 patients, whose D Dimer levels were between 5 and 25μg/ml by LPIA, were pooled for calibration material. The pooled plasma was diluted by single normal plasma into 5 levels and samples were preserved at -80℃. Measurement results of original methods were very variable with inter-assay CV around 30% at each dilution. For harmonization, a reference method was chosen and results recalculated using regression curves aligning each method with the reference method. A dramatic improvement in CV to below 8% was noted. Further evaluation of clinical usefulness was established using 16 individual samples from patients, whose D Dimer values were from 5.0 to 25.0 ug. . The harmonization procedure was able improve average CV values from 34% to 9.9%. Benefits and limitations of harmonization and the extent of improvement in variability of d-dimer assays were discussed. It was suggested that a common calibrator of pooled plasma (>80 Patients Pool) is desirable. However, there will always be problems in this area due to the variable molecular composition of the target material and the differing nature of assay antibodies. The limited supply of patient samples may also be a problem for large scale studies.

Fibrinolysis and Plasmin chaired by C Longstaff

Physiological usefulness and limitations of global fibrinolysis measurements

T Urano presented an overview and update on classical tests for global fibrinolysis, concentrating on Euglobulin Clot Lysis Time (ECLT) in a microtitre plate format following absorbance at 340 nm. Preparation of the euglobulin fraction primarily removes alpha-2-antiplasmin and allows the assessment of fibrinolysis. which is dependent on the presence of tPA and PAI-1 and the proportion of free tPA. ECLT correlates with the amount of free tPA or tPA activity and with PAI-1 but not with total tPA antigen. ECLT showed very good positive correlations with total and free-PAI-1. It was found that Ca^{2+} dramatically shortened ECLT and further investigations showed that this was due to inactivation of PAI-1 by thrombin. Other mechanisms of PAI-1 inactivation were also possible, which involved the contact system. The reproducibility of euglobulin preparation remains a source of variability when using this method.

Update on WHO international standards and SSC secondary standards for tPA antigen and PAI-1 antigen in plasma

C Longstaff provided an update on previous collaborative studies organized through the subcommittee to measure tPA antigen and PAI-1 antigen in plasma. In 2007 results from a collaborate study were reported to the subcommittee showing wide variability of PAI-1 antigen determinations from 12 laboratories using a range of assays. However, the study included 5 samples of PAI-1 pools with a range of PAI-1 and a harmonization procedure demonstrated that conversion of results between methods was possible using an appropriate regression equation. An International Standard (IS) for PAI-1 antigen in plasma would be useful for such a harmonization procedure but this should be calibrated in ng and there is a need to find a way to determine the "real" ng level of PAI-1 in plasma (rather than the apparent ng reported by each method, which are not equivalent).. Data were presented from studies using SPR (Biacore) that may provide a way of estimating the real ng in plasma samples which may provide a route to this calculation. Further work is underway.

Preparation 94/730 was established as a new IS by the ECBS in October 2007 and is now available as the 1st IS for tPA antigen in plasma with a value of 25 ng of tPA. This preparation should be useful in calibrating tPA Elisa assays and replaces a previous preparation that was used for this purpose that is no longer available. The SSC coagulation plasma secondary standard lot 3 was also included in the same collaborative study to calibrate 94/730. When results were reviewed by participants and Fibrinolysis subcommittee members it was also agreed that SSC plasma lot 3 could be assigned a value of 3.0 ng/ml tPA antigen. However, a question

was raised about "cryptic tPA" which is not detected by one commercial kit and could have introduced further variability into the study results. This was investigated further it was found that the low values determined by one kit were increased from 1-2 ng/ml up to 5-6 ng/ml if plasma samples were diluted using 2 M lysine. However, this procedure is not routinely performed and it was recommended that the original calibration of 3.0 ng/ml should stand and this has been accepted by the Working Group on Coagulation Standards.

Utility of standards for 4-nitrophenol, 4-methylumbelliferon and 4-nitroaniline for standardizing serine protease activity

C Thelwell summarized recent work at NIBSC on the application of potential standards for 4-nitrophenol and 4-nitroaniline which may be valuable in the determination of molar concentrations of active enzymes by active site titration and for the determination of kcat or katals for active enzyme preparations. Results from 2 previous collaborative studies conducted through NIBSC were reviewed in which enzyme activities or molar concentrations were determined. It appears that a major source of variability in these studies is the preparation of calibration curves for nitrophenol or nitroaniline and/or the collaborators' spectrophotometers or microtitre plate readers. This source of variability may be improved by having available standard preparations of nitrophenol and nitroaniline. Trial fills have been performed and preparations have been sent out to a number of laboratories to investigate these questions. A request was made for further participants who want to take part in this study. Ultimately it is possible that these preparations will be useful in making new IS (for example plasmin) or in dual labeling existing standards with International Units and moles or katals of activity. Furthermore, active enzymes can be titrated with inhibitors thus calibrating the inhibitor in molar units (as has been done for the 1st IS for Alpha-1-Antitrypsin). These inhibitor preparations may also serve as a route for determining molar concentrations of other enzymes.

Standardising plasmin for therapeutic use

Pete Vandeberg presented work on the standardization of plasmin activity determinations as practiced by Talecris for their commercial product currently in clinical trials. Standardisation is a relevant topic since a number of researchers are investigating the use of plasmin-related proteins for therapeutic applications including thrombolysis but also including ophthalmology and wound healing. Although there is an international standard for Plasmin (97/536), many commercial suppliers of research-grade plasmin use activities derived from different sources, including their own historical assays. This makes comparison of the doses used in published studies difficult. A study was presented examinining the application of active site titration using p-Nitrophenyl-p'-guanidinobenzoate as a titrant to assign a standard values of active mg/mL to a reference preparation of plasmin. This reference preparations, in turn, is used as a standard for a chromogenic potency assay. By using an absolute reference method for reference method assignment, the long-term assignment of potency used for the purpose of dosing can be maintained. In addition, the chromogenic assay using the reference standard is used to compare commercial research grade preparations of plasmin from a variety of different sources. These preparations were found to have a wide range of activities, and a wide range of specific activity, ranging from a low value of 0.07 mg/mg (active plasmin per total protein), up to approximately 1.0 mg/mg for clinical trial grade plasmin produced by Talecris Problems with the historical calibration of the plasmin IS and the withdrawal of the previous NIST standard for 4-nitrophenol suggest there is a need for improved or new standards in this area.

WHO and European activities on standards for plasmin inhibitor (alpha-2-antiplasmin) streptodornase and C1-inhibitor.

C Longstaff closed the session by summarizing progress on other IS and business or relevance to the Subcommittee. A new IS for Streptodornase is under development and should be available in 2009. Previous suggestions for a new standard for plasmin inhibitor (alpha-2-antiplasmin) are currently on hold.

Submitted by C. Longstaff