52nd Annual Scientific and Standardization Committee Meeting
Oslo, Norway
June 29 – July 1, 2006
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Animal, Cellular & Molecular Models

Chairman: H. Weiler, USA
Co-Chairs: S.R. Coughlin, USA; J.L. Degen, USA; P. Jagadeeswaran, USA; C. Kluft, The Netherlands;
N. Mackman, USA; T. Nichols, USA

The session had approximately 20 attendees.

The outgoing chairman of the subcommittee, Dr. Jagadeeswaran, reviewed SSC activities 2004 to 2006, including organization of the scientific program of the subcommittee during the ISTH bi-annual meeting in Sydney, Australia.

Dr. Johnson reported on the status of the Murine Parameter Project. The Project is a comprehensive, 3-part compendium of murine hemostasis, including methods to assess the status of coagulation, platelet function, fibrinolysis and thrombosis in normal mice, and a review of hemostatic function/phenotypes in genetically altered mice. The compendium has undergone successful peer review and is scheduled for eventual publication in the ISTH periodical, Journal of Thrombosis and Hemostasis.

Scientific program:

Dr. Jagadeeswaran (University of Northern Texas, TX, USA) reviewed progress in genetic approaches to hemostasis in zebrafish. Data suggest the existence of functionally diverse platelet subpopulations in zebrafish, and the existence of proteases secreted into the environment with potential hemostatic activity.

Dr. Taylor (OMRF, Oklahoma City, OK, USA) critically evaluated the interplay of hemostasis and inflammation in primate models of sepsis. A key conclusion drawn from the synthesis of many studies is that the relevant pathogenic mechanisms operating in diverse sepsis models depend on the severity of the inflammatory challenge (degree of ensuing lethality).

Dr. Nichols (UNC, Chapel Hill, NC, USA) reviewed the development of novel pig models of aortic and coronary atherosclerosis.

Dr. Stoll (Universitaet Muenster, Muenster, Germany) introduced novel genetic methods to detect genetic predictors for plaque development. Dr. Stoll reviewed the concept and approach of comparative genetics, and demonstrated the power of the approach by showing several novel candidate genes that have been identified.

SSC Agenda 2006-10:

The agenda was discussed in an open session by all attendees. Consensus was reached that the Animal Subcommittee will continue its activities according to previous schedules. The following items were identified as future projects:
1. Complete the Murine Parameter Project by achieving publication
2. Organize scientific program for the ISTH meeting in Geneva, 2007. Potential topics are comparative genomics of hemostasis. Efforts will be made to have a representative from the European Mouse Phenome Consortium, and corresponding efforts in other countries.
3. Establish a www.-based registry for genetically defined animal models with altered hemostatic function. The registry supports the identification, distribution and characterization of relevant animal models.
4. An effort will be made to integrate the subcommittees work with ongoing efforts by NIH to optimize the development of animal models for atherosclerosis/inflammation. One of the priorities will be to produce animal models and/or protocols with improved predictive power for efficacy of therapeutic intervention in clinical studies. The chairman will establish a working group that will include much needed expertise from pharmaceutical/biotech enterprise.
In a overall well attended meeting (80 (start)- 20 (end) persons) several distinguished members of the Subcommittee and key-experts in the field gave presentations in 4 sessions.

**Session 1: Activation of the coagulation cascade: the flow factor: moderator Johan Heemskerk.** Jaap Jan Zwaginga on behalf of Kjell Sakariassen reviewed data on validating experiments with the developed ex vivo flow chamber set up (starting in the Basel Baumgartner period). He reported how these studies are performed under venous and normal and high arterial shear rates (resembling stenotic sites) over surfaces supporting platelet activation (collagen) or coagulation (tissue factor/ phospholipids). Effects of antithrombotic medication directed against platelets (cyclooxygenase or ADP receptor inhibition) vs, those influencing the coagulation (Warfarin or Xa, FVIIa inhibition) are clearcut in terms of reduced thrombus formation or altered thrombus composition. The ex vivo experiments seemed to suggest that co-presence of collagen and (stenotic) shear induced platelet (procoagulant and thrombus) activation might need combinations of inhibitors to be effectively inhibited. A remark in the later discussion was that it is important now to compare these ex vitro flow data with those of flow chamber experiments in vitro.

Johan Heemskerk presented an overview of the requirements for rheological and flow experiments using whole blood to produce physiologically relevant results. He also discussed a number of practical considerations e.g. to work with small blood volumes. He further presented new data with animal and human blood, where thrombus formation on collagen was investigated under shear in the presence and absence of coagulation. In sum, he showed that thrombin took over the key role of platelet collagen receptors in thrombus formation under conditions of low (venous) shear rates, even in the absence of extrinsic coagulation activation. These results are promising in the development of standard flow protocols that incorporate coagulation.

Coen Hemker presented a model explaining under which conditions the enzymatic rate of thrombin formation or the diffusion of thrombin determines the effectivity of thrombin formation and the extent of coagulation. Diffusion is an important restricting factor in thrombin’s activity especially under conditions of flow. This implies that in venous arterial thrombi caveats are present where prothrombinase activity is rate-limiting, while at the thrombus surface diffusion is a rate limiting factor.

**Part 2: Rheologic determinants in animal models studying hemostatic mechanisms: moderator Marc Hoylaerts.** Imke Munnix presented perfusion data in which the stability of platelet aggregate formation was studied as a function of perfuming whole murine and human blood over surfaces of collagen. Whereas secondary perfusions with plasma maintained the integrity of preformed platelet aggregates, such perfusion with buffer led to gradual disaggregation and spreading of platelets over the collagen surface. Addition of ADP to the buffer could preserve the aggregate stability. Since these experiments were suggestive of active
maintained platelet activation, after the initial formation of aggregates, further experiments were
done in the presence of ADP receptor antagonists and in the presence of PI3-kinase inhibitors,
selective for the different PI3-kinase isoforms. These perfusion studies showed that activation of
was both required to explain the maintained platelety and βPI3-kinase 3 in αIIbβ3activation,
required to keep the platelet integrin receptor active configuration.

Barbara Furie compared two currently used models of thrombosis in the mouse, i.e. the laser-
induced thrombus formation and the FeCl3-induced thrombosis. The differences in the role of
coagulatiin were discussed, as well as differences relating to the exposure of reactive collagen
fibres, during platelet recruitment on injured blood vessels. A discussion ensued between the
members of the biorheology subcommittee on this issue, included Dr. Barbara Furie. Thus,
depending on intensity and length of exposure of vascular endothelium to laser light, the extent
of injury can be mild to severe. Moreover, with FeCl3, the thrombus can develop in a large area
of the vessel wall, occupying and occluding the entire lumen of the vessel. In this case, there is a
high probability that collagen is exposed. Standardization of both models is needed to permit
comparison of results between labs and to understand the role of coagulation vs. platelet
aggregate formation. Concerns were raised on whether laser-induced vascular injury leads to
denaturation of proteins such as collagen, thus altering platelet adhesion in vivo. On the same
note, a similar concern was raised about FeCl2-induced vascular injury. Although antibodies to
collagen have been used by the Furie lab to demonstrate collagen exposure in the latter case,
concerns were raised on whether these antibodies can distinguish between the native vs. the
denatured protein. Possible methods to employ, during standardization efforts include exposing
culture endothelial cells to laser light or FeCl2 and determining the extent to which collagen is
exposed. Such would require the development of antibodies that recognize collagen only in its
native state, reagents that may not exist or are not readily available.

Part 3: Flow dependent effectivity of receptors and ligands: moderator Thomas Diacovo

Dr. Marc Hoylaerts reported on Gallic acid modulation of platelet-leukocyte interactions: a
model for flow dependent inhibitor efficacy and presented on the concept that phenolic
compounds found in red wine such as Gallic acid can act as anti-inflammatories by impairing P-
selectin mediated adhesion between leukocytes and vascular endothelium. Gallic acid (GA) is a
major component of red wine (35-70 mg/L). To determine its role as possible anti-inflammatory,
Dr. Hoylaerts evaluated the ability of this compound to inhibit P-selectin mediated-adhesion both
in vitro and in vivo. Interestingly, results indicate that GA can mitigate this interaction but only
under conditions of flow. The mechanism by which this occurs is still under investigation.
Moreover, it does not interfere with the interactions between platelets and activated endothelium.
This work demonstrates that GA in wine may contribute to the anti-inflammatory effects of red
wine. Moreover, it demonstrates that certain P-selectin-inhibitors may only function under
conditions of flow.

Thomas Diacovo reported on bond kinetic alteration in the interaction between vWF and
platelets; effects on thrombus formation. The concept presented dealt with biophysical properties
of the GPIb alpha – vWF-A1 bond that must be maintained within a narrow range, otherwise the
ability to promote adequate hemostasis/ or form a thrombus will be perturbed. Examples of such
alterations in humans include type 2M and type 2B VWD, where inclusion of single point
mutations in the A1 domain of vWF decreases or enhances its interaction with GPIb alpha, respectively. By performing structure/functional analysis of the mouse recombinant A1 domain, Dr. Diacovo was able to identify a specific residue within this domain, the mutation of which reduced the lifetime of this interaction by >2-fold. Moreover, platelet accumulation on recombinant mouse vWF-A1 containing such a mutation was decreased by >60%. Using standard molecular techniques, this mutation was inserted into the mouse vWF gene. Animals were viable and displayed a bleeding phenotype distinct from that of the vWF KO animal. Collagen-induced aggregation and platelet adhesion to surface-immobilized collagen was perturbed in vitro. Moreover, laser-induced thrombus formation in the mouse cremaster muscle was impaired. This study demonstrates that the kinetics of the GPIb alpha-vWF-A1 bonds are optimally designed to function under the physiological conditions found on the arterial circulation and that perturbations in the properties of this bond can have devastating effects on hemostasis. In the subsequent discussion Dr. Farndale raised the issue on the ability of laser-induced vascular injury to expose collagen found in the ECM. Referring back to the results presented earlier by Dr. Diacovo’s which suggested this to be the case, while the work by the Furie lab showed the contrary, a discussion ensued. Points raised were the following:

1. It is evident that depending on the intensity and length of exposure of vascular endothelium to laser light that of the extent of injury can be mild to severe (i.e. thrombus contained to a small area of the vessel wall vs. one occupying and occluding the entire lumen of the vessel). In the case of the latter, there is a high probability that collagen is exposed. Thus, this model needs to be re-evaluated under multiple conditions to determine whether collagen can be exposed as is the case with the FeCl2-induced vascular injury model. Standardization of both models is needed to permit comparison of results between labs.
2. Concerns were raised on whether laser-induced vascular injury leads to denaturation of proteins such as collagen, thus altering platelet adhesion in vivo. On the same note, a similar concern was raised about FeCl2-induced vascular injury. Although antibodies to collagen have been used by the Furie lab to demonstrate collagen exposure in the latter case, concerns were raised on whether these antibodies can distinguish between the native vs. the denatured protein. Thus, considerable work needs to be done to answer this physiological relevant question. Possible methods to employ include exposing culture endothelial cells to laser light or FeCl2 and determining the extent to which collagen is exposed. This would require the development of antibodies that recognize collagen only in its native state, reagents that may not exist or are not readily available.

Part 4: Standardisation issues and subcommittee proceedings: moderator Jaap Jan Zwaginga

- Richard Farndale presented a clear overview of the role of collagen as platelet activating (GPVI, α2β1), vWF binding in hemostasis and therefore in in vitro testing. He stressed the enormous variability of commercial preparates in their effectivity, which is caused by contaminants, covalent bond modification and differences in concentration and fibril size. Insights are fastly expanding in the confiding characteristics of this triple helical more or less crosslinked molecule with glycine –x –y triplet repeats and proline as x or y or hydroxyproline as y as being most efficient in forming a triple helix. In this respect, Farndale showed function
analysis of synthetically engineered collagen peptides: mostly 9 triplet parts of the native collagens in conjunction with proximal and distal triple helix inducing glycine-proline-(hydroxyl-)proline triplet repeats. With this synthetic collagen peptide toolbox he showed that typical sequences with separate functions can be identified thus enhancing structure function insights on the collagen molecules and in vivo subtypes. Moreover (mixes of) these synthetic collagen related peptides (CRP’s) are now being considered as standard in vitro stimulus for platelet adhesion and activation/ aggregation in in vitro hemostatic tests.

- Jaap Jan Zwaginga closed the session stressing the improved biorheology network illustrated by the (new) members of the subcommittee (Hans Vink, Ton Lisman/ Peter Lenting, Shaun Jackson, Maurice Frojmovic, Scott Diamond, Patrick Andre, Arnaud Bonnefoy, Kjell Sakariassen, Philip de Groot, Richard Farndale). He furthermore gave an update on the 2 reports that were prepared as official short SSC publications in JTH with extended versions comprising extended references and tables to go on the SSC website. The present and future activities of the subcommittee were discussed and finally the subcommittee expressed the will to stimulate and effectuate synergistic work and exchange of thoughts with the animal study subcommittee for rheologic QC of animal models, the platelet physiology subcommittee in the area of flow dependent platelet assays and possibly the WP of the VWD subcommittee studying shear stress assays in VWD.
Control of Anticoagulation

Chairman: Sam Schulman, Canada
Co-Chairs: M. Greaves, UK; J. Harenberg, Germany; C. Kearon, Canada; M. Laposata, USA; J. Olson, USA; G. Palareti, Italy; A.M.H.P. van den Besselaar, The Netherlands

Chairmen: Job Harenberg and Anthon van den Besselaar
S. Schulman opened the meeting of the committee and briefed on the activities over the past year

Point-of-Care tests for prothrombin time

W Plesch presented the new CoaguChek XS System, which is being introduced on the market now and demonstrated the confirmation of calibration and results of performance testing. The mean ISI of the thromboplastin is 1.01 (CV was 1.1 – 2.0%), and it is an amperometric method. Calibration was according to WHO recommendation at 4 sites, master lot against IRP, production lots against master lot. Performance evaluation was against Innovin and RecombiPlastin with capillary or venous samples on the test strips, using 33 instruments. Accuracy 95% of data within +/- 14% of the data. Trueness rel bias –0.4%. Close agreement up to an INR of 8. ISO 17593 assessment was also done, within ISO limits more than 99%. Repeatability was very high. Master lot will last for 1 to 1.5 years.

L Poller demonstrated (on behalf of P Meijer, C Kluft, FJM van der Meer, M Keown, S Ibrahim, AMHP van den Besselaar, A Tripodi, J Jespersen) the feasibility of large scale quality assessment of CoaguChek point-of-care testing prothrombin time monitors. A system for quality assessment (QA) of the CoaguChek “Point of Care testing” prothrombin time monitor has been developed by the European Concerted Action on Anticoagulation (ECAA). Sets of five certified ECAA plasmas were tested on 539 CoaguChek monitors by experienced staff at 9 Netherlands Thrombosis Centres and results compared with certified INR. A 15% INR deviation has been classified as “significant deviation” and was found with 20.3% of the monitors with significant differences between test strip lots. One single lot was responsible for most of the variation. A conventional type of external quality assessment analysis was also applied with similar findings. The results validate the use of the single instrument QA procedure for CoaguChek users developed by the ECAA. This is the largest EQA study so far on a POC instrument. (Paper accepted in Journal of Clinical Chemistry).
W. Plesch commented that CoaguChek is intended for whole blood and that it is not sure that the 20.3% showing deviation would be seen in that case.

Miguel A. Cortés Vázquez (on behalf of E Gómez, A Hervás, R Valero, P Muñoz) described how they had performed validation of the computer decision support software TaoCheck to monitor oral anticoagulant therapy. Many clinical trials have demonstrated the utility of computer-based dosage programs to monitor oral anticoagulant therapy in outpatients. However some of them are not already validated. Therefore they carried out a prospective, randomized trial to validate the efficacy of the computer decision application TaoCheck. 56 were randomized to TaoCheck aided dosing, 62 to experienced physician dosing. No differences seen between groups re Time in Therapeutic Range (50% per group). Number of appointments was lower in control group.
Factor Xa inhibition and the monitoring thereof

**Frank Misselwitz** (Bayer Health Care) reported on the results of the clinical trials in the prophylaxis and treatment of medical and surgical VTE with a Xa inhibitor. Rivaroxaban (BAY 59-7939) is a novel, direct, oral factor Xa inhibitor, which selectively inhibits factor Xa (Ki 0.4 nM) but not other serine proteases. It is a small molecule which fits directly into the active site of FXa; and has a competitive, reversible mechanism of action. Rivaroxaban dose-dependently prolongs PT and aPTT, HepTest, and factor Xa-inhibition is strictly correlated to its plasma concentration (correlation between PK and PD 0.96 for PT). Rivaroxaban dose-dependently prolongs Prothrombinase-induced clotting time (PICT), and inhibits ETP (reases the lag time and decreases the peak in thrombin generation curves) both via the intrinsic and extrinsic pathways (collagen and tissue factor as stimuli). No need for routine monitoring, but good monitorability using PT with different PT reagents, which all highly correlate to PK but result in different slopes (ISI 1.01 with Innovin, 1.3 with Neoplastin, 2.0 with Simplastin, 2.4-2.69 for Thromboplastin C). The "steepness" of the correlation does not depend on ISI of the PT reagent. There is no effect on TT, Ecarin time or on platelet aggregation. There is not enough data to make a recommendation for a therapeutic PT range. PICT is prolonged with the drug and may also be a candidate for monitoring.

**Job Harenberg** gave an update on the clinical laboratory validation of PICT test (Pentapharm, Basel) in the monitoring and potency evaluation of heparins and pentasaccharides. Three hospitals participated. Normal range is approx 20-34 s.RVV actives F V. PICT seems to be sensitive to low-dose LMWH. PICT versus ECT in Hirudin monitoring gave an r-value of 0.94. PICT is prolonged by antithrombin-dependent anticoagulants but also by direct Xa and IIa-inhibitors. Vitamin K antagonists do not prolong PICT, and therefore PICT is useful during concomitant therapy. Dr Harenberg proposed to perform a larger standardization study with various anticoagulant agents.

**Job Harenberg** proposed an international study with collection of anti-F Xa levels from patients on therapeutic dose of fondaparinux. Patients on prophylactic or therapeutic doses will have samples collected at trough and peak. Local analysis with an anti-FXa method and/or Hepest. He aims for 200 patients with low dose and 200 with high dose treatment. A fondaparinux standard has to be used. Information on local routines for the analysis will be collected. F Misselwitz commented that both samples after first dose and after 5th dose or later is desirable to get steady state. It was decided to partly collect retrospective data, partly to go prospectively and then also save plasma for central analysis.

**Soumaya Elrouby** (ITC) reported on monitoring of low molecular weight heparin using whole blood clot based Hemonox™ method in comparison to ACT and anti-Xa assays. Hemonox™ is a new point of care test (POCT) for monitoring the anticoagulant effect of LMWHs. It is a one step whole blood coagulation test that uses the HEMOCRON® Jr. Signature+ Whole Blood Microcoagulation System with software version 2.4 or higher. The HEMONOX test has been evaluated in several clinical trials. The test showed a clear distinction between untreated and treated patients. Normal HEMONOX baseline is in the range of 70 seconds, usually below 85 seconds. Normal range is 50-76 s in volunteers, 59-81 in obese donors. A baseline value equal or exceeding 100 seconds indicates prior treatment with LMWH or heparin. Peak HEMONOX
results are quickly achieved 10-15 minutes post intravenous (IV) bolus; a clotting time >150 seconds corresponds to therapeutic anti-Factor Xa levels (>0.5U/ml) in PCI patients. The relation between HEMONOX results and anti-Factor Xa is not affected by the presence of GpIIb-IIIa antagonists. The HEMONOX response profile is patient specific and reproducible. In the non-invasive setting using subcutaneous administration, the peak is 4-6 hours post injection. The test may not exhibit the level of sensitivity to the anticoagulant effects of enoxaparin as observed using IV dosing, however the HEMONOX results may provide the clinicians with a diagnostic tool to interpret the anticoagulant response in ACS patients who progress to PCI. The HEMONOX POCT is more sensitive than the ACT test in measuring the progressive effect of LMWH in ACS and PCI patients. The HEMONOX test may also indicate a hypercoagulable state in some obese patients receiving small doses of LMWH for gastric bypass procedure.

HEMONOX vs LMWH conc gave an R=0.966 for dalteparin. HEMONOX vs anti-Xa was R=0.85-0.90 in PCI patients and 0.87 vs Heptest. Coefficient of variation usually 4.5%.

W. Jeske pointed out differences in the 1st and 2nd LMWH standards. Soon after the introduction of LMWHs, a standard to cross-reference their potency was developed. The 1st LMWH standard (85/600) was produced by nitrous acid digestion of porcine mucosal heparin and exhibited characteristics comparable to dalteparin. Because of the widespread use of the first standard, a new standard material has been introduced, which is also produced by nitrous acid digestion but whose characteristics were different. The MW profile and the biologic activities, including serpin affinity, of these two standards are distinct. As many generic versions of LMWHs are currently being introduced, a systematic study was carried out to cross-reference several commercially available generic products from India and South America against the first and second LMWH standards using amidolytic anti-Xa and anti-IIa assays. Differences were observed in the slopes of the concentration-response curves for these standards in both the anti-Xa and anti-IIa assays. The potencies of the generic LMWHs ranged from 76 to 119 U/mg in relation to the first standard and from 88 to 122 U/mg in relation to the 2nd standard. On average, the anti-Xa potency was 8% higher when calculated with the 2nd standard. The differences in anti-IIa potency were further amplified and exhibited wider variation (range 15-31 U/mg with the 1st standard and 19-41 U/mg with the 2nd standard). On average, the anti-IIa potency was 28% higher when calculated with the 2nd standard. When used to cross-reference commercially available LMWHs such as enoxaparin, the new standard consistently overestimates the anti-Xa potency by 5-10% in comparison to the first standard. Such an overestimation may lead to a reduction in dosage and have a significant impact on the clinical dosing of LMWH in various indications. W Jeske suggested that if the 2nd standard is used, it should first be cross-referenced against the first standard in order to properly assign the anti-Xa and anti-IIa activities. He also called for developing individual standards for each of the branded products. Such standards should be defined in terms of not only anti-Xa and anti-IIa activity, but also in terms of anticoagulant activity using the USP assay, activated clotting time (ACT), aPTT and Heptest.

Jawed Fareed pointed out that several generic versions of the LMWHs enoxaparin and dalteparin have been introduced in some Asian and South American countries. In addition, numerous generic suppliers have applied for the approval to sell the generic versions of
enoxaparin and dalteparin in the US and European union. Neither the US FDA and EMEA have any guidelines for the generic interchange of branded products. As a result, several substandard products have been introduced and withdrawn. The current pharmacopoeial guidelines are inadequate to accept the generic version of the branded products as these apply the older guidelines that are applicable to the conventional generic drugs. The LMWHs represent a hybrid of the biologic and chemical manufacturing processes. Moreover, these are derived from unfractionated heparin of porcine origin. There are not even proper guidelines to control the raw material, porcine mucosal heparin which is the starting material for the manufacturing of LMWHs. The European Pharmacopoeial description of each of the individual LMWHs is incomplete and US Pharmacopeial doesn’t even have a description statement regarding these LMWHs. Few of the professional societies have made any comments on this issue. As the ISTH/SSC has played a role in the biologic standardization and characterization of LMWHs, Dr Fareed proposed that ISTH/SSC on the Control on Anticoagulation, consider the development of specific guidelines for the requirements to accept a generic version of the branded product. The ISTH membership should be highly qualified to undertake this mission. Such guidelines should be based on the newly available analytical methods and characterization of the biological effects of heparins.

**Discussion**
E. Gray commented that in other studies that she had performed the comparability in e.g. a large cohort study she did not see that kind of comparability, except for one single preparation. Dr Harenberg thought that it might be necessary to have a local correction factor for the individual lab when switching to the second standard. Dr Barrowcliffe informed that the generic LMWHs were not available when NIBSC performed the study. It was therefore decided to continue the discussion afterwards whether to have a working group on the issue of how to assess the generic LMWHs and if the 2nd standard is useful.

**Joint session with Subcommitte on Plasma Coagulation Inhibitors**
*The minutes from the following part will appear in the minutes of Subcommitte on Plasma Coagulation Inhibitors*

Report on the international collaborative study on fluorogenic methods for Thrombin generation test. *E. Gray, A. Lawrie*

Pre-clinical validation of the Calibrated Automated Thrombogram using platelet rich plasma and the effect of thrombomodulin. *H. Spronk*

International multi-centre assessment of the calibrated automated thrombogram thrombin generation assay. *Y. Dargaud*

A new global assay with small amounts of recombinant tissue factor and tissue-plasminogen activator providing novel parameters to determine the overall hemostatic potential. *S. He, M. Blombäck*

Methods recording dynamics of fibrin formation. *B. Sorensen, J. Ingerslev*
Discussion

Introduction of a new registry

_Sam Schulman and Anna Falanga_ launched an international registry on recurrent VTE in patients with cancer. The registry will thus be under both the committee of Malignancy and Hemostasis and the committee of Control of Anticoagulation. It is anticipated that 200 cases will be registered over 2 years and thus results reported in Vienna at the 54th Annual SSC Meeting 2008. The objective is to register current regimens used for these problematic patients and to get a rough estimate of the efficacy and safety over 3 months of follow-up. The data collected may be used for guidelines with a low level of evidence and also form the basis for the design of appropriate trials in this field. The registry is supported by an unrestricted educational grant from Leo and deposited at ISTH. A symbolic honorarium is paid for each complete case submitted. Information on the registry, case report forms, instructions and a template for submission to the IRB/REB/ethics committee can now be found under REGISTRIES at the ISTH website.

1 July, 2006

_Chairman: Clive Kearon_

_H.C. Hemker_ (Synapse BV) discussed the need for continuous individual calibration in fluorogenic measurement of thrombin generation. Fluorogenic thrombin generation (TG) measurements are based on the thrombin-catalyzed production of a fluorescent substance. From the velocity of increase of the fluorescence signal (dF/dt) the concentration of thrombin has to be calculated. Traditionally this is done via measuring the dF/dt of a known amount of thrombin. If a concentration [T] of thrombin is added and a velocity of V is measured then the calibration factor (Cf) equals T/V (Cf = T/V). Under the conditions of a thrombin generation experiment Cf is, however, not a constant. At the end of the experiment it may be half of that at the beginning. (due to: substrate consumption and non-linearity of the fluorescent signal with the concentration of fluorescent product). Also Cf differs considerably between plasmas. In icteric- or haemolytic plasma it may be half of that in normal plasma. For this reason and also because the error brought about by non-linearity is more important at high thrombin generation than at low (more substrate split, more fluorophore produced), standardization via normal plasma does not work. The only possible solution is continuous individual determination of Cf in a parallel sample. This technique is used in the Thrombinscope method but not in the alternative Technoclone method. We therefore compared the two methods. From six volunteers we drew 12 samples in the course of one day and determined the six intra-individual coefficients of variation. In the table the CVs are rendered of the Thrombinscope method with Continuous Individual Calibration (CIC) ON and OFF and of the Technoclone in the two forms in which it is presently marketed. With CIC-ON, the CV of peak-height and initial slope were 4% and 8% resp. In the three methods without CIC the CV varied between 20% and 38%. (ETP cannot be calculated in the Technoclone method).

The presentation was followed by criticism that other manufacturers of kits for measurement of thrombin generation were not invited. S.Schulman responded that the subcommittee was approached by Prof Hemker with data that were found to be pertinent for presentation at the
subcommittee but we neither have resources or sufficient time in the program to invite all competitors to present.

*A van Hylckama Vlieg* had been invited to present studies on the predictive value of ETP measurement for VTE; first episode and recurrence. However, she had sent apologies, that she was unable to attend.

Clive Kearon introduced a session on the possible links between arterial and venous thromboembolism. He mentioned the acute situation when pulmonary embolism with increased cardiac load can unmask ischemic heart disease.

Giancarlo Agnelli Gave the first presentation on “Venous thromboembolism and atherosclerotic disease – common denominators or different diseases?” After reviewing the literature, he went into detail with the study of Prandoni et al (NEJM 2003) showing an association between VTE and carotid plaques. Agnelli’s group has shown a reduced endothelial function in patients with idiopathic DVT. Becattini in his group found increase in the rate of MI and stroke in the long-term course after unprovoked pulmonary embolism. Prandoni also showed in a cohort of almost 2000 patients that those with unprovoked VTE had a HR of 1.9 (adjusted 1.6; 1.2-2.0) for cardiovascular events. He also mentioned Young’s study that residual thrombosis on ultrasound is associated with worse survival, but this is partly due to concomitant cancer.

Sam Schulman gave the second presentation on this topic and also presented some data from the 10-year follow-up of the DURAC 1-trial. Here it was shown that patients with venous thromboembolism have a higher death rate than expected, both from all cause deaths, from cancer and from myocardial infarction/ischemic stroke. This pattern is more pronounced in patients with unprovoked venous thromboembolism and perhaps also in those with proximal DVT or pulmonary embolism (versus distal DVT). A common denominator is difficult to identify from this trial, although cardiolipin antibodies may play a role.

**Reagents for the monitoring of vitamin K antagonists**

*AMHP. van den Besselaar* gave a progress report on the revision of the 1999 WHO Guidelines for thromboplastins and plasma to control oral anticoagulant therapy. Several new developments in the control of oral anticoagulant therapy occurred after the publication of the 1999 WHO Guidelines. These should be addressed in the revision of the document.

1. The requirement of three International Reference Preparations for thromboplastins and the procedures for their replacement and calibration.
2. Requirement of real-time stability study of International Reference Preparations for thromboplastins.
3. Local-system calibration using certified plasmas.
4. Statistical procedures for calibration.

According to the 1999 WHO Guidelines, it is recommended that patient’ samples with INR values in the range 1.5 – 4.5 should be selected. Samples with INRs outside the 1.5 – 4.5 range
shall be excluded. The Guidelines include an example in which the exclusion of samples is based on measurements with the reference thromboplastin (in this case RBT/90).

The above procedure of sample exclusion can lead to a bias in the slope of the calibration line from which the ISI is calculated. Planimetric considerations predict an underestimation of the slope of the line if the exclusion of the samples is performed with one of the two thromboplastin systems only. An alternative procedure for sample exclusion is proposed which avoids the bias in the slope. The alternative procedure takes into account the measurements performed with both thromboplastins. The data of three multicenter calibration studies have been used to estimate the magnitude of the bias induced by the current WHO recommended exclusion procedure. The average bias in one calibration step is 0.5%. This is not of clinical importance but it should be realized that the bias is cumulated in each subsequent calibration step. If the average bias in one step is 0.5%, the total bias after 10 steps may be as high as 5%. The bias should be avoided by using both the reference and the new thromboplastin in the calculations.

In the discussion the issue was brought up whether for a new reference thromboplastin a correction could be made for the error incurred by the type of calculation used in the past. It was agreed that the results should be published as a report from the Subcommittee.

**A.M.H.P. van den Besselaar** (also on behalf of A. Tripodi) discussed the replacement – or discontinuation – of bovine reference thromboplastin. The second international reference preparation of thromboplastin, bovine, combined (coded OBT/79) was established in 1983 with an assigned ISI of 1.0. OBT/79 was derived from bovine brain and combined with factor V and fibrinogen and should be used to calibrate thromboplastin materials of bovine origin and combined thromboplastins of whatever origin. This recommendation was based on the experience that the calibration of a given thromboplastin is more precise if performed against an international reference preparation of similar composition and from the same species.

The stocks of OBT/79 have been exhausted recently. The question arises whether OBT/79 should be replaced by a similar material. In an ideal world with unlimited resources, the answer would be: YES. It should be realized that worldwide, human and rabbit-based thromboplastins are used on a much larger scale than bovine reagents. As far as the discussants know, only two bovine thromboplastin reagents are prepared commercially. Apart from the question whether the replacement is feasible and affordable, Dr van den Besselaar investigated if bovine thromboplastin may be calibrated against a rabbit thromboplastin with an acceptable level of precision. Two commercial thromboplastins were calibrated by their laboratory using both OBT/79 and RBT/90 (international reference preparation for thromboplastin, rabbit, plain). There was good agreement between the ISI obtained with the two reference preparations. The CV of the calibration slope with OBT/79 was 0.9 and 1.2% for the two commercial reagents, respectively. The CV of the slope with RBT/90 was 2.0 and 2.0%, respectively. Although the CV with RBT/90 is greater than with OBT/79, it is still lower than the CV which is considered by the WHO as the upper limit of acceptable imprecision, i.e. 3%. It was concluded that bovine thromboplastins can be calibrated against rabbit reference thromboplastin with acceptable precision. The calibration against OBT/79 is not needed if a CV of 3% or less can be obtained with a rabbit reference thromboplastin. There is not an absolute requirement to replace OBT/79.
Armando Tripodi (also on behalf of A.M.H.P. van den Besselaar) proposed to start the process which hopefully will be leading to the replacement of rTF/95 (the human WHO International Standard for thromboplastin). The stocks of the latter will be depleted in a few years. As in the past he proposed to ask manufacturers to submit candidates by December 2006. A maximum of three candidates will be provisionally accepted by the Subcommittee if they fulfil the requirements set in advance. The candidates could be recombinant, placenta or TF derived from cultured endothelial cells (but not brain) and the ISI should be 0.90 – 1.3 with manual technique. The three candidates will then be calibrated in an international collaborative study (involving 20 labs) against rTF/95 and RBT/05, and then the most suitable candidate will be chosen on the basis of pre-defined criteria. The entire process will end on the occasion of the SSC Meeting in 2008 where the new standard will be approved by the Subcommittee and will be submitted to the WHO Expert Committee on Biological Standardization.

A question was raised by W Plesch if we could have a back-up reference to avoid running out of material and then having only a single reference preparation. T Barrowcliffe responded that this is a luxury that we don’t have for other references. It may also be difficult to have that approved by WHO.

There were no objections to the proposal by A Tripodi and it was approved to go ahead.

Leon Poller thereafter argued for the importance of the continuation of the WHO thromboplastin IRP (combined). The WHO Prothrombin Time (PT) Standardisation Scheme is based on calibration of local PT test systems against the relevant species reference thromboplastin (human, rabbit or bovine). The provision of the replacement bovine IRP to WHO is necessary for various reasons:

1. published reports have described species INR differences on the same anticoagulated patients’ blood samples using the three current IRP. These are particularly evident with lyophilised plasma samples used in external quality assessment schemes and also increasingly for local ISI calibrations;
2. currently certification of candidate IRP has to be based on multicentre calibration of a new candidate IRP of whatever species against all three current IRP of human, rabbit and bovine origin. IRP must be stable. In the absence of the bovine IRP stability monitoring of the WHO IRP would have to be assessed solely on the comparative values of two rather than three IRP. Such studies based on results with two IRP only, have been relatively inconclusive;
3. Precision studies indicate that the rabbit IRP would not satisfactorily replace the bovine IRP. In addition, there would be no “combined” IRP as the other two IRP are “plain” reagents;

The WHO thromboplastin IRP bovine combined should therefore be replaced to maintain the present hierarchical structure of the IRP; to provide more reliable IRP stability monitoring; to provide a “combined” IRP for ISI calibration of “combined” thromboplastins; to provide more precise ISI calibration with bovine reagents and to remove species specificity problems in PT testing.
Discussions

A bovine thromboplastin will most likely not be approved by the WHO due to the BSE risk. It was therefore concluded that the main issue to consider and preferably present at the next SSC Annual meeting is a new “combined” thromboplastin, presumably of rabbit origin. Drs van den Besselaar and Tripodi will consider this.

Sam Schulman, chair.
Disseminated Intravascular Coagulation (DIC)

The session was broadly divided into 3 strands:

1. Laboratory standardization issues in DIC.

Nigel Key discussed the current situation on microparticle estimation. The majority of laboratories use flow cytometry with the others using ELISA capturing for phosphatidylserine or the target antigen of interest. There are issues around freeze thawing that need to be addressed and this is a potential joint endeavour between this SSC and the Working Group on Vascular Biology. Quality and specificity of antibodies are also a consideration in terms of determining tissue factor specificity.

Carl-Erik Dempfle concentrated on the issues surrounding accurate determination of high Ddimer levels. This is particularly relevant in the refinement of the DIC scoring systems that depends on meaningful cut-off levels of D-dimer. Work is underway on a common calibrator of the Ddimer assay with results anticipated by the end of the year. This also requires follow-on studies evaluating individual performances of each assay in clinical studies to allow for refinement. However, this work should not delay the recommendation of the current DIC algorithms.

With increasing number of publications confirming the utility of the biphasic waveform in DIC and sepsis, Tina Dutt and Colin Downey discussed the standardization work in this area. When using the MDA180 analyser and MDA reagents, there was good correlation with a CV of 0.23.

Elaine Gray discussed the laboratory assays of protein C and antithrombin in the DIC setting. Chromogenic assays appear appropriate but care is needed with choice of reagent blanks. PC deficient plasma as sample diluent gives higher results than when diluted in buffer.

2. The role of inflammatory markers in DIC testing.

Hideo Wada discussed the growing literature on high mobility group box proteins in sepsis/DIC. The highest HMGB1 levels were in patients with organ failure and non-survivors. HMGB1 plasma levels correlated with the DIC score and sepsis-related organ failure assessment (SOFA) score.

Hyun Kyung Kim investigated the prognostic value of a new parameter (the fibrinogen/CRP ratio) for predicting 28-day mortality in 1056 patients with suspected DIC and found that the area under the ROC curve of the fibrinogen/CRP ratio, for predicting 28-day mortality, showed significantly better discriminative power than did that of the fibrinogen level.

Jorn Nielsen overviewed the state of understanding on inflammatory markers in DIC. At present, he concluded that measuring soluble adhesion markers would not add to prognostication but might be helpful in pathogenic understanding.

Discussions led by Keith Hoots and Fletcher Taylor met with a lively response, with broad agreement that we are ready to submit a communication for publication to recommend use of both overt and non-overt algorithms. We will also look at the evolution of the overt scoring system by incorporating useful elements from the non-overt parameters. In addition, the emphasis will be in applying scoring longitudinally and how that might be incorporated into management issues.
Registry of Exogenous Hemostatic Factors

July 27, 2006
University of Strathclyde, Glasgow, UK

Chair: N. Marsh, Australia
Co-Chairs: R. M. Kini, Singapore; F. Markland, USA; K. Clemetson, Switzerland; T. Morita, Japan; M. A. McLane, USA

Nine members of the Registry including Co-Chairs (Kini, Markland, Clemetson, and Morita) were in attendance plus some 35 guests. Chair Marsh and Co-Chair McLane were absent with apologies.

Welcome: R. Manjunatha Kini, Co-Chair (Chaired the meeting)

1. Meeting was brought to order by the Chairman Dr. Manjunatha Kini.
2. The minutes of the last meeting were read and there were no questions.
3. Classification and nomenclature of C-type lectin related proteins (T. Morita)
   A report on the classification and nomenclature of C-type lectin related proteins from snake venoms was prepared by Dr. Morita was presented. It is a difficult task as the new proteins are being identified through proteomics and transcriptomics. Some suggestions were made to tackle the situation and improve the nomenclature and classification system. Dr. Clemetson agreed to contribute in resolving some issues.
4. Classification and nomenclature of disintegrins (M. A. McLane) A report on the classification and nomenclature of disintegrins from snake venoms was prepared by Dr. McLane with inputs from Dr. Markland and was presented to the committee by Dr. C.Paquette-Straub. The new nomenclature system was proposed based on the earlier proposal. The presentation was followed by a discussion of nomenclature. It was agreed that a report on classification and nomenclature be sent as an official communication of the Registry to SSC.
5. Organization of the International Conference – We are planning to organize the Fourth International Conference on the Exogenous Factors Affecting Thrombosis and Hemostasis as a satellite meeting to the XXII World Congress to be held in Boston, USA. Dr. McLane will be Chairman of the Organizing Committee and will present the details of the organization of the International Conference in the XXI World Congress to be held in Geneva, Switzerland.
6. The next meeting of the Registry will be held in Geneva during XXI congress of ISTH.
7. Any other matters: As there were no other matters, the meeting was adjourned.
Factor VIII and Factor IX

Chair: K. Mertens, The Netherlands
Co-chairs: J.C. Gill, USA; C. Lee, UK; J. Oldenburg, Germany; F. Peyvandi, Italy; J.M. Saint-Remy, Belgium; A. Srivastava, India; H.M. van den Berg, The Netherlands

The Chairman opened the Subcommittee meeting at 14.15 for an audience of approximately 170 attendants. He announced a few amendments to the final program and provided the timing details of the various sections in the agenda.

Section 1: FACTOR VIII CLINICAL ISSUES
Co-chairs: J.M. Saint-Remy and H.M. van den Berg

Phenotypic heterogeneity in severe haemophilia A and B: A. Srivastava

Though it has been recognized that 10-15% of patients with severe haemophilia have clinically mild disease in terms of frequency of bleeds and extent of arthropathy, the basis for this phenomenon has not been fully understood. We hypothesized that the levels of various pro- and anti-coagulant factors and functional polymorphisms in the coagulation protein genes as well as genes of cytokines involved in the inflammatory response may modulate the development of hemophilic arthropathy. A total of 114 minimally treated patients (50-150 IU/kg/year) with hemophilia A (n=94) and hemophilia B (n=19) diagnosed were evaluated as outlined above. They were categorized as ‘mild’ (<1 affected joint and < 5 bleeds in the preceding year, n=15) or ‘severe’ (>1 affected joint and >5 bleeds, n=99). Early data suggests that polymorphisms in TNFα-308 AA/AG (pro-inflammatory) (RR-3.4, p=0.037, 95% CI, 1.07-10.7), TGFβ Codon 10 CC/CT (pro-inflammatory) (RR-2.8, p=0.07, 95% CI, 0.91-8.3) are associated with clinically severe disease while MDM2 GG (RR-0.3, p=0.038, 95% CI, 0.1-0.93) tended to ameliorate severity. These data suggest that the clinical phenotype of severe haemophilia could be influenced by interactions between a variety of hemostatic factors as well as some inflammatory response proteins.

Factor VIII inhibitors and continuous infusion: I. Scharrer

Dr. Scharrer presented data also on behalf of Drs. v. Auer and Oldenburg and the German Hemophilia Society University Hospitals Mainz and Bonn. They have conducted a retrospective study to investigate the development of inhibitors after continuous infusion of F VIII in Germany. 42 hemophilia centers were questioned. 19 of these conducted 200 continuous infusions in 128 patients. 14 patients developed inhibitors (10 HR, 4 LR). 5 of these patients were suffering from severe, 1 from moderate and 8 from mild hemophilia (age between 7 months and 73 years, 11 PTT’s, 3 PUP’s). The infused amount ranged between 4300 and > 100 000 U before inhibitor development. Exposure days ranged from 4 to > 100. Regarding the genotype only 2 intron -22- inversions have been found. In Dr. Scharrer’s center no inhibitors had been found in 81 patients with major orthopedic surgery and bolus infusions compared to 2 inhibitors in 8 patients with similar surgery. In conclusion the inhibitors developed very often in mild hemophiliacs without the typical gene mutations for inhibitor development.

Feedback from EMEA Workshop on Inhibitors: R. Seitz

Prof. Seitz reported that in a CHMP class review of recombinant FVIII products and inhibitor
development the data received were heterogeneous and not comparable between products. An expert meeting was convened at the EMEA in London on 28.02. – 02.03.2006. He presented a brief feedback on this workshop; a detailed report is currently prepared. A letter has been sent to the Chair of the FVIII&IX Subcommittee, Prof. Mertens. CHMP notes the ISTH SSC sub-committee on FVIII and FIX initiatives regarding the improvement of the FVIII inhibitor assay and the establishment of the global PTP inhibitor surveillance. These will be complementary to CHMP’s ongoing work in revising guidelines on clinical investigation of plasma-derived and recombinant FVIII products. CHMP would appreciate to be informed about the outcome of the ISTH SSC sub-committee on FVIII and FIX discussions on the above topics. In the discussion dr. Mertens indicated that several of the issues are already ongoing actions of the Subcommittee. He proposed that Working Parties could be initiated to address these issues whenever appropriate.

**Global PTP inhibitor surveillance study: update : D.M. DiMichele**
The impetus for this project was derived from regulatory issues identified by the FDA and EMEA associated with potential immunogenicity of new FVIII products studied in pre-licensure PTP clinical trials. The need for harmonized international data collection on the natural history of inhibitors in PTP populations was reiterated at both the FDA (11/03) and EMEA (2/06) workshops, although no consensus definition of the PTP yet exists. International guidelines for PMS studies were reviewed as were the pros and cons of conducting this project through the ISTH FVIII/IX subcommittee. The history of this project in this committee was reviewed. Within this context, this report focused on the feasibility of national PTP database harmonization with respect to a data collection tool. A preliminary effort to do this by two established databases in the US and UK was reported on behalf of its contributors (CRM Hay, chair of the UKHCDO in the UK and Dr. Mike Soucie and the CDC in the US. Dr. DiMichele concluded that based on this effort, such harmonized data collection was feasible. This harmonization project between the US and UK will be further pursued. Furthermore, the proposed schema for collaborative data collection will be shared with other countries planning or engaged in such prospective data collection. Finally, as per the formal request to this subcommittee by the EMEA, the Chair will request approval for this project to proceed a sanctioned FVIII/IX Subcommittee activity and a formal Working Party will be established to continue this work on an international scale.

**Treatment-related inhibitors in PUPs with severe hemophilia: Rodin study : H.M. van den Berg**
The Rodin study has been developed to determine treatment related risk factors in Pups with severe hemophilia A. The Rodin study will be performed as a satellite study of the Pednet registry and it has been extended to the Rodin study group which consist of other major hemophilia centres from Europe, Israel and Canada which do not participate in the PedNet/Rodin study. The study started to include data of full cohorts of patients with severe hemophilia A born from 1-1-2000. The aim is to collect upto 1-1-2008 a total of 400 patients in the Pednet/Rodin study and another 400 patients through the Rodin study group. Data will be collected on all first 75 exposure days, which will include every reason for treatment, the dosage, peak treatment, surgery. Besides extensive data on patients characteristics. Further information can be obtained from the websites; [www.pednet.nl](http://www.pednet.nl) and [www.rodinstudy.nl](http://www.rodinstudy.nl).
Low-titre inhibitors in Canal study and Recombinant studies : E. Santagostino

Dr. Santagostino indicated that our knowledge on the natural history of low-titer inhibitors is mainly based on the data provided by the studies carried out in PUPs with hemophilia A treated with recombinant FVIII. Overall, almost half of the inhibitors detected during these studies showed titers of 5BU/mL or less and remained below this level although FVIII exposure was repeated. More than two third of these low-titer inhibitors were transient and disappeared spontaneously continuing FVIII treatment, while immunetolerance induction was started in the remaining cases. Transient inhibitors have also been reported in hemophiliacs with titers always maintained between 5 and 10 BU/mL. Transient inhibitors may represent a model for understanding the mechanisms of immunetolerance so that immunological studies in this specific setting should be implemented. Many aspects concerning the clinical management of hemophiliacs with low-titer inhibitors are still empirical because few data are available on the behaviour of inhibitor titers, FVIII recoveries and half-lives over time. In particular, the need for immune tolerance induction treatment remains controversial and should be better defined.

New methods for the assessment of FVIII inhibitors : J.M. Saint-Remy

Factor VIII immunogenicity is not limited to the detection of inhibitors but its evaluation should take into account all antibodies formed towards FVIII. Current ELISAs can easily detect FVIII antibodies but the use of such assays is far too limited. Identifying the precise epitopes recognized by anti-FVIII antibodies is important to understand the reasons/mechanisms by which such antibodies are formed. It could in addition provide some insight into possible new therapies for inhibitors. The combined transcription-translation method using a library of FVIII fragments is well suited for this purpose. The immune memory compartment of the anti-FVIII immune response can also be evaluated at both B and T cell levels. Methods such as the ELISPOT can be used on a routine basis to count the number of memory B and T cells and thereby evaluate the risk of producing an inhibitor. Such methods are also appropriate to compare the efficacy of therapies aiming at the eradication of inhibitor antibodies.

Section 2: FACTOR VIII ASSAYS AND STANDARDISATION ISSUES
Co-chairs: A. Srivastava, K. Mertens

Factor VIII inhibitor assays: technical issues: B. Verbruggen

FVIII inhibitor tests still are assays that show a very high interlaboratory variation in international survey programs despite recent described modifications. There are a number of variables that influence test outcome: pH stability in incubation mixtures, type of control sample, type of used deficient plasma, liquid handling, incubation time, FVIII content of normal pool plasma, type of Factor VIII assay / type of reagent. It is advised to use a FVIII deficient plasma that contains von Willebrand factor as substrate plasma in the assay of residual factor VIII assay in the test and control mixture. With respect to the assay of type II inhibitors the following items are important:

1. Dilutions with at least 50 % residual activity have to be analysed
2. In monitoring type II inhibitors dilutions that give identical residual activity have to be used
To increase sensitivity of inhibitor detection we have developed a FVIII inhibitor test that is ca. 20 times more sensitive than the Nijmegen-Bethesda assay. The test is independent of residual FVIII in the test sample and has a cut-off value of at least 0.04 NBu/ml, but probably better. There is a strong correlation between a decreased half life of infused FVIII and a low inhibitor titre and low titre inhibitors may therefore be of clinical importance. The putative clinical significance of low-titre inhibitors has to be validated in a bigger cohort of patients.

Collaborative Study on 1st International Standard for FVIII inhibitors: S. Raut
The international need for a reference FVIII inhibitor standard was established at the FVIII/IX Subcommittee in 2001, and the project ratified by the Subcommittee in 2003. Sanj Raut presented a progress report on this ongoing project. The aim of this study was to develop a definitive WHO International Reference Preparation for measurement of FVIII Inhibitors in plasma. 5 candidate preparations (rabbit FVIII PAb, 2 humanised MAbs I & II, human low (X) and high (Y) titre inhibitor plasmas) were assessed against 2 human inhibitor patient plasmas. Inhibitor methods and the FVIII assays were also assessed. Samples were distributed to 25 expert laboratories. Data were received from 21 labs. For all samples, results without a reference standard gave CVs between 17-34%. For the overall mean inhibitor titres, comparisons of the Nijmegen vs Bethesda assays, and comparisons of one-stage vs chromogenic methods, the candidate with lowest CVs was preparation Y. On assessing the patients’ inhibitor plasma relative to the 5 candidate preparation, once again preparation Y gave the lowest CVs of 17 & 18%. It was interesting that no marked improving in CVs were observed for the Nijmegen method compared to the Bethesda method, but a marked improvement in the CVs was observed for the chromogenic assay compared to the one-stage assay. Overall preparation Y (05/206) was the most suitable candidate material for the proposed 1st IS FVIII Inhibitor Plasma standard with an overall Bethesda Titre of 8.2 BU/vial and an overall GCV of 17.5%. Preliminary stability studies show a predicted loss of inhibitor titre potency of < 0.01% per year at

Collaborative Study SSC 8: evaluation of result: M. Lee
This presentation was intended to provide a basic statistical analysis of the results obtained from the most recent worldwide collaborative study of Factor VIII assays sponsored by the ISTH Factor VIII/IX subcommittee. In this study, four different Factor VIII preparations (three recombinant and one plasma-derived) were evaluated by 31 laboratories using their local assay (primarily chromogenic or one-stage) and pre-diluent (deficient plasma or buffer). Using analysis of variance techniques, buffer pre-diluent typically provides lower potencies for recombinant preparations, but the reverse is true for the plasma-derived material. The chromogenic assay gives a significantly higher result for full-chain recombinant Factor VIII products, but not for the B-domain deleted preparation. The combination of pre-diluent and assay is an important source of variation, particularly for B-domain deleted Factor VIII. For the plasma-derived product only the assay method really matters. This information has been used to help design the next collaborative study, SSC9, where the components of variation for the Factor VIII assay will be examined in greater detail.

Collaborative Study SSC 9: preliminary report: S. Raut
Dr. Raut reported on the Phase II SSC9 “Controlled” study following on from the Phase I (SSC8) “Field” study carried and reported last year. The aim of this study was to investigate the differences and variability observed in the Phase I study and further identify any potential
sources of variability, by following strict protocol instructions. Furthermore, it tested the ISTH/SSC recommendation by studying the chromogenic method and pre-diluting in FVIII deficient plasma (containing normal levels of VWF) provided. The effect of different operators and different day set-ups were also assessed. The same 4 samples used in the SSC8 study (3 recombinants and 1 plasma-derived) were distributed to 25 participants. Data were received from 21 labs. The results obtained gave a marked improvement in inter-lab variability with GCVs between 4-11% (compared to 14-19% in the SSC8 study). For the 3 recombinant concentrates both the chromogenic method and predilution in FVIII deficient plasma containing normal levels of VWF were particularly important. For the plasma concentrate, predilution in FVIII deficient plasma was not as important. Variability appeared not to be influenced by different operators, assays on different days or by local standards. This unique data set will be further analysed in more detail (components of variation analysis) by Martin Lee and will be reported in 2007. In the discussion Dr. Mertens noted that the results were much better than in previous studies, where other methods than the SSC recommended method were also included.

**Genetic reference materials for Haemophilia A: A. Hubbard**

On behalf of Dr. E. Gray, Dr. Hubbard announced a study that will involve reference materials for the detection of the intron 22 inversion. DNA samples are being prepared, and include an affected male, a carrier female, and the appropriate normal controls. The study will start in November 2006. Those who are interested should contact Dr. Gray (egray@nibsc.ac.uk).

**Calibrated measurement of thrombin generation in hemophilia: P. Giesen**

Dr. Giesen (Thrombinoscope, The Netherlands) reported that with the calibrated automated thrombogram™ (CAT) plasma is divided in two samples, in one sample thrombin generation is triggered and to the other sample a calibrated amount of thrombin calibrator is added. Both samples receive fluorogenic substrate and the signal is followed in a 96-well plate fluorometer. This allows proper calculation of thrombin in time such that the measured signal is corrected for donor-to-donor differences in plasma color as well as for non-linearities of the signal. It is shown that the same amount of factor VIII in different donors gives substantial different thrombin generation. At the same time a perfect relationship exists between factor VIII concentration and thrombin generation measured in one plasma. Therefore individual calibration in combination with individual measurement of factor VIII are the basis for efficient monitoring of factor VIII treatment.

**Thrombin Generation Assay: Application for measuring the haemostatic efficacy of FVIII: K. Varadi**

Dr. Varadi presented assay principles and technical details of a thrombin generation assay (TGA) have been presented. Thrombin generation is triggered by tissue factor-phospholipid complex (TF-PL) and CaCl2 in the presence of a fluorogenic thrombin substrate. The changes in fluorescence signal are converted to thrombin concentrations using a reference curve, where thrombin is added instead of plasma. The TGA is sensitive to factor VIII levels even below the detection limit of factor activity assays, demonstrated by in vitro spiking experiments and in ex vivo samples obtained after FVIII administration. The ability to measure the haemostatic effect of factor VIII down to 0.002 U/ml may help to predict the possible risk of spontaneous bleeding episodes and to tailor the prophylactic therapy. Individual plasma calibration could be used in the assay, however it is not essential, and therefore the TGA is easily applicable to any fluorescence
reader having the appropriate excitation/emission filters for the AMC-fluorophore. The assay is commercially available from Technoclone (Vienna, Austria).

Thrombin generation in hemophilia: proposal for collaborative study: E. Gray
Dr. Gray presented a brief overview of the collaborative study on thrombin generation as performed in the Subcommittee on plasma coagulation inhibitors (see minutes of that Subcommittee for summary). The next stage could be to prepare a reference plasma in order to assist in reducing interlaboratory variation. In the next collaborative study Dr. Gray is willing to include plasma samples of thrombophilia and haemophilia, the latter in collaboration with the FVIII&IX subcommittee. In the discussion several attendees stressed the potential of thrombin generation for haemophilia. The view was expressed that substitution therapy might be more appropriately expressed in terms of thrombin generation than in factor VIII levels, and that the Subcommittee should focus on this point.

General discussion: future directions
In the general discussion Dr. Mertens raised the issue how to proceed from here. There was consensus within the audience that a Working Party should be established within the FVIII&IX Subcommittee to focus specifically on haemophilia and low factor levels. This group should work in close collaboration with Dr. Gray in order to prevent any overlap with activities ongoing elsewhere. Dr. Ingerslev suggested that it might be appropriate to also include methods based on thromboelastography in this Working Party.

Section 3: FACTOR IX
Co-chairs: J. Oldenburg, K. Mertens

Factor IX gene transfer: current status: A. Nathwani
Dr. Nathwani reported also on behalf of Drs. Tuddenham, Pasi, Kay, Nienhuis and Davidoff on new technology that may raise new hope for gene therapy of haemophilia A. They proposed a distinct approach to gene therapy of haemophilia B which addresses many of the limitations of previous gene therapy trials. Firstly vectors based on adeno-associated virus (AAV) serotype 8 will be used because of the lower prevalence of pre-existing humoral immunity to this virus in humans. Secondly the genome in our vector will have a selfcomplementary (scAAV2/8-LP1-hFIXco) format to enable efficient therapeutic gene transfer with lower titres of vector. Finally, scAAV particles will be administered into a peripheral vein, which is safer and more convenient for patients with a bleeding diathesis. In nonhuman primates their approach has consistently resulted in mean stable plasma human FIX levels of 26% of normal without any toxicity even in animals with pre-existing immunity to AAV. The plan is to establish the safety and efficacy of peripheral vein administration of scAAV2/8-LP1-hFIXco in adults with severe HB. The protocol has received regulatory approval in the UK with plans to commence recruitment in 2007. Further details of this approach were presented and discussed.

Factor IX inhibitors: update J. Lusher
Dr. Jeanne Lusher gave a brief, near final report on Dr. Warrier's registry concerning FIX inhibitors accompanied by anaphylaxis (or severe allergic reactions) in persons with severe hemophilia B. Fifty-six subjects have been reported (29 from U.S. and 27 from other countries), and a few more were verbally reported to Dr. Warrier at the Vancouver meeting in May 2006,
with the promise that registry forms would be completed. The median number of exposure days was 11; med. age at inhibitor detection 19.5 mos. Among those genotyped, null mutations appear to confer a significantly increased risk. Of those with inhibitors to FIX, roughly one-half also had one or more severe allergic reactions. Of those who were desensitized to FIX and then put on an ITI regimen, success was quite poor (12%), and 1/3 developed nephrotic syndrome within 8-9 months after ITI regimen was begun.

Factor IX standardisation; replacement of 3rd International Standard: E. Gray
Dr. Gray mentioned that the 3rd international standard needs replacement. This will be done in parallel with the EP working standard replacement. Manufacturers are invited to submit materials for the standard, and participants who are willing to perform the calibration study should contact Dr. Gray (egray@nibsc.ac.uk).

Section 4: ISTH-SSC Working Party on RARE BLEEDING DISORDERS (RBDs)
Chair: F. Peyvandi
Co-chair: C. Lee

Overview of ongoing activity of working group on RBDs; F. Peyvandi
In 2004, a SSC working group on "Rare Bleeding Disorders" was established. Two years later, 58 centres from all over the world joined the International Database of Rare Bleeding Disorder (RBDD) by including data on 2665 patients. Preliminary information on distribution of affected patients in the world and the available treatment for them are reported at www.rbdd.org.

The next goal of this working group will be to prepare a specific data collection tool which could be used as a homogeneous questionnaire by a majority of the countries around the world. This will contain the most important clinical and therapeutic data useful to answer the missing information that remains unanswered in the field of RBDs. In addition, specific subcommittees will be organized with the aim to prepare evidence-based guidelines using data collected in either RBDD or other available National Registries.

RBDs in the USA (US working group on RBDs): A. Shapiro:
The rare bleeding disorder group in the United States will be developing a resource center hosted on-line by the National Hemophilia Foundation that will contain information on many rare plasma protein deficiencies. Links to existing websites, registries, and organizations related to these disorders will be included. The material developed for this resource center will be submitted for publication. In addition, the development of a national database in the United States is presently underway and a uniform platform has been adopted. Development of a database for rare bleeding disorders will be included in this national database effort. The rare bleeding disorder group in the US wishes to collaborate on an international level to further both development of international data collection and research efforts, including expansion of treatment options.

Glanzmann thrombasthenia: World distribution, mutations and founder effects: U. Seligsohn
A preliminary survey was performed in order to estimate the minimal prevalence of the disease in different populations, the mutations so far detected and founder effects. Data were extracted
from reports involving 9 unrelated patients, national and reference center registries, a database of mutations (by Debra French and Alan Nurden) or personal communications. The minimal prevalence ranged from 1:80,000 in Jordanian, 1:143,000 in Israelis and Palestinians to 1:2,800,000 in Indians, with an average global prevalence of 1:1.1x10^6. As of May 2006, 159 mutations have been reported in peer-reviewed journals, 69 in b3 and 90 in a IIb. 133 were identified in individual patients and 26 in 2 or more unrelated individuals. For 7 of the 26 mutations, a founder effect was discerned by haplotype analysis: 2 in Iraqi Jews, 1 in Palestinians, 1 in Jordanians, 2 in Indians and 1 in Manouche Gypsies in France. This preliminary survey is a rough estimate of the global problem related to this severe hemostatic abnormality, but is somewhat limited because of lack of data from China, Africa, Latin America, Eastern Europe and densely populated countries like Pakistan, Bangladesh, etc.

Menorrhagia in women affected by bleeding disorders- Proposal for an International study: F. Peyvandi
A significant number of women affected by blood coagulation diseases present with complications such as menorrhagia and post-partum hemorrhage. Previous studies on this issue are not very informative on this subject due to the heterogeneous group of patients enrolled and the different methodologies used to analyze the data. We therefore propose a multicentre prospective study which enroll a large number of female patients affected by vWD, rare bleeding disorders and carriers of hemophilia. Based on a specific questionnaire designed to collect clinical data in a homogeneous format. In future it could be available online (www.rbdd.org) for the participating centers. The results obtained will be examined by a scientific committee and then published on the space assigned to on-line studies within the URL: www.rbdd.org. The results of this project will provide information on the incidence of bleeding complications and to assess whether hormone therapy, anti-fibrinolytic and replacement therapy could have beneficial effects on bleeding symptoms and on quality of life of these patients.

Long term prophylaxis in afibrinogenemic patients: a rational based on results from single dose PK; T. Waegemans
FIBRINOGENE T-I (LFB) is a new concentrate of human plasma fibrinogen. It is derived from cryoprecipitate and its manufacturing process includes three major biological safety steps. A single dose PK study performed in 5 afibrinogenemic patients showed homogeneous results within a one-compartment pharmacokinetic model with IV infusion and first order elimination. This model was applied to simulate PK profiles at steady-state after repeated infusions. A selection of targeted parameters will allow investigators to determine individualized dosage regimens before initiating a long term prophylaxis in afibrinogenemic patients. A design of PK study will be presented, aiming at comparing the values predicted by the simulation with those observed during the prospective follow-up of patients.

Getting product to patients: Industry Options: J. Lloyd.
2 years ago, BPL identified market access, regulatory requirement and clinical trial design as constraints to manufacturing products for RBD. Over the past two years there have been some advances made. Registries and databases of rare bleeding disorders have made it easier to establish patient numbers and treatment regimes to support orphan drug designation. Further
development and support of these registries is required to aid industry in deciding whether to develop a product. Orphan drug designation and the parallel scientific advice available from the FDA and EMEA have potentially reduced the need for multiple dossiers and trials. Clinical trial design and support remains an issue, which is overcome by protocol assistance from regulators. There are still some major hurdles for industry to overcome. Further ongoing cooperation between regulatory agencies is required. The maintenance of parallel scientific advice provided by the EMEA and FDA is vital.

**General discussion and concluding remarks**

In the discussion Dr. Peyvandi raised the issue how to proceed with the Rare Bleeding Disorders now her outside funding for this project expires. The Chairman indicated that SSC activities in general would benefit from some level of funding by ISTH. This particularly includes Working Groups or Working Parties that have formally been endorsed by SSC. This issue was discussed in more detail in the audience. It was believed that ISTH, by virtue of its established authority, could drive related national or regional projects under the same umbrella. The general feeling was that ISTH could show leadership in providing funding for those SSC activities that are considered to have appropriate priority. The Chairman mentioned that this issue has been brought to the attention of the SSC Chairman. He concluded the meeting thanking all speakers for their presentations and the audience for attending and contributing to the discussions.
Factor XIII

Chair: R. Ariens  
Co-Chairs: A. Ichinose, H. Kohler, M. Maurer, R. Seitz.  
Active Members: L. Muszbek, A. Inbal, V. Ivaskevicius

Session I: Vascular biology. Ikuro Maruyama (Japan) discussed the role of nuclear DNA binding protein HMGB1, which is found intracellularly as well as in the circulation, in inflammation and multiple organ failure during sepsis. Recent data show and effect of HMGB1 on several coagulation parameters, amongst which FXIII. HMGB1 competes with thrombin for thrombomodulin binding and increases FXIII activation. Aida Inbal (Israel) presented data on the role of FXIII in angiogenesis and wound healing. Experimental models in FXIII knockout mice show reduced angiogenesis and wound healing. Reconstitution with FXIII restores these defects effectively.

Session II: Registry and standardisation. Vystas Ivaskevicius (Germany) presented the first International Registry on FXIII mutations and deficiency. The registry currently lists data from 105 patients with FXIII deficiency, including mutations in both FXIII A- and B-subunit, and details on phenotypic presentation. A website for the registry that includes submission forms and where information on the mutations can be found has been set up at www.f13-database.de. Currently most data are from patients from Europe, and submissions from the rest of the world including less developed countries are encouraged. Sanj Raut (UK) presented an update on current standardisation activities of FXIII. Data from the addendum report on assignment of antigen level to the 1st International plasma Standard for FXIII was presented. The report has been approved by the SSC, FXIII SWG (standard working group) and expert reviewers. Approval will be sought from the Business meeting to submit the report to the WHO. Protocols for an International collaborative study for the development of a standard for FXIII concentrate were presented. It is currently discussed which assays should be included in this study. Akitada Ichinose (Japan) presented an overview of the history of the FXIII standard working group, which he chairs. Issues regarding formation of rFXIII-A complexes with B in plasma were discussed in light of eventual need for a standard for rFXIII-A. Dr Ichinose expressed concerns regarding acknowledgement and financial support for the contribution of academics and scientists (who are funded by research and government grants) involved in the standardisation processes.

Session III: Measurements. Muriel Maurer (USA) presented an overview of current methodologies available to transglutaminase scientists for the measurement if these enzymes. Sensitivity and specificity of the various assays, including spectrophotometric methods, radioactive labelling, biotin/streptavidin systems, fluorescence and phage displays were discussed. Suitability for high-throughput strategies (low volume, suitability to multiwell plates) were also discussed. Dr Maurer suggested that future assay systems may be developed that make use of mass spectrometric or NMR technologies. Janos Kappelmayer (Hungary) discussed data using flow-cytometry that showed the presence of FXIII-A in the blast cells of monocytic and myelomonocytic origin in patients with acute myeloid leukemia and acute lymphoblastic leukaemia (ALL). Sixty percent of ALL cells stained positive for FXIII-A. Data were confirmed by western blots and ELISA and suggest that FXIII-A may be a useful marker in the diagnosis of...
Rainer Seitz (Germany) discussed data from a fluorescent isopeptidase assay. The assay is based on the release of a quencher by transamidase activity. The quencher is released from a synthetic peptide that is based on the sequence of alpha2-antiplasmin, a specific substrate for FXIII. Data were shown on the Km and kcat values for FXIII in plasma and FXIII/fibrinogen mixtures. The assay has a low detection limit and shows linearity with other activity assays.

Session IV: Regulation of FXIII. Hans Kohler (Switzerland) discussed preliminary data from a novel ELISA assay designed to measure the activation peptide of FXIII. In plasma, the FXIII AP signal increases within minutes from the addition of thrombin with concomitant decrease of the A2B2 tetramer signal. FXIII AP was also detected in serum. The assay system will be further characterised with regards to specificity and sensitivity. Laszlo Muszbek (Hungary) discussed novel data on the degradation of FXIII by proteases (cathepsin G, elastase) released by polymorphonuclear cells in the clot. Elastases degraded FXIII within 3 hours, along with fibrin degradation which also occurs by the same enzymes. The degradation was reversed by specific inhibitors of these enzymes. Helen Philippou (UK) showed novel data on the degradation of FXIII by plasmin. This degradation was found to be dose- and time-dependant, reversed by alpha2-antiplasmin, occurred mainly with activated FXIII, was enhanced by fibrin and was shown to occur in plasma clots. The degradation of FXIII by plasmin preceded lysis of the clots. Clots from plasminogen depleted plasma did not show FXIII degradation.
Fibrinogen

Chairman: Nicodemo Weinstock
Co-Chairmen: Moniek de Maat, Jaap Koopman, Leonid Medved, John Weisel, Dieter Peetz
Active Members: Michel Hanss, Bernhard Laemmle, Michael Meyer, Winfried Plesch, Benny
Sørensen, Michael Spannagl, Erwin Strasser

The FXIII and fibrinogen subcommittees held again a joint meeting this year. There were more
than 80 interested researchers present and the presentations were followed by lively discussions.

Leonid Medved: spoke on fibrinogen nomenclature, making recommendations based on the
commonly used terminology for the regions, modules and domains of fibrinogen. With
increasing information on fibrinogen structure now available, a more detailed description is
possible and some older terminology is no longer appropriate and should be avoided. A proposal
for fibrinogen and fibrin nomenclature will be prepared in the form of a paper for publication.

John Weisel: continued with nomenclature related to fibrin polymerization. Since the
polymerization sites in fibrin have been partially characterized and named, these names should
be incorporated into the nomenclature of the field. In addition, terminology for the intermediates
in fibrin polymerization were proposed. Finally, there was discussion of ‘crosslinking’ versus
‘ligation’ to describe the action of Factor XIIIa on fibrin.

Jaap Koopman: described the difficulties of standardization of names of fibrinogen variants,
with over a million possibilities. Therefore, it is only possible to name the most common
variants, based on primary amino acid sequence, modification of amino acids, source of
production/synthesis, and oligomeric structure. A logical system for abbreviations describing
these aspects of fibrinogen variants was proposed.

Moniek de Maat: then provided evidence on the effects of fibrinogen heterogeneity on
fibrinogen assays. In comparison of clottable protein assays, clotting rate assays, PT-derived
assays, and other newly developed assays, normal fibrinogen behaves consistently but variant
fibrinogens demonstrate different functional properties and give different results for these assays.

For all of these presentations above, the proposals will be formalized in two papers, approved by
the Fibrinogen SSC, and published in the ISTH journal, so that these aspects of fibrinogen
nomenclature will become widely known and used, Dr. Weinstock urged timely preparation and
publication of these papers.

Dieter Peetz: presented the study design of the Prevent-it (Proteomics, Genomics and Vascular
Endothelial Dysfunction) trial, an epidemiological prospective cohort study for cardiovascular
risk stratification. Primary objective of the Prevent-it trial is the development of a score for
cardiovascular risk stratification taking into account biochemical variability (including
fibrinogen), exogenous influencing factors and the extent of subclinical disease. Overall 16.400
German inhabitants between age 35 and 75 will be included and examined at baseline (complete
clinical examination) as well as 2.5 years (follow-up 1, interview) and 4.5 years (follow-up 2,
complete examination as at baseline) afterwards. Baseline and Follow-up 2 examinations include
blood sampling with acquisition of plasma/serum, DNA, RNA and platelet rich plasma aliquots stored at -80 °C to establish a large prospective biobank. Primary objective of the Prevent-it trial regarding fibrinogen measurement is the evaluation of a “standardized” fibrinogen cutoff (same cutoff for fibrinogen methods in routine use) included in a cardiovascular risk score. Prerequisite for this aim is the standardization of methods including the new first high fibrinogen reference material. This project will be performed in cooperation with Dade Behring. Fibrinogen methods to be performed are: clotting rate methods [Clauss diluted (optical/mechanical) and Clauss undiluted (optical/mechanical)], PT-derived methods (turbidimetric), immunological (nephelometric) and indirect methods (viscometry). Additionally, in a whole genome approach association studies regarding fibrinogen will be possible as samples will be measured with the Affymetrix 500k array set.

Dr. de Maat announced that she would like to cooperate in this study by measurement of selected samples (nested case-control sets) with specialized assays for fibrinogen variants.

**Winfried Plesh:** stressed that OAC-control with a point of care testing device shows some relation between fibrinogen concentration and INR, but this influence is comparable to differences seen by the Prothrombin time method. Thus INR determination by POCT gives reliable results. Some studies will be done with blood of dysfibrinogenic patients and patients having large amounts of fibrinogen subspecies.

**Benny Sørensen:** spoke on the importance of the different patho-ethiology of acquired fibrinogen deficiencies. He considered acquired reduced levels due to e.g. fibrinolysis and increased consumption or to conditions of dysfunctional fibrinogen, defect synthesis and the bleeding tendency related to different diseases like Multiple Myeloma and Autoimmune diseases. He stressed on the importance of combined conditions like reduced and dysfunctional fibrinogen and exogenic interferences with polymerization, they are often the reason for massive bleeding and specially ongoing bleedings. There are critical levels for substitution when fibrinogen is < 100 mg/dl but sometimes substitution is even helpful when the levels were > than 200 mg/dl. Further items were the dependence of fibrinogen levels as measured by different methods. It was decided to prepare a publication on suggestions how and when to substitute. Dead line for all SSC members to get in contact with Benny to give comments and suggestions will be September this year and a peer reviewed paper should be prepared as an SSC Official communication the end of November 2006. The work will be done in strong collaboration with Wolfgang Korte, Uri Seligsohn and Michael Spannagl.

**Erwin Strasser:** presented data on the effect of haemodilution. He compared different solutions like Ringer, HAES or saline on different tests specially focusing on fibrinogen. Clotting tests were compared with immunological tests (RID) and clot firmness as measured by ROTEM-technique. Clotting tests were significantly influenced by dilution but results differed with different test and different solutions. The study will go on trying to find out if and how these differences are related to the clinical situation.

**Michael Spannagl:** spoke on proficiency testing and the central role of influence the of fibrinogen. There is a large difference between proficiency testing in coagulation compared to clinical chemical parameters. In coagulation we still need consensus values and it will take time
and a lot of standardization work to get to target values, if ever. To get suitable control material - that fairly compares to a fresh pooled plasma - is difficult and partly a problem of costs. At the end of his talk he presented a new study focused on fibrinogen and patient substitution in haemostaseology intensive critical care called HICC. This study approved by the SSC Fibrinogen will begin in September this year.

**Nico Weinstock:** showed that using D-Dimer, Fibrinogen and CRP in Pulmonary Embolism(PE) the PPV (positive predictive value) could be razed from about 25% to 80%. Thus the intruding differential diagnosis of PE will be greatly facilitated. Using these algorhythm 90% of all patient can correctly classified in PE-pos. or PE-neg. avoiding the very unpractical, costly and/or dangerous Pulmonary Angiography or Helical Computer tomography.

**Nico Weinstock:** presented and discussed the value of Clauss fibrinogen. The clear message was: **CLAUSS does not measure** functional fibrinogen not even **fibrinogen!!!** Clauss measures a time of clot formation. This time depends on many - functional fibrinogen independent – factors. His suggestions were to routinely use derived fibrinogen in combination with the Clauss method. Whenever there is a difference between the two methods there will be a surprising result hided behind. “We will get a lot more of information on coagulation and coagulation defects in doing so”. Many results presented by the different speakers at this meeting support these facts.
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 anisoylazoforomyl 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 fluorescent 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 Willemsen 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-arginine 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 Longstaff 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 Biotechnology 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 3rd 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 measurement 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 trial.
The first part of the meeting, chaired by A. Falanga and L. Zacharski, addressed biological issues. E. Gray presented an update for the Working Group on TF standardization in cancer. The panel of this Group is composed by: A. Falanga and T. Barrowcliffe (Coordinators), E. Gray, N. Key, B. Osterud, K. Mann, S. Butenas, N. Mackman, J. Morrissey, F.R. Rickles. The need for creating a task force to standardize the procedures for TF measurement in malignant tissues comes from the knowledge of TF relevant role in cancer. There are many methods to measure TF in tissues as well as in circulating blood. However, the sensitivity and specificity of the available assays are variable. The specific aims and proposed activities of the TF working group are: 1. To compare measurements of TF with a variety of methods in different laboratories; and, 2. To improve intra- and inter- laboratory reproducibility by development of standardised protocols, appropriate reagents and reference materials. Gray presented the results of the pilot collaborative study aimed to investigate the suitability of a panel of candidate TF reference materials (freeze-dried): purified tissue factor, recombinant, cell lysates (THP-1, NB4). The participants of the pilot study were the 7 Working Party laboratories. Assay methods utilised were: clotting, chromogenic and antigen. So far 6 out of 7 participating labs have returned results. A report of the pilot study to participants will be done by August 2006. Future plans (Time frame – 2007/2008): 1- to assess suitability of current batch of purified recombinant TF (may be able to share a batch with Working Party on Thrombin Generation Tests), assess suitability of freeze-dried cell lysates: cell numbers, stimulated or unstimulated, excipient. Prepare large scale batches of tissue factor and cell lysate; 2- to initiate main international collaborative study on proposed candidates; 3- dependent on collaborative study results, establish candidates as ISTH/SSC references –; and 4. standardise assay methods.

The issue of circulating thrombotic markers in malignancy has expanded to a number of blood components and molecules. C. Francis presented the predictive factors for thrombosis in pancreatic cancer, particularly he presented data regarding TF expression in resected and metastatic pancreatic cancer patients, TF levels in peripheral circulation in metastatic pancreatic cancer and correlation with angiogenic factors and DVT/PE. Tissue specimens were obtained from 109 patients with resected pre-neoplastic lesions and 122 patients with resected pancreatic cancers. In addition, 10 normal pancreas specimens were evaluated. A pancreatic tumor microarray of resected pancreatic cancer specimens was constructed. Tissue sections from these microarrays were deparaffinized, rehydrated, washed and stained for TF, VEGF, and CD31 for microvessel density. Overall results of TF immunostaining showed a high percentage of positive staining in pre-neoplastic and neoplastic pancreatic tissue, whereas TF expression was not observed in the 10 normal pancreas. VEGF expression was observed in 56% tumors. Tumors with high TF expression were much more likely to also express VEGF. TF expression also correlated with microvessel density. The association of TF expression with development of clinical VTE was evaluated in a subgroup of 33 patients with resected pancreatic cancer for whom data regarding VTE was available. In this group, a strong association of TF expression
with VTE was found. Finally, median survival of subjects with tumors with low TF was higher than in those with high TF expression, however this difference was not statistically significant.

**S. Haas** presented the analysis of predictive factors from TOPIC I and TOPIC II studies on the prevention of VTE by LMWH certoparin in patients with metastatic breast (TOPIC I) or lung cancer (TOPIC II). VTE events in the breast cancer group were less frequent than expected (4%). Indeed TOPIC I was discontinued at the interim analysis because the incidence of thrombosis was very low (4%) and showed an equal rate in the LMWH certoparin and placebo groups. In contrast, in TOPIC II, the incidence of VTE events was 4.5% in the certoparin arm compared to 8.3% in the placebo arm. In these non small cell lung cancer patients an elevated pre-chemotherapy platelet count (>350,000 plts/mm^3) was associated with an increased rate of VTE and in these patients prophylaxis with LMWH was very effective, in fact certoparin reduced VTE rate from 11% of placebo to 2%.

**T.E. Warkentin** reported on venous limb ischemia in cancer patients in relation to warfarin administration. In 98 cancer patients who were treated with heparin, 9 developed lower limb ischemia. In these patients (assuming heparin followed by warfarin, started from the 2nd day of heparin treatment to reach a therapeutic INR range), a sudden decrease of platelet count was observed when heparin was stopped and patients developed limb ischemia. Tests for HIT were negative, TAT complex elevated and levels of protein C and Factor VII were low. In conclusion, cancer-associated venous limb ischemia can be associated with warfarin administration and the pathogenesis may be attributed to disturbed procoagulant-anticoagulant balance, with increased thrombin generation (warfarin fails to inhibit thrombin generation), and decreased protein C and FVII, which are particularly prone to depletion with warfarin in cancer hypercoagulability.

**CLINICAL RESEARCH UPDATE**

The second part of the meeting chaired by **M. Prins, C. Francis and A. Lee**, included an update of the ongoing clinical trials of antithrombotics in patients with cancer. In addition the designs of new clinical trials and registries was presented.

**ONGOING CLINICAL TRIALS**

**H.M. Otten** described the Trousseau study (Screening for Occult Malignancy in patients with idiopathic VTE). Trousseau study: “Screening on malignancy in patients with an idiopathic VTE: Effect on mortality”. It is a prospective, multicenter, cohort study. The objective is to see the effect on mortality of screening with CT chest/abdomen + mammography versus routine in patients with idiopathic VTE. Inclusion criteria are: Objectified VTE, No risk factor, 40 years, First VTE, No signs of malignancy at routine examination. Trousseau is ongoing and reached approximately 380 patients and interim analysis will be performed within 2006.

**I. Pabinger** updated on the CATS (Cancer And Thrombosis Study). Aims of this study are to evaluate the incidence of venous thromboembolism in cancer patients and to identify predictive parameters for the development of venous thrombosis and pulmonary embolism in patients with malignancies. It is a prospective nested case control study. Patients with newly diagnosed cancer of the central nervous system, breast, lung, kidney, the gastrointestinal or genitourinary system,
sarcoma or haematological malignancies (multiple myeloma, high and low grade lymphoma) or progression of disease after complete or partial remission, are enrolled into the study.

At today, 709 patients have been enrolled. Dr. Pabinger reported preliminary results on cumulative probability of VTE according to tumor type. In addition, in the group of glioma cancer patients a cohort study was performed in 63 consecutive patients high grade glioma. Observation started at time of surgery (patients that presented with VTE prior to surgery were not included). A 26% probability of VTE was found. Patients undergoing total tumor resection had a lower probability to develop VTE compared to partial resection, while the occurrence of VTE had no impact on survival compared to non-VTE group.

A. Falanga on behalf of G. Agnelli presented the PROTECHT trial (PROphylaxis of ThromboEmbolism during ChemoTherapy). This multicenter clinical trial evaluates the efficacy of the LMWH nadroparin versus placebo in the prevention of symptomatic venous and arterial thromboembolism in advanced cancer patients during chemotherapy. Types of cancer included: Lung, Breast, Gastrointestinal, Ovarian, Head and neck. Number of patients enrolled so far is 956 (estimated sample size: 1,200 patients). The interim analysis of the 400 pts was completed in November 2005 and no issues were raised, therefore the study continued. The interim analysis of 800 pts will be completed in September 2006. Last patient included in March 2007, preliminary data may be available in October 2007.

A. Falanga on behalf of M. Kovacs presented the catheter study. This is “A Pilot Study of Central Venous Catheter Survival in Cancer Patients Using Low Molecular Weight Heparin (Dalteparin) for the Treatment of Deep Vein Thrombosis of the Upper Extremity” (The Catheter Study). The hypothesis of this study is that treatment (dalteparin + warfarin) of upper limb DVT secondary to central venous catheters can salvage the line and allow chemo to proceed without significant VTE complications or need for a new line. Seventy four cancer pts with symptomatic acute upper limb DVT associated with central venous catheter (with or without pulmonary embolism, objectively documented) have been enrolled in the study, 64 of 74 (86%) completed 3 months follow-up. Results: DVT Outcome = 0, PE Outcome = 0, Major Bleeds = 3 (1 death). The catheter outcome results show that 63 (85%) patients could be treated with no incidents and catheters were in and remained functional. This study suggests that treatment of catheter-associated DVT can allow the line to be salvaged in the majority of patients until it is no longer needed.

R. Lecumberri updated on the ongoing study named BECAT (prophylaxis of catheter-related DVT in cancer patients). This is a multicentric, randomized, placebo-controlled and double-blind study for the evaluation of the efficacy and safety of antithrombotic profilaxis with Bemiparin (3,500 IU/day) in cancer patients with a central venous catheter. Oncohematologic pts with central venous catheter and platelets above 30,000/mm^3 are enrolled at CVC insertion and randomized to receive placebo or Bemiparin 3,500 IU/d. At days 45 and 90 Ecodoppler is performed and combined incidence of DVT-CVC related recorded.

Dr. Lecumberri also presented the CANBESURE STUDY: Cancer, Bemiparin and Surgery Evaluation, a multicentric, randomized, double-blind, placebo-controlled clinical trial to evaluate the efficacy and safety of the prophylaxis with Bemiparin 3,500 IU/d for 28 days compared to 8
days, in venous thromboembolic disease in patients undergoing oncological abdominal or pelvic surgery. It is a phase III, double-blind, multicentre study conducted in Spain.

**NEW STUDIES**

**A. Kakkar** presented the design of a the study **OVANOX (OVarian cANcer and enOXaparin)**. This is a randomized, Phase III-b placebo-controlled study of extended VTE prophylaxis with LMWH enoxaparin given pre and post-operatively and continued concomitantly with chemotherapy in patients with operable epithelial ovarian cancer. The principal objective of the study is to demonstrate the superiority of extended post-operative VTE prophylaxis with enoxaparin 40 mg subcutaneous once-daily for 3 weeks, compared to placebo, both following 1 week of initial treatment with enoxaparin 40 mg sc once-daily. The main secondary objective is to demonstrate the superiority of enoxaparin 40 mg sc for 3 weeks followed by 1.2 mg/kg in addition to chemotherapy for 18 weeks, over placebo for 3 weeks followed by no treatment, in improving disease free survival at 180 days. Other secondary objectives are: 1) compare overall survival up to 5 years between enoxaparin and placebo, in addition to chemotherapy up to 6 cycles; 2) assess the incidence of total documented VTE (symptomatic / asymptomatic, distal / proximal) up to Day 21; 3) assess the incidence of documented symptomatic VTE (proximal/distal) up to 6 chemotherapy cycles; 4) evaluate the safety (hemorrhage, death, SAE) of enoxaparin in patients with operable ovarian cancer.

**M. Prins** on behalf of **H. Buller** presented the design of the study **INPACT (INproving the Prognosis in Advanced Cancer with low-molecular weight heparin Therapy)**. It is a prospective, randomised, open-label, multicenter study to evaluate the survival in patients with Lung (NSCLC, Stage III-B), Prostate (Hormone refractory), or Pancreatic (locally advanced) cancer. Eligible patients will be randomised to: standard anti-cancer treatment, or standard anti-cancer treatment plus nadroparin. All patients will have standard anti-cancer treatment: 14 days weight-adjusted “full therapeutic” dose, followed by 4 weeks half dose, then a 4-week wash out period, finally 2-week once daily full dose + 4-week wash-out period for several cycles. This last cycle can be repeated up to 6 times in the absence of contraindications. The primary outcome is death from cancer.

**A. Lee** presented the study design of **FOCUS (Fragmin in Ovarian Cancer: Utility on Survival)**. The general objective is to identify a potentially efficacious and safe dose of dalteparin as an adjuvant agent in women receiving standard chemotherapy for newly diagnosed ovarian cancer, for phase III investigation. The study wants to determine the effect of 3 selected doses of dalteparin on CA-125 response in women receiving standard chemotherapy for extended ovarian cancer, in addition to the determine incidence of symptomatic VTE in this group of women and establish the safety (bleeding) of dalteparin when given with chemotherapy over a 3-month period. Finally the study will determine the feasibility of once daily sc injections and women compliance with self-injections over a 3-month period. Also a substudy to explore the relationships between tumour biology and activation of coagulation will be performed.

**R. Lecumberri** presented the design of a phase II prospective, randomized, open and sequential study to evaluate the efficacy and safety of Bemiparin administration on the response to treatment in patients diagnosed with limited small cell lung cancer (ABEL study). Patients with
limited SCLC will be randomized to received the standard chemotherapy treatment versus standard treatment plus bemiparin 3500 IU/d for 6 months. A follow up of 5 years will be performed and the following parameters will be evaluated: Survival (1, 2, 3 and 5 years), Response rates (complete/partial/global) after first-line therapy, Incidence of VTE, Haemorrhages, Biological markers.

**S. Noble** presented the design of a study, named FRAGMATIC. A randomised phase III clinical trial investigating the effect of FRAGMin® Added to standard Therapy In patients with lung Cancer. Aim of the study is to assess the effect of adding 6 months of daily dalteparin (Fragmin) to standard treatment for patients with lung cancer. The primary outcome is the overall survival. Multicentre randomised phase III trial, 2200 patients will be randomised to one of two groups with a 1:1 randomisation. The control Group will receive anti cancer treatment according to local practice for the patient’s histology, stage and performance status. The intervention group will be treated as control plus once daily subcutaneous dalteparin for 6 months at a prophylactic dose.

**REGISTRIES**

**S. Schulman and A. Falanga** presented the registry of Recurrent VTE in cancer patients. This registry represents a collaborative project of the two subcommittee, on “Control of anticoagulation” and on “Haemostasis and Malignancy”. Recurrence of VTE occurs in spite of adequate anticoagulation in 7-27% of cancer patients per years. There is also a high risk of major bleeding on vitamine K antagonists in patients with cancer (5-13%/year). There is no guideline for the treatment of such recurrences. This registry collects data on 200 events of this type, the treatment provided and the effect and safety thereof. The data will create a basis for future trials.

**A. Kakkar** presented the design of a Prospective Registry of Cancer and Events Involving Venous Thromboembolism (PERCEIVE). In this prospective multicentre study of newly diagnosed malignancy (Pancreas; Lung; Prostate; Breast; Colon and rectum; Ovary), patients will be treated according to local best practice, no additional tests or procedures will be required. Selected data will be collected from the patients’ clinical records. Patient progress will be monitored for up to 1 year, with special attention to medical history, VTE risk factors, treatment and outcome. Primary objective is to collect data on the clinical incidence, treatment and outcome of VTE; secondary objectives are to produce evidences to help set standards of practice to improve patients’ clinical care and expected outcome in terms of both prevention and treatment of VTE, and to identify areas of interest for future studies to investigate specific related issues.

**H.M. Otten** presented the design of the TEACH survey (Thrombo-Embolism And CHemotherapy). This is a prospective survey on the incidence of venous thrombo-embolic events during chemotherapy for solid tumors. TEACH will start in May or June 2007. Objectives are to evaluate the Incidence of DVT (symptomatic and asymptomatic) and of VTE/PE (symptomatic) in patients receiving chemotherapy by a prospective multi-center survey (Europe). Patients needed are 2000 with negative baseline US with a maximum of 600 pts per disease entity. They should have no prior major surgery within 4 weeks, no prior chemotherapy within 6 weeks, no anticoagulant scheduled treatment or prophylaxis, an a live expectancy > 3 months.
JOINT SESSION ON D-DIMER

This session was organized in collaboration with the Subcommittees on “Fibrinolysis” and “DIC”. It was well attended and very successful. Carl-Erik Dempfle updated on current state of play for D-dimer and fibrin-related markers determination in DIC. Cheng Hock Toh talked on diagnostic potential of D-dimer and fibrin-related markers in DIC. Finally, Marielle Beckers (on behalf of K. Hamulyak, H. ten Cate and M. Prins) showed the diagnostic pitfalls of D-dimer analysis in patients with cancer suspected from venous thrombosis. Promising results may come from study evaluating the predictive value of D-Dimer for occult cancer or for cancer survival, but better standardized clinical studies are required.
Lupus Anticoagulants/Phospholipid-Dependent-Antibodies

Chairman: Ph. G. de Groot (The Netherlands)
Co-chairs: M. Galli, Italy; S. Machin (UK); T. Ortel (USA); J. V. Pengo (Italy); H. Rand (USA);
G. Reber (Switzerland), R. Roubey (USA)

Genetics

Gene expression profiles are capable of identifying subtle distinctions that define important clinical phenotypes. Dr. Ortel (USA) and coworkers have recently demonstrated that gene expression patterns can distinguish patients with antiphospholipid syndrome (APS) from non-APS patients with venous thromboembolism as well as from asymptomatic individuals with elevated antiphospholipid antibody levels. Translating microarray data into a useful clinical laboratory test requires identifying the smallest number of genes that provide sufficient discriminatory power and using quantitative RT-PCR-based analyses to analyze these genes.

Coagulation

Dr. de Groot (the Netherlands) presented the results of a multi centre study on the predictive value of an LA assay that was only dependent on anti-beta2GPI antibodies for the clinical manifestations observed in APS. 325 patients positive for LA (214 patients with thrombotic complications and 72 patients with pregnancy morbidity) from 6 hospitals were included. The major conclusion was that a LA dependent on anti-beta2GPI antibodies correlates better with thrombotic complications compared to a normal LA (OD = 3.3) but that the assay was not solid enough to be performed on a diagnostic laboratory of a general hospital. A positive LA assay combined with a positive anti-beta2GPI ELISA also results in an improved detection of patients at risk for thrombosis (OR 2.5) and this combination was recommended.

Dr. Haywarth (Canada) discussed the implantation of the SSC recommendations in diagnostic laboratories 15 years after the publication. NASCOLA and ECAT laboratories were surveyed to determine if their LA testing practices conformed to 1995 ISTH SSC recommendations. High conformity was seen in following the recommendations to do two or more LA tests, based on different principles, and at least one test to confirm phospholipid dependence. However, many sites did not perform mixing studies to demonstrate an inhibitor and only a few did investigations to exclude a specific factor deficiency or an inhibitor. These data suggest that recommendations and/or practices for LA testing need updating.

Dr. Ortel (USA) discussed Point-of-Care INR Monitoring in Patients with APS. A subset of patients with antiphospholipid syndrome (APS) has antibodies that appear to interfere with determination of the PT with certain thromboplastins, complicating monitoring warfarin therapy with the INR. Point-of-Care INR monitors are being increasingly used for the management of patients on chronic warfarin therapy. As with plasma-based INR measurements, certain patients with APS have unreliable INR measurements using point-of-care meters. Patients with APS who are being considered for INR monitoring with a point-of-care meter should have correlations performed with the main laboratory plasma-based INR, with discordant results being further evaluated with additional assays prior to using a specific meter with a given patient.
ELISAs

Dr Pierangeli (USA) gave an overview of the various tests used in the diagnosis of APS and on the recommendations of the revised classification Sapporo for diagnosis of APS was first presented. There was emphasis on current and on-going problems and challenges with the various tests, recognizing that one of the major shortcomings of the aCL ELISA is the number of “false positive” results in patients with infectious and other autoimmune diseases. In an effort to address some of those issues, samples from 56 APS patients (classified according to the Sapporo criteria), from 206 patients with infectious diseases and various autoimmune disorders, and from 150 healthy controls were tested in a blind fashion in various centers utilizing: an anti-b 2 GPI, an anti-PT ELISAs, or an aCL (in-house) ELISAs, and a commercial kits for anti-b 2 GPI ELISA kit (INOVA Diagnostics, Inc.) and aPL ELISA kit (APhL ELISA, Louisville APL Diagnostics, Inc.). The tests were considered positive when results were above the cut-off points established for each assay for either IgG and/or IgM isotypes. Receiver Operating Curves (ROC) were created to compare the areas under the curve for the various tests. The nominal cut-offs for statistical significance was set at 0.05. All statistical tests were two sided. Sensitivities and specificities were determined. ROC curves showed that the APhL ELISA, that utilizes a mixture of phospholipids and b 2 GPI instead of CL, showed the best predictive value, followed in decreasing order by the anti-b 2 GPI (in-house), the aCL (in-house), the anti-b 2 GPI (INOVA) and the aPT (in house). Although the aPT assay showed a good specificity, the sensitivity was 24.07 % and was not found positive in any APS sample in the absence of a positive aCL, APhL or anti-b 2 GPI test. Either aCL and/or APhL and/or anti-b 2 GPI tests were found positive in all APS samples. This study validates the recommendations of the recently revised classification criteria for APS with respect to serological tests recommended for the diagnosis of APS (Participating centers and collaborators. Istituto Auxologico Italiano, Milan, Italy. (Prof. Pier Luigi Meroni and Maria Orietta Borghi, Department of Ob-Gyn. Univ. of Utah, Salt Lake City (Dr. D. Ware Branch and Jurhee Rice), Department of Neurology. Univ. of Texas, Health Sciences Center San Antonio. (Dr. Robin Brey and Pat Padilla), Antiphospholipid Standardization Laboratory. Morehouse School of Medicine. Atlanta,GA. (Dr. Silvia S. Pierangeli, Veronica Henderson, Dr. Hector R. Pierangeli), Clinical Research Center, Morehouse School of Medicine. Atlanta, GA. (Dr. Alexander Quarshie))

Relation between serological and clinical manifestations

Dr Pengo (Italy) discussed the presence of different type of antiphospholipid antibodies and the clinical course of patients with primary obstetric APS patients. In primary APS with pregnancy morbidity in classification category I, quite different groups of patients may be identified on the basis of laboratory tests. Triple positivity and/or a history of thromboembolism predict new TE events and new unsuccessful pregnancies.

Dr Galli (Italy) analysed the prevalence and clinical significance of various antiphospholipid antibodies in 112 patients enrolled in the the WAPS (Warfarin in the AntiPhospholipid Syndrome) study. Anti-beta2-glycoprotein I antibodies were significantly associated with all the major endpoints of the syndrome (arterial and venous thrombosis and obstetric complications), thus confirming the usefulness of their inclusion among the laboratory criteria of the APS. Antibodies to prothrombin, protein S and annexin A V appeared associated with some selected
clinical endpoints. The measurement of IgG but not IgM antibodies appeared clinically useful. The generation of laboratory profiles (by combining two or more clotting or ELISA positive tests) did not really improve the identification of patients at risk of thrombosis.

Dr. Pierangeli (USA), also on behalf of Dr. Roubey (USA) reported on the Antiphospholipid Syndrome Collaborative Registry (APSCORE): What Can we learn from this registry? The Antiphospholipid Syndrome Collaborative Registry (APSCORE) is a national, NIH-funded, multicenter disease registry in the United States. The registry is designed to support a broad range of research projects involving the etiology, pathophysiology, diagnosis and treatment of the antiphospholipid syndrome (APS). Over the past 4 years, APSCORE has enrolled approximately 900 subjects including both patients with APS and asymptomatic individuals with antiphospholipid antibodies (aPL). Patients are followed every 2 years. The registry includes a detailed clinical database, core laboratory data, and a tissue repository with plasma, serum, and genomic DNA specimens. Characteristics of the APSCORE cohort will be reviewed, including the correlation of historical aPL results with core laboratory studies, and correlation of core laboratory data at enrolment and at two-year follow-up. Registry resources (data and specimens) have been made available to the research community and 12 research projects are currently supported or under review. Mechanisms for obtaining APSCORE resources will be reviewed.

Dr. Boffa (France) presented a registry of children born to mothers with the antiphospholipid syndrome. The aim of this register is to study the immunological status of infants born to mothers with APS from birth to 5 years and to evaluate their clinical symptoms and neuro-developmental evolution during this period in relationship with the maternal disease. Up to now 75 couples “mother/child” were included. A level around 18% of prematurity and of intra-uterine growth retardation was observed. Few neo-antibodies appeared at birth. Two out of the 20 infants followed up to 2 years showed abnormal behaviour (1 confirmed autism). This addresses the question: are infants born to mothers with APS more susceptible to develop abnormal behaviour?
**Perinatal/Pediatric Haemostasis**

Chair: P. Massicotte, Canada  
Past Chair: U. Nowak-Gottl, Germany  
Co-chairs: G. Kenet, Israel; P. Matthew, USA; P. Monagle, Australia; W. Muntean, Austria; N. Schlegel, France

**THROMBOSIS**

**A. CONGENITAL HEART DISEASE**

1. Cardiopulmonary Bypass and Stroke: AKC Chan/P Massicotte  
A discussion on the status of the ISTH position paper was presented.

2. Thromboprophylaxis of Mechanical Heart Valves: Point of Care testing M. Bauman/ F. Newall  
An update about the ISTH position paper was discussed with the manuscript currently in preparation

3. Point of Care monitoring: Patient Education programs and outcomes.

**M. Bauman/ F. Newall**  
Point of Care (POC) monitoring of anticoagulant therapy represents a potential solution to the challenge of safe and effective dosing of warfarin in children. Standardized, comprehensive, education provided preceding warfarin treatment and POC monitor use positively impacts reliability of results and improves outcomes for children requiring warfarin therapy. The implementation of a formalized focused education program results in excellent correlation between lab/POC INR result $r^2 = 0.96$, increased time in INR therapeutic range (TTR) (84%) $p=0.04$, increased patient knowledge $p< 0.0001$, and improved adherence and no thrombotic or bleeding results. POC INR monitors are a safe and effective alternative for monitoring INR in children who underwent a standardized comprehensive education program, preceding patient self testing with a POC INR monitor. Long term knowledge retention and its influence on safety and efficacy will be evaluated.

**Recommendations:** The education package can be provided in English and French (when translated) to those who are interested by contacting marybauman@cha.ab.ca.

**B. RISK FACTORS FOR THROMBOSIS**

1. Recurrent Pediatric Stroke and risk factors U. Nowak Gottl/ G. deVeber:

Data on recurrent stroke from pooled international databases held at the hospital for sick kids, Toronto, University of Munster and Great Ormond Street hospital, UK were presented. The effects of thrombophilic risk factors for the risk of recurrent stroke was evaluated. Data were available from 678 patients (age range: 1 month to 21 years) followed for a median of 36 months. Recurrence rates were significantly higher among patients with cardio vascular diseases
and increased lipoprotein (a) levels. The presence of any prothrombotic risk factor doubled the risk for recurrence after adjustment for presence of cardiovascular disease, whereas the use of either antiplatelet or anticoagulant therapy significantly reduced it.

**Recommendations:** Due to the paucity of information regarding the risk of recurrent stroke in children, pooled international data collection and further collaborative studies were strongly encouraged.

2. Antiphospholipid Antibodies: Risk of thrombosis in children with APLA+: Results of the Israeli experience **G. Kenet:**

There are only few small case series regarding the issue of primary APS in the pediatric population. In Israel, a cohort of 28 patients with APS was prospectively followed for a median of 6 years. Patients were assessed for presence of any other thrombophilic risk factors and any recurrent thrombosis. The commonest presenting symptoms were thrombotic, with a small subgroup presenting with perinatal stroke. Among laboratory markers of APS, LAC prevailed. During follow up, 5 females developed SLE, 7 patients suffered recurrent thrombosis that was not associated with presence of thrombophilia, and the recurrences were inversely related to anticoagulant therapy administration. The subgroup presenting with perinatal stroke and APL antibodies had a monophasic disease with no recurrences despite lack of anticoagulant therapy. Unique features of pediatric APS and the need for proper diagnosis and early anticoagulant therapy were discussed.

**Recommendations:** The committee suggested a potential future joint session with the APL subcommittee to discuss diagnostic criteria for pediatric patients and guidelines for therapy. Pediatric Hematologists were encouraged to join the European APLA registry, collecting data about infants born to mothers with APS. To receive more information about the registry, contact Nicole Schlegel at schlegel@wanadoo.fr

3. Evidence that Tissue Factor Is the Driving Force in Childhood HUS, with Direct Implications for Therapy and Clinical Studies **E Grabowski**

Up regulation of tissue factor activity is seen on TNF α activated human glomerular endothelial cells exposed to shiga toxin on proximal tubular cells exposed to this toxin and on sections of kidney from rabbits given this toxin orally. In the last case, platelet adhesion/aggregation on the sections is tissue factor driven, as shown by full inhibition of the increase seen with shiga toxin when sections are first incubated with a monoclonal antibody directed against human rabbit factor.

These animal studies suggest that a clinical/preclinical trial to evaluate the role of the tissue factor pathway in the childhood hemolytic uremic syndrome (HUS) is warranted. The clinical/preclinical trial would also evaluate the role of site inactivated factor VIIa in blocking this pathway and suggesting a novel therapy for the syndrome.
**Recommendations**: Samples of blood and urine from patients in the acute phase of childhood HUS are required to be tested in the in vitro cell culture systems. Any interested investigators should contact Dr Eric Grabowski at eric@MGM.harvard.edu

**C. TREATMENT & PROPHYLAXIS OF THROMBOSIS**

1. **New Anticoagulants in children. G. Young**

Low molecular weight heparins have overcome some of the limitations of unfractionated heparin yet, they can not inactivate clot bound thrombin, have a long half life and do not have a specific antidote. In adults, several new anticoagulants have been licensed in recent years to prevent and treat a variety of thromboembolic complications. These agents have superior pharmacologic properties to heparin and some have shown a dramatic reduction in the risk for bleeding without compromising efficacy. These agents have properties which make them particularly attractive for evaluation in children. Data was presented on the results of 2 single centre pilot dose finding and pharmacokinetic studies using 1. bivalirudin in infants with thrombosis and 2. argatroban in children who have heparin induced thrombocytopenia (HIT). The data for dosing and safety of both agents suggests safety and efficacy in infants and children.

**Recommendations**: Properly designed studies including pharmacokinetic and pharmacodynamic studies are required with the new anticoagulant agents in children to determine safety and efficacy.

2. **Central venous lines: thromboprophylaxis: ISTH position paper S. Revel Vilk/L. Mitchell:**

An update of the manuscript status was presented; manuscript is currently in preparation.

3. **Report on Diagnosis and Treatment of DVT: Survey of SSC. (Status of manuscript) L. Bomgaars/ P. Massicotte:**

An update of the manuscript status was presented; manuscript is in preparation.

**BLEEDING**

**A. TREATMENT OF BLEEDING**

1. **FVIIa use in non haemophilic children: International Registry update. (ISTH study) P. Mathew/J. Blatny:**

Presently, n= 30 patients have been entered into the registry. Committee members were asked to increase their level of awareness and report all off-label use in pediatric patients into this registry.

2. **New study : FVIIa in IVH in premature infants P. Mathew:**

The rationale for this study was presented and a draft outline was discussed.

3. **Acquired Purpura Fulminans: The Argentinian Data M. Bonduel**
Sixty-three children with clinical diagnosis of APF related to suspected infectious diseases were retrospectively evaluated in Argentina. Neisseria meningitides was the microorganism most frequently isolated. All patients had signs of DIC, and most of them required mechanical ventilation and inotropic support.

On admission, plasma samples of 31 patients with APF associated with different infections were evaluated. There was a significant difference in PC, PS and AT levels between survivors and non-survivors. PC was significantly lower than AT or PS. In varicella associated APF (n=6) severe deficiencies of free and total PS were detected.

The patients were treated with fresh frozen plasma, protein C concentrates (n=9), or recombinant APC concentrate (n=1). No bleeding events were observed. Despite the therapies used, high percentages of mortality and severe morbidity, in some groups of patients, were found.

**Recommendations**: More data should be collected in APF in children to elucidate possible therapeutic measures which should ultimately be tested in clinical studies.

### B. ANTIPLATELET THERAPY

1. ASA resistance (ISTH study update) M Rand/M Albisetti

The clinical efficacy and dosing of ASA in children have not been well studied. The Working Party of ASA ‘Resistance’ of the Platelet Physiology Subcommittee of the SSC has concluded that: 1) a clinically meaningful definition of ASA ‘resistance’ needs to be developed, based on data linking ASA-dependent laboratory tests to clinical outcomes in patients; and 2) the correct treatment, if any, of ASA ‘resistance’ is unknown. (Michelson et al., J Thromb Haemost 2005;3;1309).

Studies in Canada, the USA and Switzerland are now ongoing to explore ASA ‘resistance’ in children; in one of these, a prospective study of ~120 children with arterial ischemic stroke, 20% are ASA ‘resistant’ based on laboratory testing. Several other centres with appropriate patient populations have been identified.

**Recommendations**: To continue to work with the Platelet Subcommittee in this area. To complete studies in children to provide data to clarify the definition and clinical relevance of ASA resistance in children.

### C. PREDICTORS OF BLEEDING

1. Tests used to predict bleeding in children before adenoidectomy and tonsillectomy: ISTH questionnaire results G. Kenet/ N. Schlegel

Due to the lack of information there is currently no consensus regarding pre-operative screening questionnaire and screening tests for bleeding in young children undergoing various surgeries. Prior to SSC meeting a pediatric bleeding proposed questionnaire has been submitted to committee members for approval and comments. Following the correspondence, it has been
suggested to use the VW questionnaire (with some pediatric modifications) as baseline screening for any child and for parents of younger children as well. The score of this questionnaire should be added to the score of surgical procedure (differentiating high and low risk surgeries, with regard to bleeding potential) and the score of abnormal laboratory screening tests (to be obtained for every child with high-risk bleeding history or high-risk surgery as well as selected cases upon clinical experts definition) in order to stratify the risk of bleeding for patients undergoing various surgical procedures.

**Recommendations:** A sub group led by G. Kenet/ N. Schlegel will work upon further definitions of bleeding score. The committee members have agreed to prospectively collect data of patients referred for peri-operative screening according to questionnaire and score suggested. Contact gili.kenet@sheba.health.gov.il if you are interested in participating in the sub group.

2. Pre op PFA-100 screening in 500 children **W. Muntean**

Peri-operative screening of 500 children referred for Hematology consultation due to potential high-risk bleeding history was performed by using 2 assays: PTT and PFA-100. Neither prolonged PTT, nor prolonged closure time of PFA-100 were associated with operative bleeding in this group despite a number of children with prolonged PTTs and closure times. This may have resulted due to limitations of both tests, as well as non-reliability of the screening questionnaire as administered by non-expert physicians.

**Recommendations:** To try to further define the risk for bleeding through questionnaires and laboratory predictors as suggested above.
Plasma Coagulation Inhibitors

Chair: Elaine Gray, UK
Co-Chairs: F. Bernardi, Italy; K. Suzuki, Japan; H.C. Whinna, USA

WHO International Standards

Chair: HC Whinna

Proposed international standards for Protein C. E Gray

Twenty laboratories from 10 countries took part in a collaborative study to assign potency values to 2 proposed World Health Organisation (WHO) international standards: the 2nd International Standard for Protein C, Plasma, Human (02/342) and the 1st International Standard for Protein C, Concentrate, Human (04/252) and also to calibrate the Scientific Standardisation Committee (SSC) secondary plasma standard Lot#3 for Protein C functional activity and antigen. The proposed candidates were assayed against the 1st International Standard for Protein C, Plasma, Human (86/622) and locally collected normal plasma pools (n = 38). Intra-laboratory variability (GCV) was found to range from 0.3 – 21.3%, with the GCV for the majority of laboratories being less than 10%. Good inter-laboratory agreement, with the majority of the GCV being less than 10% (GCV range = 1.4 – 15.6 %) was also obtained. Comparison of results against local plasma pools with results against the 1st IS for Protein C, Plasma, Human, 86/622 showed significant differences between estimates. Considering the demonstrated stability of the 1st IS for Protein C, the more likely reason for the discrepancy is the change in Protein C levels in normal plasma pools over time. In order to preserve the continuity of the international unit, it was therefore proposed that the potency values for the proposed WHO 2nd IS for Protein C, Plasma, Human 02/342, the proposed 1st International Standard for Protein C, Concentrate, Human, 04/252, the SSC Lot#3 be based on overall mean value obtained from assays relative to the 1st International standard for Protein C, Plasma, Human, 86/622 only. All participants agreed with the proposal for the assignment of potencies to the candidate 2nd IS for Protein C, Plasma. Nineteen out of the 20 participants agreed with the recommendation for the Protein C, Concentrate. However, due to discrepancy of performance of the candidate and their in-house material, one participant would like the proposed standard to be labelled with both the clotting and chromogenic values. The recommendation to establish the concentrate standard will be deferred until results from further study is available to resolve this issue.

Proposed International Standard for Protein S, Plasma. T Hubbard

Twenty laboratories from 11 countries have participated in the collaborative study to calibrate the proposed WHO 2nd IS Protein S Plasma (03/228) for total antigen, free antigen and function. Estimates of intra-laboratory variability were acceptably low with geometric coefficients of variation (GCV) below 10 % for 80/96 data sets. Only 5 out of a total of 189 assays were excluded from the analysis. The inter-laboratory variability (GCV) for estimates relative to the WHO 1st IS (range 3.33 - 9.02 %) was lower for each parameter compared to estimates relative to the local normal plasma pools (range 7.88 - 13.16 %). Largest inter-laboratory variability was found for the estimates of free antigen, particularly those incorporating PEG precipitation steps. There were no significant differences between the estimates calculated relative to the WHO 1st
IS and the local normal pools for any of the three parameters and the overall mean estimates were extremely close (total antigen 0.83 vs 0.82; free antigen 0.81 vs 0.81; function 0.77 vs 0.75 respectively). These results are very encouraging in that they have confirmed the original definition of the IU and are also consistent with the stability of the WHO 1st IS and its suitability for the calibration of the proposed WHO 2nd IS. It is proposed that the WHO 2nd IS Protein S Plasma (03/228) be assigned the mean estimates calculated relative to the WHO 1st IS as follows:

Total antigen 0.83 IU/ampoule; Free antigen 0.81 IU/ampoule; Function 0.77 IU/ampoule

All 20 participants have agreed to the proposed potencies and it is planned to submit the calibration to WHO ECBS in October for formal establishment.

Replacement of 2nd International Standard for Antithrombin, Concentrate. E Gray

The stock level of the 2nd International Standard for Antithrombin, Concentrates is running low and has to be replaced within the next 18 months. There is a call for donation of candidate materials and participants for the forthcoming collaborative study which will be initiated in November 2006.

Reference Materials and Methods for Antithrombin

Update on activities of the ISTH/IFCC Joint Committee on Standardisation of Coagulation tests (C-SCT): Working Party on reference materials and methods for antithrombin: Pilot study on primary reference methods for antithrombin. E Gray/CM Jackson

A pilot study has now been initiated to evaluate a primary method for antithrombin activity. This is based on the titration of antithrombin activity against factor Xa. Purified antithrombin and human and bovine factor Xa are now being sourced as critical reagents for this assay.

Joint session with the Control of Anticoagulation

Global Coagulation/Haemostatic Tests
Chair: HC Whinna, Dr van den Besselaar


An international collaborative study involving 39 laboratories was carried out to investigate the sources of variability in thrombin generation tests. It was concluded that the concentration and source of trigger (tissue factor and phospholipids) were the major determinant of intra- and inter-laboratory variability. Pre-analytical variables also influence the comparability of the test. By normalising the results against a “reference” plasma, the variability could be reduced. The Working Party (WP) on Thrombin Generation Tests therefore propose to develop and evaluate a reference plasma for thrombin generation test and to assess the concentrations and sources of tissue factor for use in the study of different clinical plasma samples. The WP would like to work
with the experts in the FVIII/FIX subcommittee to define TGT protocols for the testing of haemophilia plasma.

**Monitoring endogenous thrombin generation in healthy individuals and patients after a first acute myocardial infarction (Clinical validation of the Calibrated Automated Thrombogram) R van Oerle/ H Spronk**

Several studies have shown a persistent hypercoagulable state following and acute chest syndrome, by coagulation activation markers. Theoretically, quantification of endogenous thrombin generation potential (ETP) may offer a more detailed analysis of the intrinsic properties of an individual’s plasma with regard to hypercoagulability, but none of the commercial methods have been rigorously validated in normal individuals as well as in patients. We performed a series of pre-clinical standardization studies of the calibrated automated thrombogram in normal volunteers and applied this method in consecutive patients with a first acute myocardial infarction (AMI).

Thrombin generation studied in healthy volunteers (n=139) showed significant differences in peak height, time to peak and time to tail between males and females, whereas lag time and endogenous thrombin potential (ETP) were comparable. Over a three month period repeated measurements showed unaltered thrombin generation.

Thrombin generation was studied in 55 patients after a first AMI on admission, after 4 days, 3 and 6 months. On admission, patients showed increased thrombin generation: ETP-ratios were elevated compared to healthy persons (1.238, SD:0.264 vs. controls 1.041, SD:0.155, 95%CI:1.015-1.067) and peak values were (1.479, SD:0.344 vs. controls 1.007, SD:0.177, 95%CI:0.977-1.037). Antithrombotic treatment with low molecular weight heparin (LMWH) dose dependently suppressed ETP and peak height (r²=0.772, p<0.0001 and r²=0.808, p<0.0001, respectively). ETP and peak height remained elevated as compared to normal persons at 3 and 6 months and showed time variation effects in contrast to normals. In conclusion, preclinical studies confirm the reproducibility and stability in time of CAT-analysis and the method is suitable for detecting hypercoagulability in patients after a first AMI.

**International multi-centre assessment of the calibrated automated thrombogram thrombin generation assay. Y Dargaud**

The objective of this study was to assess inter-laboratory variations of the Calibrated Automated Thrombogram (CAT) results and also intra- and inter-assay imprecision of the test within 5 European centres with proven experience in TG measurements. A large variability of ETP results between centres were found when the centre were using different sources and concentrations of tissue factor and phospholipids. Results are incomparable and multi-centre clinical studies can not be designed. When a standardized protocol was used by all the centres the variability could be limited. Under these test conditions, contact factor inhibition improved the intra-assay CV in all centres. There is a need for experienced operators and for the use of the same version of the software will help to reduce variability. These results emphasize the requirement for a standardized protocol using standard reagents before organizing multi-centre clinical trials and before a wider application of CAT in clinical laboratories.
A new global assay with small amounts of recombinant tissue factor and tissue-plasminogen activator providing novel parameters to determine the overall hemostatic potential. S He/M Blombäck

Dr He described an assay for the Overall Haemostatic Potential with plasma containing recombinant tissue factor, t-PA and phospholipid. The expected findings from the commercial plasmas with coagulant deficiencies and samples with high activity of PAI-1 indicate that the new method can detect physiologically relevant actions, as regards fibrinogen clotting and fibrin digestion regulated by thrombin generation and plasminogen activation respectively. Increased coagulation and decreased fibrinolysis, as well as the induced changes by heparin/a thrombin inhibitor in the thrombotic cases suggest that the new approach may determine hypercoagulation and monitor anticoagulating therapies. Decreased overall haemostatic potential based on low levels of coagulation and high level of fibrinolysis was also found in the haemophiliacs, showing a possibility that a criteria in addition to the FVIII/FIX concentrations may be created according to the assay results for better selecting patients who really need the regular prophylactic treatment. Ongoing studies will further explore haemostatic disturbances in more clinical materials, to confirm whether this simple approach can become a laboratory tool in clinical routine.

Methods recording dynamics of fibrin formation. B. Sørensn/J. Ingerslev

Traditional plasma coagulation analyses, such as the PT and APTT usually only provide information of the early start of clot formation. However, following the initiation of clot formation, there is a rate specific dynamic development of the clot. During recent years thrombelastometry has been used extensively to visualise the dynamic properties of continuous whole blood clot formation. In our center a thrombelastographic model, employing minute amounts of tissue factor as activator, has been explored to demonstrate phenotype heterogeneity of patients with severe haemophilia, dose titration response to rFVIII, rFVIIa, aPCC in haemophilia, evaluation of anticoagulants and modalities for reversal, detection of tarumatic and dilutional coagulopathy and methods for reversal as well as detection of hypercoagulation. Continuous profiles of plasma clotting can be obtained from several coagulation instruments. Adopting simple signal processing, including differentiation and filtration, dynamic profiles and parameters of e.g. APTT plasma clotting analysis can be generated. This presentation summarize the application of dynamic APTT clotting parameters in patients with haemophilia as well as patients with an episode of verified venous thromboembolism. In summary, the maximum velocity of APTT induced plasma clotting reflects more heteroneity amongst patients with severe haemophilia A (FVIII:C < 0.01) than standard APTT clotting times. Individualized in vitro rFVIII spiking experiments may serve as an additional laboratory tool for selecting appropriate dose regimens. In patients with a history of verified venous thrombosis, our data suggest that the maximum velocity of APTT plasma clotting represent a stronger predictor for hypercoagulation than standard APTT measures. Ongoing prospective studies aim at evaluating the clinical correlation and feasibility.
The 2006 SSC plasma kallikrein-kinin subcommittee was attended by approximately 20 individuals. Several discussions were presented, as outlined below:

Dr. Alvin Schmaier discussed the interactions of high molecular weight kininogen with endothelial cells, and its binding to a multiprotein complex consisting of uPAR, gC1qR and CK1. The activation of prekallikrein on cell surfaces by prolylcarboxypeptidase was reviewed and updated. The mechanisms by which BKB2R deficient mice are protected from thrombosis was discussed in detail. The working hypothesis for this observation involves increased levels of Ang >II interacting with AT2R, which are upregulated in several tissues, leading to vasodilation, increased NO and PGI2. Also, preliminary evidence suggests that BK1-5 may impair platelet function.

Dr. Thomas Renne discussed clinical studies on hereditary angioedema, including a new Type III variant that may result from a Thr347Lys mutation in FXII, leading to increased FXIIa proteolytic activity. The remainder of the presentation focused on thrombosis in the FXII deficient mouse, which is not prone to increased bleeding, but fails to form occlusive thrombi. The latter observation appears to result from failure to stabilize the initial platelet aggregates at the site of vessel damage. These mice also have markedly smaller infarcts in and induced stroke model, with less vessel occlusion. The relationship of these observations to the human situation was discussed at length by the audience.

Dr. David Gailani update his work on mice deficient in FXII, FXI or FIX, in particular those deficient in FXI. These mice are protected from thrombosis to an extent caused only by high concentrations of heparin in the range of 200 U/ml. These animals display both decreased fibrin deposition as well as decreased platelet consumption in thrombosis induced by FeCl3. Interesting studies designed to test the hypothesis that FIX deficiency would protect against the pathology of plg deficiency were presented, based on crosses of mice deficient in both. These mice did have increased survival compared to plg deficiency alone. However, FXI deficiency worsens the outcome in plg deficient mice, and crossed mice develop a marked inflammatory process in the lung. Finally, a discussion of the genetics of human FXI deficiency was presented, with findings suggesting that specific FXI mutations may impair FXI secretion in a dominant manner by forming heterodimers with normal FXI monomers and impairing cellular secretion.

Dr. Robert Colman was scheduled to speak but was unable to attend the SSC meeting.

Dr. Keith McCrae presented additional information concerning the mechanisms by which two chain HK impairs angiogenesis and causes apoptosis of proliferating endothelial cells. This process is associated with enhanced cellular oxidant stress, and may also involve activation of cellular MMPs. Studies describing kininogen deficient mice were also presented. The first of two kininogen genes have been deleted, yielding viable mice. Studies suggest that this gene is solely
responsible for plasma kininogen, and that though gene 2 is transcribed it appears to yield little if any protein. Possible reasons for this were discussed. Future studies with these mice were discussed with other members of the subcommittee.

Dr. David Pritchard discussed studies of several forms of FXIIa in acute cardiac ischemia. Two forms of FXIIa were measured-FXIIaA and FXIIaR. The latter is available only after release by chemical treatment of plasma. The biochemical nature of these forms of FXIIa have not yet been characterized in detail. However, in the RACS study, upper quartile levels of FXIIaR are strongly associated with risk of recurrent MI within 30 days. Moreover, high levels of FXIIaA are associated with increased risk for 30 day mortality as well as death and recurrent MI at 1 year. The implications of these findings and the biochemical nature of FXIIaA and FXIIaR were discussed at length by the subcommittee.

Dr Zia Shariat-Madar discussed the implication of sepsis to activation of the KKS. In particular he reported that HK binds with significant affinity to bacterial LPS at physiological pH. This is blocked by the HKH20 peptide. The sentinel observation was that LPS appears to increase the expression of prolycarboxypeptidase by human endothelial cells, suggesting one mechanism whereby the kallikrein-kinin system may undergo enhanced activation in sepsis.

At the conclusion of the meeting the possibility of developing a FXII plasma standard was raised. Dr. McCrae will discuss these further with several subcommittee members and the subcommittee will consider this objective over the coming year.

Submitted by Keith McCrae 7/01/06
Platelet Immunology

Chair: T. Warkentin (Canada)
Committee Co-Chairs (in attendance): A. Greinacher, Y. Gruel, V. Kiefel, H. Kroll
Committee Co-Chairs (not in attendance): M. Murphy, G. Visentin

The program was divided into three parts: (I) Alloimmune Thrombocytopenia, (II) Autoimmune Thrombocytopenia, and (III) Drug-Induced Thrombocytopenia.

ALLOIMMUNE THROMBOCYTOPENIA (Chairs: H. Kroll, T. Warkentin)

P. Metcalfe: Proposal for adoption of an anti-HPA-3a reference reagent by the ISTH. Anti-HPA-3a is the third or fourth most common cause of neonatal alloimmune thrombocytopenia (NAIT), but the pathogenic anti-HPA-3a alloantibodies can be difficult to detect. Therefore, there was interest in the development of a reference anti-HPA-3a reagent. A reagent (03-190) was prepared from plasma obtained from a mother with two babies born with severe NAIT due to anti-HPA-3a. Forty-nine laboratories from 23 centers participated in the evaluation of this material, including internationally-recognized laboratories (Kiefel, Kroll/Santoso, Murphy, Kaplan, Ouwehand, Aster/Curtis). Studies were aimed at determining the extent to which the participating laboratories could identify the antibodies, including minimum titer of detectability. Almost all of the labs (except for two) could detect the antibodies. Dilutions of anti-HPA-3a up to 1:64 were detected. It was determined that a minimum dilution of 1:8 should be detected by a laboratory (if not, the assays used are not sensitive enough). Prior to the meeting, the draft document (prepared by P. Metcalfe) describing the performance of this reagent among the laboratories was circulated to committee members to review.

Vote by committee members on proposal to adopt anti-HPA-3a reference reagent (NIBSC 03-190) by the ISTH: FOR (Chong, Imbach, Kaplan, Kiefel, Kroll, Greinacher, Gruel, Smith [in absentia], Warkentin); AGAINST (none against).

C. Kaplan: Predictive value of anti-HPA-1a alloantibody concentrations for severity of fetal alloimmune thrombocytopenia. About 1 in 800 live births is affected by NAIT. Severity of thrombocytopenia increases with subsequent pregnancies. The MAIPA was used to test for a correlation between the antibody concentration in the MAIPA against severity of thrombocytopenia. In the method reported, a correction factor for hemodilution is performed (using albumin levels). Significant correlation was seen between anti-HPA-1a concentrations of >250 AU/mL and fetal thrombocytopenia, irrespective of the gestational age. The sensitivity and negative predictive values were improved when the testing was performed before 28 weeks of gestation. The correction factor for hemodilution is important to perform. Of interest, significant decline in antibody levels occurs toward the end of pregnancy (presumably reflecting increase in transplacental passage of the antibodies).

C. Kaplan: New mutation altering HPA-1a genotyping. Laboratory diagnosis relies on testing for alloantibodies (usually by MAIPA) and assessing parental antigen incompatibility. In a case presented, no maternal antibody was detected by MAIPA, but there was apparent HPA-1b incompatibility shown by genetic testing (mother: HPA-1b/b, father HPA-1a/a). However, by
platelet typing by MAIPA, the mother was found to be heterozygous. Platelet genotyping by PCR-RFLP also showed the mother to be heterozygous. A silent mutation was found within the antisense primer of the PCR-SSP that led to false typing assignment (Bertrand et al. Transfusion 2006; in press). This illustrates the importance of using complementary methodologies to avoid false diagnosis of incompatibility.

H. Kroll/S. Santoso: Registry of alloimmunization against low-frequency HPAs and atypical anti-HPA-3a. A new alloantigen (Swi a) has been recently described (Kroll 2006), representing GPIa 3347 (T to C) substitution. It would be useful to have a registry on these (and others to be discovered) rare alloantigens. The speaker indicated that he is working with committee members to develop such a registry (ongoing work). Recently, a questionnaire was submitted regarding interest in such a registry regarding rare alloantibodies, as well as with atypical anti-HPA-3a alloantibodies. A questionnaire regarding these issues was sent to 52 labs; to date, 6 labs responded.

Technical issues of anti-3a alloantibody detection were discussed, including time of test platelet storage (which leads to loss of reactivity with some anti-sera). The speaker indicated that he will work with committee members to establish a registry of alloimmunization to these rare (low frequency) alloantigens.

AUTOIMMUNE THROMBOCYTOPENIA (Chairs: B. Chong, V. Kiefel)

P. Imbach: Update: Activities of the Intercontinental Childhood ITP Study Group (ICIS). An overview of ITP pathogenesis was presented, highlighting the involvement of many aspects of the immune system. An historical perspective of IVIgG therapy was also summarized, and the dramatic growth of this therapy was emphasized (currently, 57 tons/yr sold world-wide). ICIS Registries I and II are closed. Currently, the Splenectomy Registry and the PARC-ITP Registry are open. More than 2000 patients were entered into Registry I; a slight male predominance was seen, which was greatest in children under 1 year of age. The natural history of ITP was such that 11% of patients still had a platelet count <20 at 1 year follow-up. Standardized approaches to management (e.g., systematic approaches to treatment of three stages of bleeding severity) were discussed.

F. Rodeghiero; on behalf of the Working Group on Thrombocytopenias of the European Hematology Association. Heterogeneity of terminology and clinical definitions in adult ITP: A critical appraisal from literature analysis. The speaker demonstrated the magnitude of terminology heterogeneity and definitions for ITP in the literature, and ways to achieve consensus. A total of 1247 papers were retrieved (2000-2005). Definitions of ITP (platelet count thresholds, time-related parameters, grade of severity) were reviewed. First-line therapies were reviewed. Definitions were assessed for various factors, including definition of ITP, platelet cutoff for initial treatment, platelet levels to define response, time to assess response, bleeding score, platelet level to define chronic phase, time point to assess for meeting the criteria to diagnose “chronic” ITP, criteria for splenectomy, definition of response to splenectomy, etc. Numerous differences in criteria were found, with no literature consensus. For example, for starting treatment, platelet count thresholds varied widely (<20, 3/21 publications; <30, 13/21; <50, 3/21; other criteria, 2/21). Similarly, complete and partial response criteria varied widely in
the literature, as did timing of assessment for first-line therapy, and duration of response (e.g., short term defined as 3-10 days, and long term defined as 3-12 months). Re: definition of chronic ITP, platelet thresholds ranged from <50 to <100 to <150 to < “normal range”, and duration of thrombocytopenia ranged from >6 weeks, to >3 months, to >6 months, to >2 years. All of the items reviewed showed considerable heterogeneity in definition. Thus, there are considerable limitations in the interpretation of the ITP literature. The speaker reported on plans to develop consensus on these issues by a Working Group on Thrombocytopenias of the European Hematology Association, with various planned upcoming meetings (Sep 2006 ICIS Meeting; Nov 2006 Vicenza meeting Proposal and Discussion on Topics and Methodology; Dec 2006 ASH Congress; June 2007 EHA Meeting Presentation of Final Terminology Proposal. The participants were listed as: Rodeghiero [Chair], Provan [Co-Chair], Godeau, Fenaux, Imbach, Bussel.

B. Chong [on behalf of the Working Party]: Definition of ITP (integrated clinical and laboratory definition: an update of current activities). Dr. Chong gave the presentation on behalf of J. Bussel, who was unable to attend. The starting point for a definition of ITP is that of thrombocytopenia caused by platelet-reactive autoantibodies, and that is fundamentally based upon clinical exclusion of other causes of thrombocytopenia. Discussion was held regarding which specific conditions need to be excluded? (HIV, HCV, splenomegaly, congenital thrombocytopenia, etc.). Should response to treatment be a criterion? (but then, what if treatment is not required). The speaker advised generating a list of disorders to be ruled out, along with a guide to what tests ought to be performed. Parameters for performing bone marrow aspirate were discussed, e.g., age >60 years, no response to treatment, prior to planned splenectomy or certain immunosuppressive therapies. Studies of “phase III” assays for platelet glycoprotein-associated assays show sensitivity in the 50-66% range, and specificity in the 80-90% range. Important unresolved issues include absence of a “gold standard” laboratory assay for diagnosis of ITP, lack of agreement on which specific non-ITP causes of thrombocytopenia need to be excluded, diagnostic value of phase III assays, differences between adults and children. The speaker presented the framework of a potential diagnostic algorithm that incorporated important steps as formalized exclusion of non-ITP causes of thrombocytopenia, performance of a phase III assay, and response to immunosuppressive therapy.

V. Kiefel; Impact of abciximab therapy on autoantibody detection in thrombocytopenic patients. The speaker showed that about 70% of patients have elevated GP-PAIgG (by MAIPA, SZ21) if they were tested soon after receiving abciximab. However, there was no good correlation between any drop in platelet counts and the level of GP-PAIgG post-abciximab. In theory, such antibodies could react against either abciximab itself, or against a new antigen (ligand-induced binding site [LIBS]). In all cases investigated, preincubation with human Fab showed nearly complete absorption. The speaker speculated that natural anti-abciximab/GPIIbIIIa IgG reacts with the papainic cleavage site of the drug.

DRUG-DEPENDENT THROMBOCYTOPENIA: D-ITP (Chairs: V. Kiefel)

A. Greinacher (on behalf also of R Aster, B Curtis, B Chong, H Kroll, S Santoso, T Warkentin): Registry of drugs causing immune thrombocytopenic purpura (D-ITP syndrome by serum exchange.
Numerous drugs are suggested as causing immune thrombocytopenia, but the “true” list of causative drugs may be much smaller. It was proposed that a systematic approach to identify drugs that cause D-ITP—in which confirmatory diagnostic testing in at least two labs (with serum exchange)—is crucial for “proving” that a drug causes immune thrombocytopenia (high specificity is assumed). This concept was examined by an exchange of several putative drug-induced thrombocytopenia sera (vancomycin, quinine, trimethoprim-sulfamethoxazole) between the McMaster and Greifswald laboratories. Concordant results were seen with each of the sera tested (though techniques varied, i.e., flow cytometry in McMaster, MAIPA whole platelet ELISA in Greifswald). Issues addressed include: serum shipping, ethics (anonymity of patients whose serum is tested is required, including avoiding even use of the date-of-birth, given the rare nature of these reactions; however, this raises issues on how to be sure distinct sera are being studied); use of generic versus brand names; how to handle drugs; how to report results, including on a registry database (positive vs negative only? quantitative data including all control data?); appropriate controls to use, especially when a known positive serum may not necessarily be available (normal serum control; platelet-reactive alloantibodies when drug-dependent serum is not available?); what sort of clinical information should be available in the registry?

B. Chong: Drug-induced immune thrombocytopenia.

Dr Chong presented some of his viewpoints regarding D-ITP, including the concept that current assays are both specific and sensitive (provided that the correct drug and drug metabolites are studied), and if patient serum is used after cessation of drug. Either drug-dependent and patient serum-dependent PAIgG or GP-specific assays can be used, although the latter assays are more sensitive. Use of indirect (serum-based) assays is preferred, compared with using patient platelets when thrombocytopenia is present. For quinine-induced thrombocytopenia investigated in his laboratory, 8/13 sera tested positive with flow cytometry, whereas all 13 reacted against GPIb-IX, and 3/13 reacted against GPIIb/IIIa, in the GP-specific assay.

Regarding ELISAs for HIT, the cutoff influences the sensitivity and specificity of the assays. For the functional tests, international and Australian surveys previously reported that expert labs can perform functional assays for HIT antibodies well, but not necessarily other (non-expert) labs.

HEPARIN-INDUCED THROMBOCYTOPENIA (HIT) (Chair: V. Kiefel)

T. Warkentin and A. Greinacher: Gender and HIT. Data from three studies were presented showing a female predominance in HIT. The common odds ratio is ~1.5-2.0. Also, analysis of data from individual patient studies shows that there are three separate risk factors for HIT: (a) type of heparin (UFH > LMWH), (b) type of patient (surgery > medicine); and (c) gender (female > males). The implication is that in some settings (e.g., females receiving postoperative UFH thromboprophylaxis) the impact of using LMWH (to prevent HIT) is most effective.

T. Warkentin, A. Greinacher, Y. Gruel, R. H. Aster, B. Chong: Conceptualizing the sensitivity-specificity tradeoffs of laboratory testing for D-ITP: influence of antibody classes. The concept was presented that current PF4/heparin ELISAs and washed platelet activation assays are inherently very sensitive for detection of HIT antibodies, but lack
specificity because non-pathogenic antibodies are frequently generated in heparin-treated patients, and because not all patients even with “strong” HIT antibodies develop thrombocytopenia. ELISAs inherently have lower specificity than platelet activation assays, since the former are more likely to detect non-pathogenic antibodies. This situation is the reverse of laboratory tests for D-ITP, in which sensitivity is not 100% (because some cases are caused by metabolites that may not necessarily be included in the diagnostic test system) but specificity is believed to be high. The implications of these concepts regarding evaluation and interpretation of diagnostic tests was presented. An advanced manuscript discussing these concepts has been prepared.

A. Greinacher: Discrepancies in PF4/polyanion-immunoassays and platelet activation assays in HIT: influence of antibody classes. Important issues in test discrepancy were pointed out. For example, sometimes commercial immunoassays have used degraded PF4 in which the antigenic complexes were lost (this problem is not necessarily shown by using a “positive” control, as these are not HIT sera, but usually monoclonal anti-PF4 antibodies). A small number (~0.4%) of samples referred to the Greifswald laboratory are positive in the washed platelet activation assay (HIPA test), but negative for IgG antibodies in the PF4/heparin ELISA. Results of seven discrepant sera (HIPA positive but PF4/heparin ELISA) were further investigated—after IgG depeletion, the HIPA test became nonreactive. This suggests that an IgG against another (non-PF4/heparin) antigen was likely present. The data further suggested that ELISA OD titers are useful for predicting risk of HIT. There is also information that IgA and IgM antibodies do not interfere with the platelet activation assay. The conclusion is that the “combination of a washed functional (platelet activation) assay and an antigen assay is still the gold standard.”

T. Warkentin, A. Greinacher, Y. Gruel, B. Chong: Proposal for HIT scoring system. The historical context of scoring systems for HIT was presented. Published evaluations of the ‘4 T’s’ clinical scoring system were shown. It is evident that this scoring system has a high negative predictive value, although the positive predictive value varies among different clinical settings. The working party on HIT is preparing a manuscript dealing with this scoring system.
Platelet Physiology

Chairman: A. Michelson, USA
Co-chairs: J. Bennett, USA; M. Cattaneo, Italy; C. Cerletti, Italy; C. Hayward, Canada; D. Kenny, Ireland; J. Lopez, USA; D. Nugent, USA; P. Nurden, France; S. Watson, UK

The meeting focused on the standardization of the measurement of platelet function in patients. The topics and speakers were:

- turbidometric platelet aggregometry (Lisa Jennings, Memphis, U.S.A.)
- whole blood platelet aggregometry (Stan Heptinstall, Nottingham, U.K.)
- VerifyNow (Rob Hillman, San Diego, U.S.A.)
- platelet function analyzer (PFA)-100 (Catherine Hayward, Hamilton, Canada)
- impact cone and plate(let) analyzer (David Varon, Jerusalem, Israel)
- flow cytometry (Alan Michelson, Worcester, U.S.A.)
- thromboxane metabolites (Paola Patrignani, Chieti, Italy)
- other methods (Paul Harrison, Oxford, U.K.)

Official publications of the SSC Platelet Physiology Subcommittee in the past year were:


Registries of the SSC Platelet Physiology Subcommittee are:

- Bernard-Soulier Syndrome (Dermot Kenny)
- Glanzmann Thrombasthenia (Debbie French)
- Non-immune Thrombocytopenia (Amy Geddis/Jim Bussel)

It was resolved to form a Working Group on Platelet Aggregation for the purpose of attempting to gain consensus on how to measure platelet aggregation in the clinical laboratory.

Alan D. Michelson, M.D.
Chair, Platelet Physiology Subcommittee, SSC/ISTH
Predictive Variables in Cardiovascular Disease

Chairman: P.J. Grant, UK
Co-Chairs: G. Lowe, UK; V. Salomaa, Finland; A. Tosetto, Italy

Around 50 people attended the session

1. **Professor Peter Grant** (Leeds, UK), as outgoing chairman, introduced the meeting by summarising some of the activities of the sub-committee and by providing a brief overview of the complexities of the cardiovascular disease phenotype.

2. **Professor Niko Marx** (Ulm, Germany) presented data on the role of various circulating and cellular components of the inflammatory cascade in the pathogenesis of cardiovascular disease. A general discussion followed on the importance of inflammation in these processes and the evidence as to whether inflammation was causative or an ‘innocent bystander’.

3. **Professor Gordon Lowe** (Glasgow, UK) presented a review of meta-analyses of haemostatic variables and risk of coronary heart disease. The Fibrinogen Studies Collaboration has established that fibrinogen shows an independent association. Weaker associations had been established for von Willebrand factor, fibrin D-dimer, t-PA antigen, Factor V Leiden and the prothrombin mutation. To date, there were no significant associations reported for other haemostatic SNP’s, hence further studies of haplotypes might be preferred.

4. **Dr Angela Carter** (Leeds, UK) presented a talk on “The Potential of Proteomic Technologies to Discover Novel Risk Factors for Acute Coronary Syndromes”. This talk followed on from previous presentations at the SSC discussing the application of proteomics for identification of predictive variables for cardiovascular disease. The presentation focussed on recent work from Leeds to establish methods for analysing the protein components of ex-vivo formed plasma clots and FXIII substrates. Data were presented from these studies to show that the methodologies employed had so far identified both known and novel clot components. Novel clot components and FXIII substrates included complement proteins, lipoproteins and proteins involved in iron homeostasis. Discussion followed about the details of methodology and the relevance of the novel proteins identified in the context of cardiovascular disease, particularly in light of earlier presentations on the validity of more established inflammatory and haemostatic cardiovascular risk factors. Broader discussions about the future application of proteomics to cardiovascular research also took place.

5. **Dr Moniek de Maat**, (Rotterdam, The Netherlands) The genetics of Cardiovascular Disease: SNP and Haplotype Analysis

6. **Dr Licia Iacoviello**, Campobasso, Italy The genetics of Cardiovascular Disease: Where Next?

Both these talks focussed on the potential for genetics in understanding the pathogenesis of coronary artery disease. Both presentations agreed that the use of haplotype analysis provided greater opportunities for rigorous data than SNP analysis alone and some of the theoretical background to the use of haplotype analysis was presented. A lively discussion ensued around
the topic of the clinical utility of genetic analysis and the areas of research in which it would provide valuable information.

Summary

Professor Grant summarised the meeting and concluded by stepping down as the current SSC chairman
Von Willebrand Factor

Chairman: A. B. Federici, Italy
Co-chairs: T. Abshire, USA; G. Castaman, Italy; J. Di Paola, USA; J. Eikenboom, The Netherlands; E. Favaloro, Australia; A. Goodeve, UK; D. Lillicrap, Canada; R. Schneppenheim, Germany

Summary of Approvals and Working Parties:

1. Final report of the WP on VWF Assays in VWF in VWD Diagnosis; data to be reported on line www.vwfassays-in-vwd.com (C.A. Lee & A. Hubbard)
2. Continuation of the WP on Standardization of Multimeric Analysis, with more laboratories (U. Budde and C. Mazurier)
3. Continuation of the WP on the use of suitable reagents for VWF:CB (collagen binding assay) has been approved (L. De Marco, E. Favaloro, A. Hubbard, R. Seitz)
4. Final report of the WP on VWD classification; publication submitted to JTH (E. Sadler & the panel of VWD experts)
5. Final report of the WP on Standardization of methods for mutation and expression studies will continue (A. Goodeve, L. Hilbert, D. Lillicrap, R. Schneppenheim)
6. Progress report of the WP on development of new improved assays for ADAMTS-13 (R. Schneppenheim)
7. Progress report of the WP on requirements for shear-stress related VWF assays to be used in clinical diagnosis of VWD (Z.M. Ruggeri & A. Reininger)
8. Progress report of the International Prospective observational study on Biological response and clinical efficacy of DDAVP in VWD type 1 and 2 with reported data directly on line into the web site www.ddavp-in-vwd.com (A.B. Federici, G. Castaman, S. Lethagen)
10. New WP on pre-analytical variables in VWD diagnosis (U. Budde & E. Favaloro)
11. New WP on the use of prophylaxis in VWD (T. Abshire & E. Berntorp)
12. New WP on the clinical & molecular markers of VWD type 3 with frequency of alloantibodies: a joint project of ISTH-SSC and WFH (A.B. Federici & P. Giangrande)

The VWF Subcommittee was attended by about 160 attendees at the first session and by about 130 at the second session. They actively participated in the discussion of the various topics provided in the program. The following represents the minutes of the program.

1) WP on VWF Assays (J. Eikenboom, The Netherlands)
Final report of the Working Party on VWF assays in VWD

The organization of the study was reported by Dr C.A. Lee. Thirty two laboratories from 17 countries have participated in a study to evaluate VWF assays in the diagnosis of von Willebrand Disease (VWD) with the objective of determining the best diagnostic repertoire for VWD. Each laboratory received 11 or 12 individually coded samples comprising 6 VWD patient samples
with known genotype (types 1/2N, 2A, 2B, 2M, 2M smear, 2N) and 2 normal control samples (Group O and non-Group O) with 3 or 4 coded duplicate samples. All plasma samples were lyophilized in 0.5 ml aliquots and comparisons with frozen aliquots have indicated that the multimeric profile has not been affected by the lyophilization procedure. Laboratories were requested to perform the following 5 assay methods: VWF:Ag, VWF:RCo, VWF:CB, VWF:FVIIIB and multimeric analysis. Samples were dispatched in May/June 2005 and results were returned by November 2005. Laboratories returned details of methodology together with a diagnosis and supporting laboratory data for each sample. The results have been decoded, sorted according to sample and dispatched for analysis by expert laboratories designated for each method.

Dr K. Friedman reported on the VWF:Ag assay. A diversity of reference calibrators was used (3rd-5th WHO standard). There was also variation in the reference ranges used (population based or ABO blood group stratified), in the size of the population surveyed for range definition, and in the lower limit of the range. Furthermore, the use of concordance ratios is unreliable when the VWF:Ag is low.

Dr E.J. Favaloro reported on the VWF:CB assay. The VWF:CB assay is effective in identifying loss of HMW multimers associated with type 2A and 2B VWD. VWF:CB (especially type I/III mixture) collagen based) is in this respect better than VWF:RCo. The results are, however, dependent on the source of the collagen used. VWF:RCo is better in identifying type 2M with a functional defect. VWF:CB does not replace VWF:RCo, but could possibly replace the need for multimers in selected cases.

Dr C.A. Sabin reported on the statistical aspects of the study. The diagnostic accuracy was high for 2A, 2N and normal individuals, but poor for 1/2N compound heterozygote, 2B, 2M and 2M smear individuals. The diagnostic accuracy was higher in the labs performing all assays. There was a good agreement (kappa value) between repeated diagnosis, however this is a measure of agreement and not of accuracy as consistently incorrect diagnoses give a high kappa value. Additional information was provided by Dr. Budde and Castaman on VWF Multimers and VWF:RCo assays.

VWF collagen binding versus VWF:RCo activities

Dr. A.B. Federici informed the audience that Dr. De Marco could not attend but he is still working on the isolation and characterization of human collagen type VI; he probably will report on this issue in Geneva. Dr. Federici presented data on the relationships between VWF:CB and VWF:RCo in a sample population of patients with VWD types 2A, 2B, 2M followed at the Angelo Bianchi Bonomi Hemophilia Thrombosis Center of Milan, all characterized by RIPA and by specific mutations. Patients with VWD type 2M showed RIPA > 1.2 mg/ml, VWF:RCo/Ag < 0.7 VWF:CB/Ag > 0.7; patients with VWD type 2B showed RIPA < 0.8 mg/ml, VWF:RCo/Ag < 0.7 VWF:CB/Ag < 0.7; patients with VWD type 2A showed RIPA > 1.2 mg/ml, VWF:RCo/Ag < 0.7 VWF:CB/Ag < 0.7. Collagen type I seems more sensitive than collagen type III in this setting of experiments. Therefore, differential diagnosis of VWD 2A, 2B, 2M can be obtained by using these three tests together.
Conclusions. After discussion, there was agreement to publish the data of the WP on VWF assay in VWD diagnosis in a peer review Ms (future submission to JTH after presentation for formal approval by the ISTH-SSC on VWF in Geneva) and to go on with the WP on multimers and on collagen to standardize the minimal requirements and the appropriate collagen reagents for these two assays.

2) Proposal of WP on standardization on pre-analytical variables in VWF assays (G. Castaman)

Dr U. Budde and E. Favaloro presented data on the role of different temperature and processing-freezing-thawing of blood/plasma samples on VWF:RCo, VWF:CB and VWF multimers. After discussion there was a consensus that it is important to provide written recommendations about the best procedure to process blood and to prepare and store plasma samples to be devoted to VWF tests.

3) Final report of the WP on VWD Classification (David Lillicrap, Canada)

The final proposal for an updated VWD classification was presented by Evan Sadler and was formally approved by a unanimous vote Dr. Sadler will submit the Ms to JTH within two weeks.

4) Progress report of the WP on Molecular Biology and Expression Study (Anne Goodeve, UK)

Dr. L. Hilbert presented results of a survey; SSC/ISTH Working Party on VWF Molecular Biology and Expression Studies. The objective of this Working Party is to make a survey on the methods used for the identification and expression of von Willebrand disease (VWD) mutations. In 2005, 11 laboratories involved in 2 multicenter studies on type 1 VWD (European MCMDM-1VWD and Canadian projects) filled out a four page questionnaire to collect data on methodology. The major results from this pilot survey were presented during the SSC meeting in Sydney. A second questionnaire, with additional questions on both transient and stable transfection, was then sent not only to the participants from the pilot survey, but also to 21 other laboratories who had published data in this field. 22 laboratories from 15 countries responded and 19 laboratories completed the questionnaire. This international survey allows recommendations to be made on the conditions for the identification of a von Willebrand factor (VWF) mutation and the construction of a mutated expression vector. At present, no recommendations may be drawn for stable transfection because little data (4) is available. The 12 responses concerning transient transfection of VWF were heterogenous, so new participants must be recruited, and further discussion must take place before the best conditions are fixed. All interested parties, please contact hilbert@lfb.fr to participate. Dr. Hilbert will prepare a written report to be published as SSC Communication

Dr. S. Haberichter presented VWF pro-peptide assay for identification of type 1 VWD patients with decreased VWF survival. The decreased survival of VWF in plasma has recently been identified as a novel mechanism for type 1 VWD. We report four families with moderately severe type 1 VWD characterized by low plasma VWF:Ag and FVIII:C levels, proportionately low VWF:RCo, and dominant inheritance. A decreased survival of VWF in affected individuals
was identified with VWF half-lives of 1-3 hours, while the half-life of VWF propeptide (VWFpp) was normal. DNA sequencing revealed a single (heterozygous) VWF mutation in affected individuals, S2179F in two families, and W1144G in two families. We report preliminary data establishing the normal range of VWFpp and VWFpp/VWF:Ag for each blood group. We demonstrate that the ratio of steady-state plasma VWFpp/VWF:Ag can be utilized to identify patients with a shortened VWF half-life. An increased ratio distinguished affected from unaffected individuals in all families. A significantly increased VWFpp/VWF:Ag ratio together with reduced VWF:Ag may indicate the presence of a true genetic defect and decreased VWF survival phenotype. This phenotype may require an altered clinical therapeutic approach and we propose to refer to this phenotype as type-1C VWD.

Proposal for the future work of this WP; Molecular screening recommendations in type 1 VWD

Discussants: A Goodeve, D Lillicrap, P Collins

Dr. P. Collins presented the UK Haemophilia Centre Doctors’ Organization investigation into the molecular pathogenesis of type 1 VWD. Of 40 families diagnosed by UK Haemophilia Centers to have type 1 VWD 8 (20%) families were found not to have type 1 VWD following review. Six families were re-diagnosed to have type 2 VWD, 1 family was found to have a platelet storage pool disorder, and 1 family was determined to be unaffected. In the remaining 32 families direct DNA sequencing of the essential regions of the VWF gene identified a total of 11 different candidate mutations in index cases of 20 of the 32 confirmed type 1 VWD families. These included R1205H (4 families), Y1584C (8 families), R924Q (3 families), 2 other missense changes, 4 candidate splice site mutations and a single nt deletion. No candidate VWF gene mutation was identified in 12 of the 32 type 1 VWD index cases (38%). Linkage analysis showed that in 13 of the 32 families it was likely that VWD segregated with the VWF gene. In 8 families, VWD did not segregate with the VWF gene. Y1584C was present in 8 families but did not co-segregate with VWD in all cases. It was associated with blood group O in 95% of cases. In the rest of the cohort 70% of patients were blood group O.

Dr. A. Goodeve presented results of the MCMDM-1VWD study. 150 families with type 1 VWD were analyzed for mutations, and were identified in 105 (70%). A total of 124 candidate mutations were identified, of which 75 were different and of which 55 were novel. 18 (12%) index cases had 2 of more candidate mutations. 80% of the mutations were missense alterations, and these were located throughout VWF. Several changes were recurrent; R854Q (5 cases), R924Q (4 cases), C1130F/G/R (7 cases), R1205H (10 cases), R1374C/H (6 cases) and Y1584C (13 cases). Blood group O contributed significantly to low VWF, being present in 65% of index cases. In families lacking an identified mutation, blood group O was present in 78%, and in 95% of 19 families lacking a mutation and demonstrating non-linkage to VWF. Only a small proportion of families had candidate mutations demonstrating fully penetrant VWD.

Dr. D. Lillicrap presented a summary of the Type 1 VWD Canadian Cohort Study. One hundred and twenty three index cases and their families were studied and putative mutations identified within the VWF gene in 63% (n=78) of index cases leaving 37% (n=45) with no identified changes. These changes comprised 50 different putative mutations; 31 (62%) missense mutations, 8 (16%) changes involving the VWF transcriptional regulatory region, 5 (10%) small deletions/insertions, 5 (10%) splicing consensus sequence mutations, and 1 nonsense mutation.
Twenty-one of the index cases had more than one putative VWF mutation identified. Although a total of 50 different mutations were identified in this study, twelve of the mutations occurred in multiple index cases. These twelve mutations were found in 63 (51%) of the index cases and, with one exception, these were missense mutations.

Presenters suggested that mutation screening of the VWF gene has limited general utility in genetic diagnostic and family studies in type 1 VWD. If genetic studies are performed, the incomplete penetrance and variable expressivity of type 1 VWD must be taken into account. Unless linkage of VWD phenotype with the VWF gene can be clearly demonstrated, the results of any genetic family studies should be interpreted with caution.

5) Progress report on Shear-stress Assays in VWD (Emmanuel Favaloro, Australia)

Dr. Z. M. Ruggeri discussed the general concepts for the use of shear-stress tests as a measurement of VWF function. He described the most recent data about the basic mechanisms on VWF-platelet interactions in different shear-rates conditions, from relatively low to high shear rates and about the role of VWF with different multimers on platelet adhesion (only dimeric VWF A1 domain) and platelet-platelet interactions (all the VWF multimers). He is positive about future applications of these methods in a more clinical setting to determine VWF defects in patients with VWD and suggest to make a joint WP with the SSC on biorheology.

Dr. A. Reininger in his presentation suggested that future diagnostic tools for the clinical application in VWD patients building on these new insights may therefore include various blood flow assays at elevated shear rates, and – although complicated – direct, real-time visualization and quantification.

Conclusion: Contacts with the SSC on Biorheology and vascular biology should be organized to make final statements about the application of these tests on VWD diagnosis

6) WP on ADAMTS-13 Assays (Reinhard Schneppenheim, Germany)

Dr. R. Schneppenheim organized a very interesting session with presentation of the most recent work of several laboratories actively working on ADAMTS-13 assays.

a. Comparison of FRETS-VWF73 to full-length VWF as a substrate for ADAMTS13 activity measurement in human plasma samples presented by Dr A Veyradier

The FRETS-VWF73 fluorescence assay was compared with a home made IRMA using full-length VWF for the measurement of ADAMTS13 activity in plasma samples from a cohort of 64 patients with thrombotic microangiopathies (TMA) including 41 acquired thrombotic thrombocytopenic purpura (TTP), 3 inherited TTP and 20 hemolytic uremic syndrome (HUS) and also from 20 normal subjects. Both methods were correlated (y = 0.94x + 1.71, correlation coefficient 0.97). With both methods, all normal subjects and most HUS patients showed ADAMTS13 activity higher than 50% although all TTP patients exhibited an ADAMTS13 activity lower than 5%. Compared to the IRMA method, the FRETS-VWF73 assay exhibits reliable results to measure low, moderately reduced as well as normal levels of ADAMTS13.
activity. Accuracy and reproducibility of the assay combined with its short incubation time make the method highly attractive and appropriate for the clinical screening of patients with TMA.

b. ADAMTS13 activity measurement: comparison of FRETS-VWF73 and other static assays presented by JA Kremer-Hovinga

Comparison of a slightly modified FRETS-VWF73 assay with older static assays using a full-length VWF substrate showed a good agreement between the ADAMTS-13 activity determined by FRETS-VWF73 and the older assays (Kremer Hovinga, Mottin and Lämmle, J Thromb Haemost. 2006;4:1146-8). However, the presence of hemoglobin in a patient’s sample constituted a problem in the FRETS-VWF73 assay and preliminary data indicate that discrepancies in ADAMTS-13 activity between different assays may be observed in rare cases of acute thrombotic microangiopathies.

c. Japanese experience of novel ADAMTS13 activity-ELISA on patients with TMA and liver transplantation, presented by Dr M. Matsumoto

The group developed a convenient and highly sensitive ELISA for ADAMTS13 activity. This ELISA precisely determined the half-life of infused plasma ADAMTS13 activity in USS patients to be between 2.3 and 3.5 days. A rapid fall in the level of ADAMTS13 activity after liver transplantation was found. Platelet transfusions in respective patients are therefore not recommended, but plasma infusions to supply ADAMTS13 after liver transplantation.

d. ADAMTS13 activities and genetic polymorphisms in the Japanese general population, presented by Dr. K. Kokame

Plasma ADAMTS13 activities in the Japanese general population (N = 3,616) were measured by the FRETS-VWF73 assay. The values did not show a Gaussian distribution, suggesting that some kind of factor should affect the activity. The activities in males were lower than those in females. In both genders, the activities became lower with age. The plasma VWF level was not related to the ADAMTS13 activity. Comprehensive search revealed that Japanese people have six common missense polymorphisms. The P475S polymorphism significantly lowered plasma ADAMTS13 activity.

e. Biosynthesis, secretion and regulation of ADAMTS13 protease in human vascular endothelial cells-relevant to TTP presented by Dr X. Long Zheng

Zheng and his colleagues have shown that ADAMTS13 is synthesized and secreted from arterial, venous and microvascular endothelial cells; ADAMTS13 is secreted apically in polarized endothelial and epithelial cells; the apical secretion of ADAMTS13 depends on the CUB domains and their association with lipid rafts. Mutations in the CUB domains of ADAMTS13 may disrupt the signal directing polarized secretion, thereby leading to congenital ADAMTS13 deficiency and thrombotic thrombocytopenic purpura. Our data suggest that ADAMTS13 produced in the vascular endothelial cells may contribute significantly to the plasma levels of ADAMTS13 protease.
f. Podocytes express ADAMTS13 in normal renal cortex and in a patient with TTP presented by Dr. M. Manea

This study showed intracellular ADAMTS13 expression in podocytes in normal renal tissue and in the kidney of a TTP patient with the compound heterozygous mutation P353L and P457L. Expression of the mutants revealed impaired secretion and activity. Podocyte-derived ADAMTS13 may serve as a local protective mechanism in the high-shear microcirculation of the glomerulus. Deficient activity could result in the formation of platelet plugs in the glomeruli as seen in TTP.

g. Action of recombinant ADAMTS13 on recombinant mutant VWF: VWF type 2A with decreased proteolysis and VWF type 2B with enhanced proteolysis presented by R. Schneppenheim

The enhanced proteolysis of VWF in patients with classic VWD type IIA is due to an intrinsic susceptibility of mutant VWF to ADAMTS13. VWF type 2B is proteolyzed to a similar extent, however the underlying mechanism has not been experimentally elucidated. The results of a study on ADAMTS13 proteolysis of recombinant VWF 2B and VWF IIE, the latter being a phenotype with decreased proteolysis suggest that in a static assay both mutants are proteolyzed roughly normal. Therefore, the different phenotypes are most probably due to different function under flow conditions: enhanced proteolysis of VWF type 2B as a result of enhanced affinity to platelets and decreased proteolysis of VWF IIE due to its poor functional activity in platelet dependent hemostasis, respectively.

h. Could Chloride binding to VWF explain the occurrence of TTP in subjects with normal levels of ADAMTS13? presented by Dr. R. De Cristoforo

Physiological concentrations of NaCl inhibit the hydrolysis of VWF by ADAMTS-13. It was demonstrated that chloride ions specifically bind to the A1 domain of VWF. Cl- ions stabilize a “super-folded” conformation of VWF through binding to the A1 domain. The affinity of Cl- for the VWD R1306 mutant was reduced and its hydrolysis by ADAMTS-13 accelerated. These findings showed that Cl- ions control allosterically the availability of the Y1605-M1606 bond to the proteolytic attack of ADAMTS-13.

After a fruitful discussion among the participants and the audience, Dr. Schneppenheim proposed issues of ADAMTS13 and VWF of current interest:

a. to define the ideal ADAMTS13 functional assay to be equally applicable for individual samples and for large cohorts of patients, respectively
b. to further characterize ADAMTS13 with respect to i) other substrates apart from VWF, ii) the biological significance of alternative ADAMTS13 splice products, iii) its local distribution in different tissues and cells
c. the role of ADAMTS13 in conditions like sepsis, stem cell transplantation and other microangiopathies
d. therapeutic issues like the development of rhuADAMTS13
7) Progress report on WP on Acquired von Willebrand syndrome (J. Di Paola, USA) Dr U. Budde presented the technical problems on the measurements of auto-antibodies in AVWS: he showed that the use of plasma-derived or recombinant VWF used to capture auto-antibodies from the patients serum or plasma can be critical for the assay due to the role of blood groups. He proposes to use recombinant VWF from insects. Dr. C Mazurier proposes to use plasma-derived VWF from donors with blood group O.

Dr. K. Mertens, on behalf of Dr. J van Mourik, reported on the significance of the measurement of the plasma concentration of the pro-peptide of VWF (VWAgII) in the diagnosis of acquired von Willebrand syndrome (AVWS). An increased steady state pro-peptide/VWF ratio is clearly indicative of decreased survival of mature VWF, as encountered in AVWS or certain congenital VWF defects, and useful as an index to assist in the diagnosis of abnormal VWF catabolism. The molar concentration of plasma pro-peptide was assessed by calibrating pooled normal plasma against recombinant pro-peptide purified to homogeneity. This approach allows straightforward standardization of pro-peptide assays.

Dr R.R. Montgomery reported his experience with his assay with VWF pro-peptide that was tested together with native VWF:Ag in normal individuals with different blood groups. This assay will be commercially available soon. After a general discussion, a standardization of these assays was proposed for the use in AVWS and also in patients with different forms of inherited VWD.

8) Progress reports and new proposal of WP and clinical surveys (T. Abshire, USA)

Dr. G. Castaman presented the progress report of the WP on Biological response versus clinical efficacy of DDAVP in VWD type 1 and 2; 128 patients have been enrolled. The dead line for enrollment is postponed to 31st December 2006. The study is recruiting patients through the web site – www.ddavp.in.vwd.com.

Dr. A.B. Federici proposed a new WP on the clinical & molecular markers of VWD type 3 with frequency of alloantibodies to be organized as a joint project of ISTH-SSC and WFH.

Dr. T. Abshire proposed a new WP on the use of prophylaxis in VWD. This project is composed by a retrospective survey and by a prospective study on the use of different VWF concentrates in the secondary long-term prophylaxis of hemarthrosis, epistaxis and G I bleeding in severe VWD patients with a VWF:RCo < 20 U/dL.

9) Other reports and proposals, concluding remarks (A. B. Federici, Italy)

Dr. A. B. Federici presented the concluding remarks of the entire two sessions, summarizing all studies and proposals to be reported next year in Geneva.
Women's Issues in Haemostasis and Thrombosis

Chair: Marilyn Manco-Johnson

Attendance: approximately 80 people attended the meeting

Manuscripts:

Recommendations for testing women for hemostasis: awaiting reviews and approvals

Proposal for a scoring system for thrombophilia and pregnancy outcome in women to be applied to prospective outcome studies

Dr. Brenner will submit his analysis of the scale derivation in 94 Israeli women to JTH as an independent manuscript. The scale will then be placed on the web as a WP project for validation in a multinational study. The strong predictive value of previous thrombosis which is incorporated in this scale was supported by evidence presented by Dr. Conard in women with AT deficiency. The collaborative multicenter study validating the obstetrical scoring system will hopefully be submitted as a recommendation paper from this subcommittee.

Dr. Blomback presented data on correlates of recurrent miscarriage in women. She determined overweight body habitus and obesity predisposing to secondary miscarriage, and certain thrombophilias predisposing to primary miscarriage. The inclusion of recurrent early miscarriage in a scale of maternal risk factors was agreed upon.

Recommendations for evaluation and management of menorrhagia in women.

This manuscript will be reviewed for submission as an SSC publication.

It was discussed that a detailed descriptive study of women with bleeding disorders is currently funded by the US CDC and preliminary data will be presented to this Subcommittee next year. The US data will characterize women with bleeding disorders who are referred to a hemostasis center.

Dr. Shoshana Revel-Vilk will develop a community-based study of adolescents with menorrhagia in order to determine whether predictors for a bleeding disorder can be applied to an adolescent population to increase the yield of coagulation screening. She can be contacted at shoshanav@hadassah.org.il.

Registries:

Pregnancy in women with mechanical heart valves. A James, USA
Dr. James presented a literature review of pregnancy outcome in 76 women with mechanical heart valves as a background to the current registry. Her review is currently in press (J Mat Fetal and Neonatal Med, 2006). The prospective registry is up and running. There have been three case entries and several other centers are in the process of obtaining ethics approvals. It is anticipated that results will be presented to the Geneva SSC meeting.

Pregnancy in women with genetic antithrombin deficiency. **J Conard, France**

This registry is on the web and now includes 51 women from several centers in France and one other country. Participants were urged to join the registry. An update will be presented in Geneva. Dr. Conard may be contacted at jacqueline.conard@htd.ap-hop-paris.fr

Outcome of ovarian hyperstimulation syndrome. **B Konkle, USA.**

This registry is near completion and will be on the ISTH website along with sample protocol and ethics consent forms in the near future.

New Business

Diagnosis of Pulmonary embolism in pregnancy: **Matilde Nijkeuter, The Netherlands**

In this proposal pregnant women with clinically suspected pulmonary emboli will be studied with helical CT and if the study is negative, anticoagulation will be withheld and the woman will be followed for three months in order to determine the safety of the approach. This multicenter study has been initiated in the Netherlands and has enrolled 20 cases. From 100 to 400 cases will be needed to determine the safety of withholding anticoagulation from pregnant women with normal findings on helical CT, based upon an expected rate of positive PE ranging from 5 to 20%. There was some controversy expressed regarding the use of CT in pregnancy and the difficulty to enroll adequate numbers. Nonetheless, the study is endorsed by the subcommittee and potential participants are encouraged to contact Dr. Nijkeuter at Matilde.nijkeuter@lumc.nl.

Proposal for a registry on Mirena IUD in women with menorrhagia: **Rezan Kadir, UK**

Dr. Kadir presented data regarding use of the Mirena IUD to control menorrhagia in women as a whole, and in subsets of women with confirmed bleeding disorders. While the Mirena IUD has been generally effective and safe, its use in women with bleeding disorders may be accompanied by a higher rate of break-through bleeding and side effects resulting in discontinuation. Response to the registry was positive and it will be posted on the ISTH website. Dr. Kadir may be contacted at rezan.abdul-kadir@royalfree.nhs.uk.

There was a lively discussion provoked by Dr. James presentation on pregnancy in women with mechanical valves relating to the use and requirements for monitoring LMWH during pregnancy. It was decided to form a WP on anti-Xa activity monitoring during pregnancy. Dr. Charlie Hay of the UK, Dr. Aima of Greece and Dr. Sandy Duncan of the US volunteered to organize the WP. They may be contacted at: haemophilia@man.ac.uk; aima09@hippocratio.gr and aduncan@emory.edu.
Dr. Manco-Johnson suggested that the current recommendation not to test asymptomatic women prior to use of oral contraceptives or hormonal replacements may be modified if there were a way of improving risk prediction for hormonal-related DVT. Dr. Brenner proposed a study using global assays to predict risk of thrombosis in asymptomatic women presenting for oral contraception or hormone replacement therapy. A lively discussion ensued and it was decided to invite Dr. Rossing and others to speak further on this at the Geneva meeting.

Dr. Manco-Johnson suggested inclusion of neonatal thrombotic disorders including neonatal stroke in the registry of Dr. Brenner to expand detection of potential perinatal risks conferred by thrombophilia or maternal thrombosis. It was decided to embark on this as a joint activity with the Perinatal and Pediatric Subcommittee with Dr. Manco-Johnson acting as a liaison with Dr. Gili Kenet of Israel. Dr. Manco-Johnson can be contacted at marilyn.manco-johnson@uchsc.edu.
Working Group on Coagulation Standards

Co-Chairs: Jane Lenahan and Tony Hubbard

Status of Lot #2

Dr Hubbard reviewed the despatch of Lot #2 from May 2001 up to expiry at the end of June 2006. Lot #2 has been used steadily over the past 5 years and a total of 43,700 vial have been despatched to 41 different organisations in 12 different countries. Approximately 22,000 vials have been used in QA surveys by UK NEQAS and CAP. There are approximately 1100 vials of Lot #2 remaining.

Assessment of the stability of Lot #2 has been carried out using three different approaches:

- accelerated degradation study over 6 years on FVII and FVIII activity has produced robust mean predictions of loss which did not exceed 0.011% per year for vials stored at -20 °C,
- a real-time comparison of FVII and FVIII activity in vials stored at -20 °C vs vials stored at -70 °C for 6 years has indicated no relative loss in the -20 °C vials,
- a comparison of estimates for Lot #2 obtained in the original calibration exercise (in 1999) and those obtained from re-assays during the calibration of Lot #3 (in 2005) has indicated no significant differences for any of the analytes.

The overall conclusion from this data is that Lot #2 has been extremely stable during its lifetime and we should expect similar stability for Lot #3 considering the similarity of its manufacture.

Calibration of Protein C for Lot #3

Dr Gray presented the calibration for Protein C which was carried out as part of the collaborative study for the proposed WHO 2nd IS Protein C Plasma. Combination of 25 estimates for Protein C function produced an overall mean of 0.89 IU/ml with low inter-laboratory variability (GCV) of 4.2 %. Combination of 9 estimates for Protein C antigen gave an overall mean of 0.89 IU/ml and inter-laboratory variability of only 2.2%. It was proposed that the value of 0.89 IU/vial be assigned to Lot #3 for both analytes. Calculation of estimates for Lot #3 relative to the proposed assigned values for the WHO 2nd IS produced identical mean estimates of 0.89 IU/vial for function and antigen. This indicates that the proposed assigned values for Lot #3 will not require re-assessment when the proposed WHO 2nd IS Protein C is established. Lot #2 was also included in the calibration exercise and there were no significant differences between the estimates obtained in the present study and the original calibration in 1999. This indicates that Lot #2 has been extremely stable over the last 6 years.

Status of Lot #3

Dr Hubbard presented details of the manufacture of Lot #3 followed by a summary of the calibration for 19 analytes which involved over 1000 separate assays and is now completed. Results from the initial stability testing, based on Factors V, VII, VIII and XI, have indicated FV
to be the most labile with a mean predicted % loss per year at -20 °C of 0.184%. An expiry date of “end December 2014” for Lot #3 was proposed; this was based on the upper 95% confidence limit of loss for FV which indicated a “shelf-life” of approximately 11 years. However, based on the current despatch of Lot #2 it is likely that stocks of Lot #3 will be exhausted by 2012.

A draft label for Lot #3 was presented which recommended storage between -20 to -40 °C; this was included to discourage storage in -80 °C freezers. However, it was noted that long-term studies on Lot #2 had indicated no loss of activity at -70 °C. The Working Group agreed that the recommended storage temperature should be amended to -20 to -70 °C.

A draft “Instructions For Use” for Lot #3 was presented. This includes details of the manufacturing together with information on storage and reconstitution as well as “Caution”, “Liability” and “Safety” sections. The “Customer Feedback Form” will also be included as an integral part of the IFU. The final version will include both NIBSC and ISTH logos.

The minimum order size for Lot #3 will remain at 100 vials and shipping will continue by courier at the cost of the customer. The price of Lot #3 will be $5 per vial. Labelling and despatch of Lot #3 is scheduled to commence in July 2006.

**Calibration of Lot #3 for tPA antigen**

Dr Longstaff presented the results of a collaborative study to evaluate the calibration of Lot #3 (and a potential new WHO IS, 94/730) for tPA antigen. Estimates for tPA in Lots #2 and #3, relative to kit standards, were associated with large inter-laboratory variability (GCV >60%) and this was slightly improved by the re-calculation of estimates relative to a common standard, 94/730. There were clearly reproducible differences between methods. However, the inter-laboratory variability of estimates for Lot #3 could be greatly reduced if estimates were recalculated using a correction factor for each different method. Despite the overall large inter-laboratory variability similar mean values of approximately 3 ng/ml and 25 ng/ml were obtained for Lot #3 and 94/730, respectively, in different studies. Dr Longstaff indicated that the proposal to calibrate 94/730 (and Lot #3) will be reviewed by the Fibrinolysis sub-committee. Pending this outcome the proposal to calibrate Lot #3 will be circulated to the Executive Board of the Working Group.

**Data from proficiency studies and use of Lot #3 as a support tool by EQA schemes**

Dr Kitchen presented data from EQA surveys on FVIII, FIX and Protein S estimation which demonstrated how inter-laboratory discrepancies can be linked to the use of different reference plasmas and particular reference plasma/method combinations. The inclusion of the SSC Standard as a test sample in EQA schemes not only provides information on the validity of the assigned values on the SSC Standard but also a check on the calibration of commercial calibrants. Tests on Lot #2 indicated significant differences between some commercial calibrant plasmas in the estimation of FV and FXI. Several EQA studies testing Lot #2 for FVIII:C have returned values higher than the assigned and this appears to be related to the failure of manufacturers to assimilate the re-alignment of the IU for FVIII:C which occurred with the establishment of the WHO 4th IS FVIII/VWF Plasma. These data indicate that the SSC
Standard is a very valuable tool in the resolution of potency estimation problems which may be related to inaccurately calibrated commercial references. It was proposed that a small amount of the SSC Standard (Lot #3) could be made available to QA organisers for use in problem-solving. The Working Group agreed to this use in principle, however, it will be necessary to explore the feasibility of this use with regard to the number of vials required and the logistics of despatch before approval can be sought from the ISTH Executive Committee.

**Lot #3 and the JCTLM database**

De Gray reviewed the terms of reference for the JCTLM Working Group which will allow Lot #3 to be registered as an “internationally certified reference material” and thereby be exempt from the requirement for CE marking. Lot #2 was nominated in Cycle I of the exercise and Lot #3 will be nominated in Cycle IV. Dr Gray indicated that evidence of commutability for Lot #3 may be required for all 19 analytes in the testing of patient samples as part of this process. This would be an enormous undertaking and Dr Gray will confirm the exact requirements.