58th Annual Scientific and Standardization Committee Meeting

Liverpool, UK
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Animal, Cellular, and Molecular Models of Thrombosis

29 June 2012

Chairman: Susan Smyth (USA)
Co-chairmen: Cecile Denis (France), Tom Knudsen (Denmark), Nigel Mackman (USA), Toshiyuki Miyata (Japan), David Motto (USA), Eva-Maria Muchitsch (Austria), Timothy Nichols (USA), Hugo Ten Cate (The Netherlands)

8:15 Tim Nichols (UNC-Chapel Hill): ARFI ultrasound method of detecting bleeding and documenting hemostasis in the hemophilic and VWD dogs

This presentation described a novel method for detecting hemostasis in dogs. Following a needle puncture injury, bleeding is monitored by acoustic radiation force impulse (ARFI) ultrasound, which allows for reproducible measurements of both the time to hemostasis and rate of hemorrhage. The model has been used to characterize bleeding and hemostasis in dogs with hemophilia A and in dogs with von Willebrand disease (VWD). In hemophilia A dogs, the time to achieving hemostasis is prolonged while rate is not affected. Conversely, in VWD dogs, the rate of hemorrhage following puncture is increased. The model has also been used to monitor the effects of gene therapy on bleeding and hemostasis.

8:45 Tom Knudsen (Novo Nordisk AS): Pharmacokinetics and pharmacodynamics of recombinant canine FVIIa in dogs

This presentation focused on differences between canine FactorVIIa and human FVIIa. Canine FVIIa and canine tissue factor were produced recombinantly. Canine-human cross-species FVIIa-TF interactions were described. Canine FVIIa displays more activity than human FVIIa. Moreover, while both human and canine FVIIa bind similarly to canine TF, canine FVIIa displays reduced ability to interact with human TF. Discussion centered around the implications of these findings and how differences may impact the ability to provide prophylaxis in dog models.

9:15 Henri Sprock (Maastricht University): pro-hemostatic agents in a pig model of coagulopathy

This presentation described use of a pig model of liver injury and blood loss to study therapeutic approaches to trauma-induce coagulopathy. The benefits of prothrombin complex concentrate (PPC) were discussed. PCC increases thrombin generation and reduces blood loss and mortality.

9:45 Eva-Maria Muchitsch (Baxter Innovations GmbH, Austria): assessment of thrombogenicity

The need for a standardized approach to studying thrombus formation was discussed. The Wessler test, which involves induction of venous stasis typically with a clamp and collection of clot, was used as an illustration. A proposal was presented to distribute a questionnaire to the community to collect information on current practices related to venous stasis models. The questionnaire will request information in the following areas: general questions, method-specific questions, thrombus fixation, and scoring of clot. The subcommittee voted to approve the questionnaire for general distribution and to
request its distribution to ISTH members. The information from the questionnaire will be reported in a Subcommittee publication along with recommendations to standardize the method. With this information, it may be possible to test the performance of a standardized model.

10:30 Hugo ten Cato (Maastricht University): Coagulant proteins in murine models of atherosclerosis

This presentation described thrombin potential at difference stages of atherosclerosis. Data was presented that the coagulation state correlates with extent of atherosclerosis. For example, thrombin-anti-thrombin complexes predict coronary artery calcium score. Additionally, genetic manipulations that result in a pro-coagulant phenotype are associated with accelerated atherosclerosis in mice. Finally, activated protein C and direct thrombin inhibition with dabigatran reduce accelerated atherosclerosis in cuff-injury models. The implications of these findings for therapeutic approaches to atherosclerosis was discussed.

11:00 Susan Smyth (University of Kentucky) Contributions of platelet secretion to coronary vascular remodeling

This presentation discussed the role of platelets and inflammatory cells in coronary artery remodeling. Contribution from cargo released by platelets, in particular, was suggested. Additionally, there was a discussion of the optimal methods for blood collection from mice to prevent ex vivo release of platelet granule content, as ex vivo release of granule content from platelets can artificially elevate plasma levels of proteins such as TGFβ1 and platelet factor 4 and potentially others.

11:30 Alexandra Schiviz (Baxter Innovations GmbH, Austria): Bleeding phenotype and coagulation variables of BALB/c, C57BL/6 and 129S1/Sv mice

This presentation reviewed the literature that supports genetic influence of the bleeding phenotype in hemophilia in humans and animal models. A comparison of the phenotype observed in the absence of FactorVIII in different genetic backgrounds of mice was presented. A comparison of bleeding times following tail-tip loss in wild-type mice of different genetic backgrounds (strains) was provided. The ability to predict bleeding times with thromboelastogram, performed on samples of blood collected from inferior vena cava, was also presented. The discussion focused attention on the influence of genetic background on bleeding.

The presentations were followed by planning for the 59th Annual SSC Meeting with XXIV ISTH Congress.
Biorheology

29 June 2012

Chairman: Michael R. King (US)
Co-chairmen: Lawrence Brass (USA), Shaun Jackson (Australia), Owen McCarty (USA), Keith Neeves (USA), Armin Reininger (Switzerland), Misuhiko Sugimoto (Japan)

8:00-8:05 Michael King (Cornell Univ., USA): Welcome and Opening Comments

Part 1: In Vitro Assays of Thrombosis and Haemostasis Under Flow (Moderator: M. King)

8:05-8:25 Armin Reininger (Baxter Healthcare, Switzerland): Platelet aggregates as prerequisite for fibrin polymerization under flow.

Insight into the initial mechanism of arterial thrombus formation induced by vulnerable human atherosclerotic plaques can help to improve current antithrombotic strategies. Rupture of atherosclerotic plaques causes arterial thrombus formation that might lead to myocardial infarction and ischemic stroke. Atherothrombosis is considered as an inseparable tangle of platelet activation and coagulation processes, involving plaque components such as tissue factor (TF) and collagen as well as blood-borne TF and coagulation factor Xlla (FXIIa). A combination of anticoagulants and antiplatelet agents is the present treatment.

Human atheromatous plaque material exposed to blood under flow conditions and at physiological calcium/magnesium concentrations showed that plaque induced thrombus formation occurred in two discrete steps. The rapid first phase of platelet receptor GPVI-mediated platelet adhesion and aggregation onto plaque collagen occurred within 1 min. The second phase of coagulation started after a delay of more than 3 min with the formation of thrombin and fibrin, and was driven by plaque TF. Coagulation under flow conditions occurred only in flow niches provided by platelet aggregates, with no evidence for a role of blood-borne TF and FXIIa. Inhibition of platelet GPVI but not of plaque TF blocked plaque-induced thrombus formation. Thus, plaque seem to foster thrombus formation by first inducing platelet activation via collagen, followed minutes later by TF-induced coagulation occurring on the activated platelet surface.

Discussion focused on the preparation (freezing) of plaque and TF materials, use of corn trypsin inhibitor, and radial formation of fibers in the presence/absence of flow.


Using an in vitro perfusion chamber system combined with confocal laser scanning microscopy, fibrin generation was evaluated within platelet thrombi generated under whole blood flow conditions. The extent of intra-thrombus fibrin generation, detected by fluorescently-labeled anti-fibrin specific monoclonal antibody, was evaluated as a fibrin/fibrinogen ratio. In perfusion of antibody-induced hemophilic whole blood, anti-hemophilic potentials of various (immobilized or soluble) clotting factor products can be precisely evaluated in vitro.
Discussion was on the absence of CTI, whether patient treatment conditions were recreated in the hemophilia model, and what is the significance of the immobilized VWF and FVIII.

8:45-9:05 Keith Neeves (Colorado School Mines, USA): Fibrin formation under flow correlates to FVIII levels in hemophilia A in a tissue factor-collagen flow assay.

A microfluidic TF-based flow assay was able to discriminate clinical phenotype, particularly for >1% FVIII. TF-based flow assay is more sensitive than than static assays.

Discussion was again asking about the absence of CTI in experiments, also the high TF concentration used. The audience was intrigued by the value of flow in assessing different patient risks, independent of F VIII concentration. The collagen density was discussed.

9:05-9:25 Owen McCarty (Oregon Health Science Univ., USA): Characterization of the physical parameters of mass, volume and density of platelet aggregates and thrombus formation.

We have developed a label-free imaging technique to characterize the volume and surface area coverage of platelet aggregates and thrombi formed under shear. Platelet aggregates were formed by perfusing anti-coagulated whole blood over fibrillar collagen. Thrombi were formed by perfusing calcified whole blood over fibrillar collagen in the presence of coagulation. Platelet aggregates and thrombi volume and surface area coverage were quantified using a Hilbert transform differential interference contrast (DIC) microscopy technique (HT-DIC). Our data show that platelet aggregates and thrombi formed at a shear rate of 200 s⁻¹ had similar volume and surface area coverage. At a shear rate of 1000 s⁻¹, both the volume and surface area coverage of platelet aggregates significantly increased as compared to low shear conditions. In contrast, the volume of thrombi formed in the presence of coagulation remained the same at both low and high shear rates. Utilization of this HT-DIC imaging technique can allow for insights into the composition of thrombi and the ability to determine composition fluctuations of thrombi under various conditions.

Discussion was on whether DIC can be used to quantify fibrin deposition, the presence of phospholipid (together with TF) on the surface, observation of fibrin alignment with flow, compared to previous speakers, and whether the Ab inhibitor also interferes with F XI.


The functions of endothelial cells, including those influencing haemostasis and inflammation, have been shown to be modulated by exposure to different levels or patterns of shear stress. Studies of responses to shear in vitro have utilised many devices, culture conditions, cell types and readouts (molecular or functional). Here, an overview of these methodologies and effects of variation in them will be discussed, in an attempt to define what variables or outcomes it might be possible or desirable to standardise.

Discussion was on whether blood was assayed on cultured endothelium, and complications involved in that. A question on transmigration with and without chemoattractant was also discussed.

**Part 2: Multiscale Modeling and High Throughput Assays of Thrombus Growth Under Flow**

(Moderators: A. Reininger and M. Sugimoto)

High throughput platelet phenotyping involves "pairwise agonist scanning" where a set of 74 calcium responses allowed training of a neural network (NN) model of platelet calcium mobilization for each individual donor. Each NN model was then imbedded into a kinetic Monte Carlo/finite element/lattice Boltzmann simulation of platelet deposition under flow. Simulations predicted the measured clot buildup dynamics for each donor for PPACK-treated whole blood flowing over collagen at 200 1/sec and 1000 1/sec wall shear rate in the presence of indomethacin, iloprost, or MRS-2917 (a P2Y1 inhibitor). This approach links platelet phenotype to overall dynamics within a clotting event under flow conditions.

Discussion was on the neural network training of the model to determine platelet adhesion parameters, and what is the form of the thrombin that is sensed with the new thrombin sensor, and also what is the hemacrit effect in the model.


Dr. Susanne de Witt presented on behalf of Dr. Johan Heemskerk data on a multi-substrate flow device, equipped for multi-parameters measurement of whole-blood thrombus formation at venous and arterial shear rates. She compared 42 different thrombogenic substrates, and used systems biology approaches to determine the requirements of an effective thrombogenic surface. She concluded that for full thrombus formation a surface needs to fulfill the following requirements: platelet adhesion via GPIb (high shear only), platelet adhesion via one of the integrins, and platelet activation via GPVI or CLEC-2.

Discussion was on whether the bioinformatics approach has implications for standardization, i.e., are there 3-4 critical readouts, or an ideal surface to use. A question on alpha2beta1 signaling was discussed. It was discussed whether spot location was randomized or not.


A structural and biophysical approach is essential to understanding the dynamic interplay between a receptor and its ligand. However, the ability to test hypotheses based on such studies in an appropriate biological system is key to not only understanding the clinical relevance of these findings but also for the development of computational models that more accurately simulate complex processes in a living animal. Mouse models have broadened our understanding of the role that platelet adhesion molecules and signaling pathways play in hemostasis and thrombosis. However, they have yet to be used to decipher the dynamic interplay amongst the biophysical properties, hydrodynamic force, and biology for a receptor-ligand pair critical for hemostasis and thrombosis: the human platelet receptor GPIb alpha and the human A1 domain of von Willebrand Factor (VWF-A1). We are now taking the next important steps in defining the biological relevance of this interaction by generating a mouse containing the human A1 domain to test hypotheses based on in vitro biophysical studies. We believe such studies to be invaluable in the development of sophisticated computational algorithms designed to simulate platelet-vessel wall interaction in health and in disease states such as atherosclerosis. Multiscale
computer simulation may ultimately be applied in predicting the in vivo efficacy of therapies designed to limit pathological clot formation.

Discussion was on differences between the full human VWF-A1 mouse, versus the point mutation mouse, any differences in thrombus formation with human platelets is slight.


Vascular injury triggers two intertwined processes, platelet deposition and coagulation, and can lead to the formation of an intravascular clot (thrombus) that may grow to occlude the vessel. Formation of the thrombus involves complex biochemical, biophysical, and biomechanical interactions that are also dynamic and spatially-distributed, and occur on multiple spatial and temporal scales. We have developed a spatial-temporal mathematical model of these interactions and looked at the interplay between physical factors (flow, transport to the clot, platelet distribution within the blood) and biochemical ones in determining the growth of the clot. In particular we have looked at how factors that may reduce movement of fluid, platelets, and/or coagulation proteins within the growing thrombus would affect the growth and spatial structure of the thrombus. We see that these effects can be profound and that they suggest a possible physical mechanism for limiting thrombus growth.

Discussion was on 2-D vs. 3-D simulations and symmetry boundary conditions, were the simulations for constant flow or pressure, and there was no platelet detachment.


THIS TALK WAS NOT PRESENTED. HOWEVER, THE FOLLOWING SUMMARY WAS PROVIDED:

We developed a deterministic family of coagulation models which simulate thrombin activation by both the extrinsic and intrinsic pathways under quiescent flow conditions. Members of this family can be thought of as an average of a few patients, thus, each member represents a small patient group. We linked the molecular behavior of the cascade e.g., the robustness and fragility of interactions or species with different patient subpopulations using a concept we’ll introduce called a patient fragility link matrix (PFLM). Together, we showed how to model and analyze the behavior of patient subpopulations using deterministic model ensembles, and we showed how to compare the molecular behavior across these patient populations.


Most hemostasis-related diseases arrive from abnormalities at the molecular level. We have developed a multiscale model that focuses on the qualitative/quantitative behavior change at the cell/tissue level caused by mechanical and chemical abnormalities of surface receptors and ligands and physical abnormalities of individual platelets. The simulation is based on the successful Adhesive Dynamics algorithm pioneered by Hammer, King and others, and incorporates bond kinetic models such as the Bell model, or so-called catch-slip model of bond dissociation. Both kinetic models accurately recapitulate platelet translocation via GPIb-VWF binding, except at the highest shear stresses where the Bell model
predicts a sharper increase in velocity. The simulation shows that the peripheral edge of the platelet is the most common site for tether bond formation, rather than the flat sides. Simulations of flow chamber tethering experiments demonstrate the consistency between our molecular level understanding of bond dissociation under force, and cellular scale observations of platelet recruitment at sites of injury. Next steps for the model include patient-specific simulations for alterations of bond kinetics associated with VWD, the influence of platelet size, and the inclusion of other receptor-ligand pairs such as integrin:collagen and VWF:GPIIb/IIIa.

Discussion was on the deformability of platelets, and potential future simulations in which platelet microparticle generation is considered.
Control of Anticoagulation

30 June 2012

Chairman: Walter Ageno (Italy)
Co-chairmen: Trevor Baglin (UK), Rebecca Beyth (USA), Elaine Hylek (USA), John Olson (USA), Gualtiero Palareti (Italy), Henry Watson (UK)

The program of the meeting was divided in two distinct parts, the first on conventional anticoagulant drugs and the second on the novel oral anticoagulant drugs.

The meeting started with a first session aimed to provide an Update on the laboratory monitoring of the vitamin K antagonists.

Ton van den Besselaar from the Netherlands reported on the activity of Validation of certified plasmas for ISI calibration and INR derivation.

Certified plasmas for ISI calibration and INR derivation are now commercially available. Discrepant ISI and INR values have been observed with certified lyophilized plasmas demonstrating their non-commutability and limitations. Validation of certified plasmas by comparative testing of fresh patients samples is required according to SSC-approved guidelines (JTH 2004; 2:1946-53). Validation may restrict the use of certified plasmas.

Saied Ibrahim, from the UK, reported on INR Simplified: The PT/INR Line.

The Prothrombin Time / International Normalised Ratio (PT/INR) Line is a INR determination method using a set of 5 ECAA lyophilised plasmas providing local INR (Clin Chem 2010;56:1608-17, J Thromb Haemost 2011;9:140-8). ISI and MNPT can be determined by plotting certified INR of the 5 ECAA plasmas against local PT of the same plasmas obtained with the automated test systems and fitting a linear regression line. PT/INR derived ISI and MNPT can be obtained from the slope and intercept estimates of the linear regression line with the following calculations:

\[ \text{PT/INR derived ISI} = \frac{1}{\text{slope}}, \text{PT/INR derived MNPT} = \exp(\text{intercept}) \]

Finally, Ton van den Besselaar reported on Intra-individual variation of INR in warfarin-treated patients: consequences for desirable analytical precision

The biological intra-individual variation of INR was assessed in 245 selected stable warfarin-treated patients. The mean intra-individual variation was 9.0%. Analytical performance goals could be derived from the mean intra-individual variation. For a therapeutic range of 2.0-3.0 INR, the desirable and optimum analytical imprecision is <4.5% CV and <2.25 CV, respectively.

At the end of the first session, Elaine Gray from the UK reported the results of the 3rd WHO-SSC collaborative study on International Standard for Low Molecular Weight Heparin.
Twenty-two laboratories from 13 different countries participated in a collaborative study to value assign the 3rd International Standard for Low Molecular Weight Heparin against the 2nd International Standard for Low Molecular Weight Heparin. Two candidates, sample A (NIBSC code 11/174) and sample B (NIBSC code 11/176) were included in the study. Inter-laboratory agreement was good for both candidates, with sample B giving lower variability than sample A. It is therefore recommended that the proposed 3rd International Standard for Low Molecular Weight Heparin (11/176) be assigned with potencies for anti-Xa (1068 IU/ampoule) and anti-IIa activity (342 IU/ampoule), relative to the 2nd International Standard for Low Molecular Weight Heparin (01/608).

The second part of the meeting started with a first session on the laboratory monitoring of the new oral anticoagulant drugs (NOACs).

Trevor Baglin from the UK presented the results of a recommendation paper proposed by the Subcommittee on Control of Anticoagulation on *When and how to measuring new oral anticoagulants (NOAC)*

New oral anticoagulants are given at fixed dose with no requirement for monitoring as pharmacokinetic and pharmacodynamic responses are reliably predicted in patients with adequate renal function who are not taking other interacting drugs. However, in some clinical circumstances in specific patients measurement of their anticoagulant effect will be required. Such clinical scenarios may include bleeding, before surgery or invasive procedure, suspicion of overdose, identification of sub- or supra-therapeutic levels in patients taking other drugs that are known to significantly affect pharmacokinetics, identification of sub- or supra-therapeutic levels in patients at extremes of body weight, patients with deteriorating renal function, assessment of compliance in patients suffering thrombotic events whilst on treatment. This recommendation paper considered two types of coagulation assay for each drug: one to be readily available in most laboratories and easily performed for use in an emergency or urgent clinical scenario, the second to be able to give quantitative results to be used to determine drug levels in non-urgent clinical situations.

Michel Meyer Samama from France and Armando Tripodi from Italy discussed *Which tests should we use?*

The objective of Prof. Samama was to show that we have appropriate and validated tests for anti-Xa activity measurement which are superior to PT (available everywhere at any time) and Hemoclot (dilute thrombin time) or Ecarin CT for Dabigatran. Two or three sets of plasma calibrators can be used. Available results (literature and a personal study) demonstrate important inter-individual variability in the response to treatment which suggests that the potential usefulness of laboratory measurement has been underestimated. Laboratory results in rare cases of life-threatening bleeding (4 patients leading to 3 deaths) were presented. These cases were very characteristic and different from usual results observed in treated patients. Reversal with hemodialysis in a Dabigatran treated patient was also shown.

Prof. Tripodi discussed the choice based on the following characteristics: availability (ready for use even in less specialized hospitals), standardization (comparison of results obtained with different reagents),
linearity (test response versus drug concentration), and responsiveness (slope of the dose-response curve). Based on these characteristics, a pragmatic solution could be: dilute thrombin time or ecarin clotting time for dabigatran and PT for rivaroxaban. A brief discussion followed on how to express results.

Elaine Gray was subsequently asked to report on the Collaborative study on the determination of the anticoagulant effects of dabigatran.

The scope of the study is to evaluate the sensitivity, intra- and inter-laboratory variability of assay methods for measurement of dabigatran in platelet poor plasma. Seven types of commercial assay kits will be included. The participants of the study will be requested to carry out determination of dabigatran in plasma samples spiked with unknown amount of dabigatran against dabigatran calibration curves. Due to MTA issues which will be resolved soon, this project has been delayed. It is hoped that the project could be initiated in the next couple of months and the results could be reported in the next SSC meeting.

In the second session on the new oral anticoagulant drugs, clinical experiences with the NOACs were presented.

Jan Beyer from Germany presented a talk on Major orthopaedic surgery, venous thromboembolism, atrial fibrillation: reports from daily clinical practice

Prospective trials have shown that rivaroxaban thromboprophylaxis is highly effective and safe in patients undergoing major orthopaedic surgery (MOS). However, patients treated under trial conditions are different from unselected routine patients, which may affect efficacy and safety of thromboprophylaxis. The impact of switching from parenteral to rivaroxaban thromboprophylaxis in daily care is therefore unclear. In a large monocentric, retrospective cohort study in 5,061 consecutive patients undergoing MOS we performed a comparison of thromboprophylaxis with LMWH, fondaparinux and rivaroxaban with regard to rates of symptomatic VTE, bleeding and surgical complications and length of hospital stay. Before we introduced rivaroxaban in our orthopedic department, we made sure that physicians and nursing staff were trained on the specifics of rivaroxaban, especially with regard to timing of first dose, contraindications and on the importance of ensuring patient compliance. Similar to the standards with parenteral thromboprophylaxis, nurses were instructed to give rivaroxaban tablets personally to the patient and separately from other oral medications. Nurses were further instructed to advise the patient to take this tablet immediately and time of intake was documented and signed by nursing staff. As a result of our strict training before introducing new oral anticoagulants in VTE prophylaxis we found a reduction of total VTE rates with rivaroxaban compared to LMWH or fondaparinux in our registry. Furthermore, we also found a reduction in bleeding and surgical complications in rivaroxaban-treated patients, which contributed to a reduction of the length of hospital stay. Since new oral anticoagulants have been approved for VTE treatment and stroke prevention in AF in Germany since autumn 2011, we are also interested in the efficacy and safety profile of NOACs in these populations from daily care. In October 2011, a large monocentric registry of VTE and SPAF-patients receiving NOAC was introduced in the district of Dresden,
Germany. Up to 2000 patients will be included and prospectively followed for up to 3 years and rates of outcome events such as arterial or venous thromboembolism, bleeding and death will be evaluated. Furthermore, management of NOAC therapy such as transition from VKA to NOAC or peri-interventional “bridging” management will be studied.

Elaine Hylek from the US and Giancarlo Agnelli from Italy discussed Gaps in trials and current practice in patients with atrial fibrillation and venous thromboembolism.

Both speakers addressed the challenges of translating the efficacy of novel therapeutics from clinical trials to effectiveness in clinical practice. Issues relating to patient selection and exclusion criteria were discussed. Important pharmacokinetic differences among these agents were reviewed and their relevance to patients in practice. The dearth of information from trials to guide physicians through commonly encountered clinical situations will reinforce the need for longer term real world surveillance via registries.

Finally, Gualtiero Palareti from Italy presented an SSC project: an international register on anticoagulated patients.

It was proposed that the SSC Control of Anticoagulation launches a registry to prospectively collect data on patients treated with the new oral anticoagulants, aiming at evaluating the risk/benefit ratio of these agents in a real life setting. An electronic registry that prospectively collects data on patients treated with anticoagulants is already active in Italy (START-Register). An English version of the registry is almost ready for use. The START-Register is designed solely for observational purposes to create a database that can be used to help design and execute collaborative clinical studies. It is not intended to have any influence on the treatment of the single patients included. The START-Register is a fully independent project. Public and private institutions, Foundations, Associations, individuals, and companies interested in the question of anticoagulation (manufacturers of drugs or other goods and services) are invited to help funding the creation and running of the Register via special funding programmes. Supporter for the START-Register is any Society, Foundation, Association, individual, and company who offers a contribution for the setting up and management of the Register.

The third and last session on the new oral anticoagulants dealt with Bleeding in patients receiving NOACs: problems and possible management.

Pieter Kamphuisen from the Netherlands provided an Update on current evidences from the real world and presentation of ongoing studies.

A comprehensive overview of available evidences on reversal strategies for each new oral anticoagulant, on the results of recently completed clinical trials, and on currently ongoing studies was provided.

Sam Schulman from Canada presented some Suggestions for the management of invasive procedures and bleeding events with the NOACs.

For surgery it is adviseable to stop the treatment at a time point that takes into account the half-life of the drug, the creatinine clearance (especially for dabigatran) and the anticipated risk of bleeding.
Essentially, for low-risk procedures it is acceptable to have a residual low level of the anticoagulant drug in circulation, whereas for high risk surgery it should be virtually eliminated. The resumption of anticoagulation needs to take into account the almost immediate onset of effect and can be the same day for low-risk procedures but has to be delayed 2-3 days for high-risk procedures or if there is active bleeding. For surgery that generates bowel paralysis it may be necessary to bridge with a heparin until able to take oral medications. Major bleeding should, with the lack of available and validated antidotes, primarily be managed with supportive measures (hold the anticoagulant drug, identify bleeding source and use local hemostatic measures when possible, transfuse blood as needed, or platelets to reverse antiaggregants). For anti-factor Xa agents it is possible that prothrombin complex concentrate will be useful to reverse the effect but clinical verification is needed. For dabigatran active charcoal can be used for oral overdose within 2 h, hemodialysis can remove dabigatran and a few case reports indicate that activated prothrombin complex concentrate can be effective to stop bleeding, but this requires further validation. For both types of drugs specific antidotes are under development.

Henry Watson from the UK discussed the Management of obese patients with the old and new anticoagulants

Current evidences on the optimal dosing regimens to be used in obese patients treated with the low molecular weight heparins were presented. Potential problems with the use of the new oral anticoagulants and available data from PK/PD studies were discussed.

At the end of this session, Walter Ageno from Italy summarized ongoing projects from the SSC recommendation for the guidance on the management of patients treated with the NOACs

The Subcommittee on Control of Anticoagulation of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis is committed to the production of specific recommendations focusing on the management of patients starting on the NOACs, on the laboratory measurement of the NOACs, and on the use of reversal strategies for the management of patients with bleeding complications or requiring urgent invasive procedures. This first recommendation focusing on practical aspects related to patient selection, use of concomitant drugs, follow-up modalities, and assessing of patients adherence has been submitted for publication. The second on the laboratory measurement has been presented this morning by Trevor Baglin and is nearly completed. The third on the management of bleeding is led by Mark Crowther from Canada and is currently underway.

At the end of the meeting, Walter Ageno provided an Update on research activity on unusual site thrombosis.

The SSC Control of Anticoagulation has promoted an international registry on splanchnic vein thrombosis, which was aimed to collect prospective information on the baseline characteristics, the management and the clinical history of patients with splanchnic vein thrombosis. Patients enrolment has been recently completed with the inclusion of 613 patients from 12 countries. A report on the baseline characteristics of the enrolled population, with particular focus on treatment strategies, is expected to be submitted soon. A prospective 2-year follow-up is currently underway, and should be completed in the first months of 2014.
Disseminated Intravascular Coagulation

28 June 2012

Chairman: Hideo Wada (JP)
Co-chairman: Satoshi Gando (Japan), Hyun Kyung Kim (Republic of Korea), Jorn Nielsen (Denmark), Jecko Thachil (UK)

**Dr. Carl-Erik Dempfle** presented “Fibrin related markers”. He introduced soluble fibrin (SF); fibrin complex with fibrinogen. There are 3 assay kits with each monoclonal antibody for SF from Japan. The monoclonal antibody IF43 recognizes E-domain and measures soluble fibrin complex (SFC). The second assay kit which consists IF 43, measures SFC and fibrinogen degradation products (FbDP), indicating that this assay correlates with fibrinogenolysis. The third assay kit contains with J2-23 which recognizes Aα5O2. The three SF assays are not closely correlated. The use of the FDP assay has been reduced in European countries but the measurement of FDP has continued in Japan. There are many FDP assay kits in Japan and the diagnosis of DIC has used FDP. FDP assays should be standardized in Japan. D-dimer is the fragment of D-dimer related compounds. There are more than 50 different assay kits for D-dimer. D-dimer was previously used to exclude pulmonary embolisms. Therefore, the D-dimer assay range was too small for DIC or trauma. In particular, a correlation with a high concentration of D-dimer might be required. D-dimer is also not specific for intravascular fibrin formation, and reflects extravascular fibrin. The correlation between SFC and D-dimer is weak in DIC. The standardization of D-dimer has three problems; the primary calibrator (and common calibrator), diluted buffer and reference procedure.

**Dr. Jun Mimuro** presented “Impact of Recombinant Soluble Thrombomodulin (rhTM; Thrombomodulin Alfa) on Disseminated Intravascular Coagulation” from the results of post market surveillance. He first introduced the basic data on rhTM and the results of the phase III trial in Japan. There was a 16.2% difference in the resolution from DIC between rhTM and heparin. No adverse events such as bleeding associated with hematological malignancy were observed. The post market surveillance was carried out in a general practical setting with randomized trial and 380 U/kg of rhTM was administered. This study; enrolled 3636 patients (2516 with infectious diseases and 1120 with hematological malignancies). These patients were classified into 3 groups; a phase III matched group (group 1), a phase III non-matched group (group 2) and a phase III non-matched concomitant drug group (group 3). The resolution rate from DIC and survival rate of infectious diseases and hematological malignancy were same in the phase III in group 1 but both were low in group 2 and 3. There was no significant difference in the bleeding adverse events associated with infectious diseases among the phase III and group 1-3. Bleeding adverse events increased in hematological malignancies, in comparison to phase III.

The chairman reported 4 on-going projects and new projects. Project 1 investigated the relationship between DIC and trauma. The report was submitted to JTH by Dr Gando. Project 2 is the establishment of DIC treatment guidelines. The British, Japanese and Italian DIC guidelines are discussed and standardized. Several meetings allowed us to prepare a draft of standardization of the three guidelines. This paper will be submitted to JTH between 2012 and 2013. Project 3 is the standardization
of D-dimer. The standardizatin of D-dimer is on going. The adequate cutoff value of D-dimer for DIC has been published in some kits. An adequate cutoff value will be established for most assay kits. Project 4 is the establishment of non-overt DIC diagnostic criteria. Numerous modified non-overt DIC diagnostic criteria have been published. These criteria were evaluated and discussed. Project 5 is a proposal for a new mechanism of the onset of DIC.

Dr Satoshi Gando presented “DIC and COT/ACOTS in trauma”. DIC has a fibrinolytic phenotype during the early phase of trauma, and is associated with hyperfibrin(ogen)olysis and a consumptive coagulopathy, contributing to massive hemorrhage. This type of DIC changes at later stages of trauma into a DIC with a thrombotic phenotype, which gives rise to organ dysfunction primarily due to fibrin clot deposition. Therefore, both types of DIC are among the main determinants for the prognosis of severely injured trauma patients. The Educational Initiative on Critical Bleeding in Trauma (EICBT) recently announced new disease entities at the early stage of trauma: Coagulopathy of trauma and acute coagulopathy of trauma-shock (COT/ACOTS). He focused on DIC with a fibrinolytic phenotype and proposes one concept and six considerations to discuss the hemostatic changes at an early stage of trauma. The similarities and differences in the mechanisms, diagnosis, and treatments between DIC and apparent COT/ACOTS are discussed. More information based on well-powered and appropriately controlled prospective clinical studies is needed to clearly define the major pathologic hemostatic mechanisms contributing to coagulopathy after trauma. However, the current available evidence leads to the conclusion that COT/ACOTS is not a new concept but is a disease entity similar or equal to DIC with a fibrinolytic phenotype.

Dr. Shinichiro Kurosawa presented “DIC and Thrombotic microangiopathy: What are the differences and the shared features”. He introduced a hemolytic uremic syndrome (HUS) animal model. The Shigatoxin (STx) is produced from E-coli. HUS due to STX has often been reported and it sometimes has a poor outcome. There is no specific treatment and only supportive therapy for HUS associated renal failure, etc. A mouse model does not show thrombocytopenia nor glomerular injury but LPS cause both thrombocytopenia and glomerular damage. He established a mouse HUS model injected with LPS and STx. STx causes thrombocytopenia and acute kidney injury with micro thrombi in a non- human primate (baboon) model of HUS. The baboon HUS model injected with 10 ng/ml STx survive but those injected with 50 ng/ml die. The plasma TNF levels are low but IL-6 levels increase. These findings suggest that STx effects ER stress causes HUS and LPS effects on TLR4 and causes DIC.?The murine HUS model required LPS and STx and the primate HUS model caused by STx does not require LPS.

Dr. Jecko Thachil presented “Fibrinogen, pathological coagulation and DIC?”. He introduced the protective role of fibrinogen in DIC. A low fibrinogen level is associated with a poor outcome in DIC and increased histone trigger DIC. Histones increase platelet aggregation and correlate with an increased thrombotic risk. Fibrinogen binds to histone-3. Histones without fibrinogen do not increase aggregation of platelets but histones and 0.5 g/L fibrinogen cause maximum platelet aggregation. Histones and 2.0g/L fibrinogen reduced platelet aggregation. Histones pre-incubated with fibrinogen decrease platelet aggregation. Histone-3 or -4 reduces cultured endothelial cell survival but histones with fibrinogen prolong the survival. Mice injected with histones die but those injected with histones and fibrinogen did not die.
Dr. Jorn Dalsgaard Nielsen presented “Topics on diagnosis of DIC”. He introduced hyper-fibrinolytic type DIC and hypo-fibrinolytic type DIC. Sepsis is a “hypo-fibrinolytic type DIC” and has a poor outcome due to organ failure. APL, an Australian brown snake bite, contact with Lonomia Caterpillas and amnionic fluid embolism are “hyper-fibrinolytic type DIC” and may be required anti-fibrinolytic therapy such as tranexisam acid.

Dr. Hyun Kyung Kim presented “The prognostic value of thrombomodulin expression on monocytes subsets?” She examined the TM expression on monocytes in 98 patients with DIC.

The monocytes with high positive CD14 and negative CD16 are the classic type monocytes (CM). Those with high positive CD14 and positive CD16 are as inflammatory type monocytes (IM). Those with dim CD14 and positive CD16 are called dendritic-cell like monocytes (DM). The expression of TM is high in IM cells. The patients with increased expression of TM on monocytes had a poor outcome. The expression of TF and TM on monocytes was high in the patients with overt-DIC. Cultured monocytes stimulated by LPS and IL10 form different percent of CM, IM and DM. IM increases after stimulation and then decreases, IM increases and CM decreases in vitro culture. Finally, inflammatory stimuli up-regulate the expression of TM on monocytes.

Dr. Ikuro Maruyama presented “DAMPs/PAMOs in DIC”. Pathogen associated molecular patterns (PAMPS) have a stranger signal and a damaged associated molecular pattern from inside cells (DAMPS) have danger signal. PAMPS are peptidoglycans such as LPS, and binds on TLR4 to activate NF-κB and increase plasma IL-1 and IL-6 levels. Leukocytes release NETs to PAMPS. DAMPS includes HMGB-1 and histones. HMGB-1 activates RAGE, TLR 9 and TLR2 and thereby cause cell shape change, inflammation, an acceleration of glomerular fibrin deposition and DIC. TM binds HMGB-1 to induce an anti-inflammatory effect.
Registry of Exogenous Hemostatic Factors

Chairman: Jan Rosing (NL)
Co-chairmen: Kenneth Clemetson (CH), Manjunatha Kini (SG), Francis Markland Jr (US), Takashi Morita (JP), Mary Ann McLane (US)

Manjunatha Kini chaired the session as Jan Rosing could not attend the meeting.

Six members of the registry were in attendance plus about 8-12 guests.

Welcome: Manjunatha Kini, Chair

Introduction of new members of the registry: Dr. Geoff Isbister, Australia; Dr. Robson Monteiro, Brazil; Prof. B. S. Vishwanath, India; and Dr. Soichi Takeda, Japan;

Minutes of the last meeting (Kyoto 2011) was approved.

Publication of the subcommittee
None

New inventories / activities

1. Nomenclature / classification / activities of L- amino acid oxidase will be considered. Ken Clemetson is working on it and the first draft will be ready for Amsterdam meeting.
2. Classification and nomenclature of exogenous factors from hematophagous animals. The project is too big and complicated. Ivo Francischetti will attempt come up with a strategy by Amsterdam meeting.
3. Educational programs: Two international workshops on "Thrombosis and Hemostasis: Discovery and Development of Tools and Therapeutics” have been planned. The first one will be held at Federal University of Santa Catarina, Florianopolis, Brazil (November 12-15, 2012) and the second one will be held at University of Mysore, Mysore, India (December 8-11, 2012). These workshops are sponsored ISTH and respective institutions. The workshops will be conducted by Ken Clemetson, Ivo Francischetti and Manjunatha Kini along with respective local scientists.
4. Guidelines for treatment: Gerhard Johnson, Guidance Committee, ISTH asked whether we could come with guidelines for treatment of snakebite victims. Geoff Isbister is considering to set-up a committee to come up with the guidelines.
5. Fifth International meeting on Exogenous Factors Affecting Thrombosis and Hemostasis: It will be organized as the satellite meeting after the World Congress of the ISTH in Amsterdam in 2013. Prof. Jan Rosing, Chair of the Organizing Committee, informed that the meeting will be held in Maastrict (July 5-6, 2013). We are planning for 100-120 participants.
6. Scientific presentations: Two presentations were made.
   a. Functional genomics of salivary gland proteins – Ivo Francischetti
   b. Structural basis of coagulation factor V recognition for cleavage by RVV-V – Soichi Takeda

Any other business
None
Next meeting
The subcommittee agreed to meet in Amsterdam (2013)
The meeting was adjourned at 1.45 pm.
Factor VIII and IX

29 June, 2012

Chairman: Flora Peyvandi (Italy)
Co-Chairmen: Jan Astermark (Sweden), Kathelijn Fischer (The Netherlands), Claude Negrier (France), Steven Pipe (USA), Midori Shima (Japan), Leonard Valentino (USA)

09.30 REPORT ON SSC-FVIII&FIX ACTIVITY 2011-2012 – Flora Peyvandi (Italy)
Dr Peyvandi opened the SSC session by thanking Dr. Alok Srivastava for the outstanding work done in the last 4 years as chair and co-chair of this subcommittee, and Drs. Charles Hay, Johannes Oldenburg and Prof. Edward G Tuddenham for their valuable contribute as co-chairs; and she welcomed Dr. Steven Pipe as new co-chair wishing him a fruitful collaboration.

She reported about the state of the art of the FVIII&FIX SSC:

1. Recommendation submitted as Official SSC Communications to JTH:
   - Project on Consensus definitions in rare bleeding disorders (Chair: F. Peyvandi)
2. International Standards under submission as Official SSC Communications to JTH:
   - 2nd WHO International Standard for Factor VII concentrate
   - 4th international standard for Factors II & X, concentrate
3. Reports posted on the ISTH website for further comments and discussion
   - Project on Consensus definitions in haemophilia (Chair: V. Blanchette)
   - Project on Pharmacokinetics (Chair: P. Collins)
   - Project on Potency labelling of clotting factor concentrates (Chair: A. Hubbard)
   - Project on Standardization of methods for performing the clot wave form analysis (Chair: M. Shima)
   - Project on Standardization of methods for performing the thromboelastogram (Chair: G. Young and M. Chitlur)
4. Report soon available on the ISTH website:
   - Project on Standardization of methods for performing the thrombin generation test (Chair: C. Negrier)
5. On-going project:
   - Clinical trial design for haemophilia (Chair: D. DiMichele)

Dr Peyvandi presented her proposals shared with the co-chairs and invited participant to propose others.

Dr Peyvandi presented two new projects:

- Standardisation of laboratory tests for evaluation of FVIII / FIX inhibitor by-passing agents (using global assays) and novel drugs (using one stage/chromogenic and global assays), chaired by Drs Andrew Lawrie (UK) and Armando Tripodi (Italy)
- Bleeding score in haemophilia: a prognostic tool for clinical outcome, chaired by Drs Elisa Maria Mancuso (Italy) and Alberto Tosetto (Italy).

09:40- 10.20 PROJECTS REPORTS I
Session Chairpersons: Guy Young (USA) and Alok Srivastava (India)
Dr Young introduced the session.
Three years ago, the SSC formed 3 Working Parties (now called Project Groups hereafter PG) aimed at providing standardized methodologies for global hemostasis assays. The SSC chose to focus on 3 assays: clot waveform analysis, thromboelastography, and thrombin generation assays. The PG chairs were selected by the SSC FVIII/FIX subcommittee and the chairs formed their respective groups. Each group was tasked with reviewing the literature on their specific assay and to recommend a standardized approach for the methods of each of these assays as it applies to hemophilia and rare bleeding disorders. The groups have recently completed their work and presented their final recommendations.

09:40 Standardization of whole blood viscoelastic measurement of clot formation and clot stability; Meera Chitlur (USA)
Thromboelastography is based on the assessment of the viscoelastic properties of whole blood during the dynamic process of clotting. There are two devices that are currently available, the TEG®5000 Analyzer and ROTEM® Gamma. The devices rely on the same principles and while there are some mechanistic differences, the clot parameters that are produced are essentially the same. The following are the basic recommendations for using the devices that will be discussed.

Blood collection: Blood should be collectedatraumatically with no or minimal tourniquet application into either standard sodium citrate (kaolin or INTEM) or into citrated tubes with pre-loaded corn trypsin inhibitor (CTI) 0.1 mg/mL for EXTEM or tissue factor triggered assays. The sample should be run within 2 hours of collection.

Recommendations for the Kaolin/INTEM Method: Intrinsic system activation is simple and has been the standard method for thromboelastographic analysis in the operating room setting for many years. For the (TEG®5000), 1 mL of whole blood should be transferred into the kaolin vial and 340?L then transferred into the TEG cup into which 20?L of calcium chloride had been placed. For the ROTEM®Gamma, the blood is transferred directly into the device for automated pipetting. Since both reagents are supplied by the manufacturers, their potency is standardized ensuring reliable results.

Recommendations for the Tissue Factor method: Tissue factor has been used as an activator since it is deemed to be a more physiologic representation of the in vivo coagulation process and has been adopted for use in hemophilia. Utilizing the commercially available TF Innovin®, 2 dilutions have been studied, a high concentration of approximately 0.35pM (1:17,000 dilution) and a low concentration of approximately 0.15pM (1:42,000 dilution). Which concentration is best remains unresolved. We suggest performing prelimin ary studies with both dilutions to determine the most appropriate one for the specific study one is performing. Once prepared, 20?L of the dilute TF is placed in the specimen cup, followed by 20 ?L of calcium chloride for recalcification of the citrated blood sample. Since the maximum volume in the specimen cup cannot exceed 340 ?L, 300 ?L of blood is added instead of 340 ?L as with Kaolin in the TEG®5000. The same procedure may be applied to both the TEG®5000 and the ROTEM®Gamma with manual pipetting. Otherwise, for the ROTEM® Gamma, the EXTEM reagent may be used as the extrinsic pathway activator, but it is important to be aware that the results may differ from that obtained using Innovin® described above as the activators are not identical.

Comparison of the TF and Kaolin methods: This remains a much debated and controversial question since both activators have strengths and weaknesses. The advantage of kaolin or INTEM is that the method is very simple and the reagents are standardized. The main disadvantage is the non-physiologic nature of contact system activation. The advantage of TF is the physiologic activation of clotting while the disadvantages include the lack of standardization of the potency of the TF and the requirement with
the TEG®5000 of making a "home-made" TF reagent using commercial TF intended for other uses. In a study comparing kaolin to TF, kaolin performed as well as a low concentration of TF and better than a higher concentration.

**Conclusion:** This WP has concluded that thromboelastography can be performed with either of the available instruments and that both the contact activation and tissue factor activation methods continue to be used in clinical trials. Further research to determine a correlation between the laboratory results and clinical outcomes are required before these assays can be recommended for clinical use.

**09:50 Standardization of methods for performing the thrombin generation test; Claude Negrier (France)**
Abstract will be soon available

**10:00 Standardization of methods for performing the clot wave form analysis; Midori Shima (Japan)**
Clot waveform analysis (CWA) is based on the continuous monitoring of light transmittance or absorbance during routine aPTT. The clot waveform can be illustrated using the various aPTT reagents, 0.025mM CaCl2, and reference plasma. However, the reagent validated for FVIII or FIX measurement is recommended. Furthermore, it is possible to perform tissue factor triggered CWA. The complete clotting process recorded in the CWA is categorized into the three parts; the pre-coagulation phase, the coagulation phase and the post-coagulation phase. Pre-coagulation is described as the first segment of the trace, from the beginning of the signal to the onset of coagulation. Usually, this phase is horizontal, except in waveforms in patients with DIC induced by septicemia. After the onset of coagulation, light transmittance is decreased in association with the formation of fibrin and is defined by a slope in the waveform. At the end of coagulation, light transmittance tends to stabilize and is characterized again by a linear segment. The advantages of utilizing CWA are provided by the quantitative assessment of various parameters derived by mathematically processing the waveform data. Slope 1 is initial slope in the pre-coagulation phase, before the onset of coagulation. tmin2 is the time at the onset of coagulation, and min2 is the minimum value of the second derivative of the transmittance. The absolute value of min2 (|min2|) reflects maximum coagulation acceleration. tmin1 is the time at the mid-point of the coagulation phase. min1 is the minimum value of the first derivative of the transmittance. The absolute value of min1 (|min1|) reflects the maximum coagulation velocity. tmax2 is the time at the end of the coagulation phase, and is recorded at the maximum deceleration rate of coagulation. Max2, is derived from the second derivative. Delta is amplitude of signal change, and this value reflects fibrinogen concentration. Several currently available coagulometers can be utilized for CWA if the raw data of light transmittance or absorbance can be extracted. Relative transmittance is utilized to derive the waveform, and this is readily calculated using standard statistical software. The first derivative of the relative transmittance is then used to describe coagulation velocity, and similarly, further calculation of the second derivative gives a measure of coagulation acceleration. These values can be calculated easily using straightforward statistical algorithms provided in computer programs such as Microsoft Excel

**DISCUSSION**
Questions and comments from the audience:
It should be appropriate to standardize the global assays in other haemorrhagic disorders than haemophilias.

**10:20-11.00 PROJECT REPORTS II**
Session Chairpersons: Flora Peyvandi (Italy) and Michael Makris (UK)
Dr. Makris introduced the session.
The Project reports II Session presented the deliberations around the clinical trial design in haemophilia. The small number of patients, especially of children with haemophilia B, poses particular problems to manufacturers, the haemophilia community and regulators. The first two presentations addressed the new developments in clinical haemophilia trial regulations in the USA and Europe. The final presentation was an update of the discussions by the project group on the optimal design of haemophilia trials.

**FDA and EMEA regulatory: clinical trials requirements:**

10:20 New regulation of FDA for rare disorders; Nisha Jain (USA)

Under the Federal Food, Drug and Cosmetic Act a rare disease or condition means any disease or condition which affects less than 200,000 persons in the United States, or affects more than 200,000 in the United States and for which there is no reasonable expectation that the cost of developing and making available in the United States a drug for such disease or condition will recovered from sales in the United States of such drug. Since the implementation of the Orphan Drug Regulations FDA has reviewed over 3,350 requests for orphan-drug designation of drugs for rare diseases and conditions. FDA now proposes revisions to this regulation. These revisions include (1) demonstration of an appropriate “orphan subset” of persons with a particular disease or condition that otherwise affects 200,000 or more persons in the United States, for the purpose of designating a drug for use in that subset; (2) eligibility for orphan-drug designation of a drug that is otherwise the same drug for the same orphan indication as a previously approved drug; (3) eligibility for multiple orphan-drug exclusive approvals when a designated orphan drug is separately approved for use in different subsets of the rare disease or condition; (4) requirement for demonstrating clinical superiority for the purpose of orphan-drug exclusive approval; (5) requirement for submitting the name of the drug in an orphan-drug designation request; (6) required drug description and scientific rationale in a designation request and other minor revisions. These revisions are expected to clarify the existing regulations.

10:30 New European guidelines for clinical trials in rare disorders; Anneliese Hilger (Germany)

The EU-requirements on clinical development for Factor VIII and Factor IX products are laid down in guidelines and core Summary of Product Characteristics. The guidelines cover clinical investigations to be conducted pre- and post-marketing authorisation. Guidance is also provided for authorised products where a significant change in the manufacturing process has been made. Clinical trials, addressing efficacy and safety are required in patients of all age groups for an application for a marketing authorisation. In addition, depending on the type of factor product (e.g. novel protein modifications) studies in previously untreated patients should be performed to investigate efficacy and safety in this specific patient population. In view of the limited availability of patients suffering from haemophilia, data from pre-authorisation studies only are considered insufficient to estimate all aspects of therapy with factor FVIII/IX products, especially with respect to immunogenicity. Therefore, to collect additional clinical data and to ensure consistency in the long-term between the outcome from pre-authorisation clinical studies and from routine use, a post-marketing investigation should be performed. The clinical development for factor FVIII/IX products should follow a stepwise approach in order to have some experience in adults and older children before investigating younger children. The clinical investigation in children needs to be supported by an approved paediatric investigation plan. The guidelines exist since more than 10 years and have been recently revised to fulfil progressing legal, scientific and regulatory requirements. The new guidelines became into operation in February 2012.

10:40 Clinical trial design for haemophilia; Donna DiMichele (USA)

Dr. Peyvandi informed the audience that unfortunately Dr. DiMichele was not able to reach the session to present her results. Dr. Srivastava made a brief summary of the on-going activity of this project.
The Clinical Trial Design for Hemophilia is a project group (PG) of the Factor VIII/IX Subcommittee of the SSC. The PG began its deliberations in February 2011. The aim of this PG is to develop a set of recommendations for the optimal design of pre- and post-authorization clinical studies and trials for new clotting factor concentrates (CFCs) for hemophilia A and B. Clinical trial design recommendations will be based on four priority considerations: 1) the harmonized safety and efficacy data required by regulators for product registration; 2) the post-licensure information on product safety and efficacy required by all stakeholders; 3) the realistic number of eligible and available study subjects for pre- and post-registration studies in hemophilia A and B; and 4) the availability of innovative clinical trial design strategies and models that may be suitable for rare diseases such as hemophilia.

In an effort to ensure that its recommendations are relevant and based on scientific rationale and evidence, the PG is seeking guidance from all stakeholders. Its deliberations within the PG itself are being informed by clinical investigators, immunologists, clinical trial methodologists, and representatives of the FDA and EMA. The PG is also soliciting input through consultation from hemophilia physicians, patients and the biologics industry.

Small clinical trials, such as those conducted in the rare bleeding disorders, require specific approaches to clinical trial design and statistical evaluation. Therefore the initial approach to this task has been to consider the basis for existing requirements while exploring alternative clinical trial methodology (e.g. Bayesian and Adaptive Design) and statistical modeling for the design of pre-licensure trials for new unmodified and novel FVIII, FIX and FVIII/FIX bypassing therapeutics in PTPs. PUP studies will subsequently be considered. With the goal of study optimization, the PG is examining the impact of these alternative strategies on the type and number of subjects, as well as the CFC exposure days required to achieve the current safety and efficacy endpoints for product authorization.

As part of this exercise, the group will also evaluate the statistical targets for the pre-licensure determination of product safety (defined by neoantigenicity) for both novel and unmodified FVIII and FIX CFCs. Additionally, the PG is considering the feasibility of using post-licensure studies to validate current immunological definitions of neoantigenicity and to study emerging immunological biomarkers of treatment-related antibody development for future incorporation into exploratory clinical trial design models.

The PG is examining the current tenets of clinical efficacy determination in a similar way. In collaboration with the Definitions PG, this PG is pursuing the potential implementation of more precise definitions for subject inclusion criteria and clinical outcome endpoints as a way to maximize data generation on clinical efficacy in pre-registration studies. Furthermore, the group will consider the possible role of surrogate markers (e.g., pharmacokinetics) in ascertaining clinical efficacy in pre-registration trials when complimented by mandatory rigorous data collection on clinical effectiveness through prospectively designed post-licensure studies.

All activities are ongoing and the PG’s final report will be presented at the SSC meeting in 2013.

DISCUSSION
Questions and comments from the audience centred on the following areas:

1. Number of requested patients affected with haemophilia B and other rare disorders should be differentiated from haemophilia A, since these disorders are less prevalent compared to haemophilia A.
2. It is necessary to gain different request from regulatory bodies on the number of patients to involve.
3. The regulators indicated they will consider a staged process of trials prior to registration.
4. Regarding the safety and immunogenicity of products in PUPs study, there was some discussion whether it is reasonable to treat PUPs for 50 exposure days or it is better to make it shorter and follow them with post-marketing trial.
5. The definition of an exposure day when dealing with products with prolonged half-life was questioned; no answer to this comment.
6. It was pointed out that major problems with inhibitors, such as those seen in the Netherlands in the early 1990s, will be easy to detect in phase 2/3 trials. However, to detect subtle changes will need very large numbers and the trial requirements are far too small to detect this. The number of minimum exposure days has been discussed since there is no evidence for this choice.

11.10 – 12.00 PROJECTS REPORTS III
Session Chairpersons: Steve Kitchen (UK) and Yesim Dargaud (France)
Dr. Dargaud introduced the session.

11:10 Using pharmacokinetics to individualize treatment: update; Peter Collins (UK)
Prophylaxis in severe haemophilia A is usually prescribed on the basis of weight and this strategy has been shown to result in good short and long term outcomes.[1,2] However, because the pharmacokinetics (PK) of factor VIII (FVIII) varies between patients, weight-based dosing results in markedly different FVIII trough levels between individuals.[3,4] Furthermore, because half-life increases with age[5] the trough levels achieved by standard weight-based prophylaxis are, on average, higher in adults than children.[3] Trough FVIII is related to breakthrough bleeding during prophylaxis, although the level that is appropriate for an individual is likely to vary dependent on numerous patient-related and environmental factors. However, if tailoring prophylaxis to an appropriate trough level is desirable then knowledge of the patient’s PK will be useful. Furthermore, tailoring prophylaxis using individual PK, in addition to observed bleeding pattern and activity, is likely to result in more cost effective treatment and potentially expand access to prophylaxis in countries where health care budgets are constrained.[6] The standard way to measure FVIII PK requires a washout and [8-10] FVIII measurements over a 48 hour period.[7] Calculation of the half-life and assessing the implications of this result for dosing prophylaxis is a complex undertaking beyond the ability of most haemophilia centres. Population PK and Bayesian analysis can be used to measure FVIII PK without a washout using a sparse sampling schedule. Measuring FVIII levels 3 times in a 48 hour period gives almost as much information as the full [8-10] point schedule.[8] This information can be used to estimate the effect of prophylactic regimens on trough FVIII levels and tailor the regimen to a target level appropriate for the individual. These techniques have been used routinely for many years to monitor and adjust the dose of drugs such as aminoglycosides.[9]

Recently a population PK model of rFVIII (Advate) has been published.[5] This model covers both adult and paediatric patients and can therefore be used to measure PK with sparse sampling in all age groups. The model is suitable for all standard rFVIII preparations but cannot be used to measure PK in people with low titre FVIII inhibitors or for assessing longer half-life molecules. The introduction of this population PK model into routine practice is limited because, at present, user friendly software that can be used by haemophilia centres in not available. A potentially useful programme called TCIwork® has been developed at Otago University and initial studies suggest that it may be applicable to haemophilia.[10] However, because the programme does not use age as a covariate in the analysis, it is
not suitable for all patients with haemophilia. The programme is currently being updated to allow age to be included and this programme is being investigated in the context of haemophilia.

A manuscript describing the measurement of PK with sparse sampling using population PK and Bayesian analysis has been prepared and submitted for review. An appendix will be made available giving detailed instructions on how to download, install and use TCIworks once the updated version becomes available.

Conclusions: FVIII PK can be measured by Bayesian analysis and population PK models using about 3 samples taken at appropriate times after an infusion. No washout is needed and the times of the samples can be flexible. This information can be used to help dose prophylaxis in haemophilia A and potentially make dosing much more cost effective. Simple to use software to calculate PK and prophylaxis dosing is potentially available but is being updated so that it is applicable to haemophilia. This software will need to be validated in routine practice. Studies on the use of PK tailored prophylaxis are required to establish the safety and efficacy of this approach. A document describing population PK as it applies to haemophilia has been posted on the ISTH website for comments and this will be revised after the meeting on the basis of any feedback.

References

10. Björkman S. Evaluation of the TCIWorks Bayesian computer program for estimation of individual pharmacokinetics of FVIII. Haemophilia 2010 (e-published 22 August)
11:20 Potency labelling of clotting factor concentrates: update; Anthony Hubbard (UK)
Since development of the World Health Organisation International Standards (WHO IS) for Factor VIII (FVIII) and Factor IX (FIX) Concentrates all plasma-derived and recombinant therapeutic concentrates have been labelled in International Units (IU) defined by in vitro biological activity. The development of new products, with novel properties introduced through structural or chemical modifications (e.g. truncation, pegylation, fusion), may challenge this traditional approach and present different routes of potency labelling with the risk of discordance between licensing authorities and subsequent confusion to users. In order to address this issue the project group has considered the options for the potency labelling of new FVIII and FIX concentrates and have drafted recommendations for manufacturers and regulators. The project has also attempted to reconcile the methods used for potency labelling with the local practices for post-infusion testing by clinical laboratories. The draft recommendations are presented in four sections and are summarised below:

1. Manufacturer’s characterisation of new product potency. This section recommends a thorough characterisation of the new products by clotting and chromogenic methods, relative to the WHO IS, in order to determine if statistically valid estimates are possible and to identify methods discrepancies and/or the influence of different reagents. Valid assays of modified products relative to the WHO IS, in terms of parallel and linear dose/response relationships, supports potency labelling in IU. Where there are methods-based potency discrepancies it will be necessary for licensing authorities to agree on the approach to potency labelling. Where assays relative to the WHO IS are invalid it may be necessary to label in product-specific units defined by a product reference.

2. Calibration of manufacturer’s product reference. Depending on the validity of assays relative to the WHO IS, the product reference should be calibrated in IU relative to the WHO IS or in product-specific units.

3. Manufacturer’s pharmacokinetic studies. It is recommended that pharmacokinetic studies should include both clotting and chromogenic methods with potency estimation relative to both the product reference and a plasma reference. The pharmacokinetic study should provide information on the relationship between the infused dose, based on the labelled potency, and the expected measured recovery in the patient when different methods and references are used on post-infusion samples. This information should be made available to clinicians and may allow the use of local methods and reference materials.

4. Post-infusion testing in clinical laboratories. Although the optimal approach to quantification of post-infusion samples requires testing relative to a product reference it is recognised that this may be difficult to implement in a routine clinical laboratory. Local assay systems could be used subject to manufacturer’s guidance on the interpretation of assay results.

11:30 Consensus definitions in haemophilia; Alok Srivastava (India)
This project group charged with the mandate of developing definitions of clinical events and endpoints in clinical studies has completed its work as described last year. It has carried out an extensive review of the existing literature combined with discussions with a range of stakeholders including expert physicians around the world, patients, industry representatives and regulators. Apart from endorsing the existing definitions of the severity of the condition and high and low titre inhibitors, new definitions are being provided for significant inhibitor titres as well as transient and persistent inhibitors. Factor replacement protocols including different types of prophylaxis are also being defined. In addition joint bleeds, rebleeds, target joints and muscle bleeds are being defined. Responses to treatment including that of joint bleeding and adequacy of surgical hemostasis are being covered.
11:40 Consensus definitions in rare bleeding disorders; Flora Peyvandi (Italy)
Over the past five years, the “Consensus definitions in Rare Bleeding Disorders” Project focused on the need for comprehensive data collection regarding the association between laboratory phenotype and bleeding severity in RBDs. Different networks and registries, established in Europe (European network of Rare Bleeding Disorders [EN-RBD]), United Kingdom (United Kingdom Haemophilia Centre Doctors' Organisation registry [UKHDCO]), USA (The North American Rare Bleeding Disorders Registry [NARBDR] and the American Thrombosis Hemostasis Network [ATHN] Rare Coagulation Disorder database) and India started to collaborate with an initial task of understanding what data are currently available through each system. We report the results of a review of available data that explored the association between residual plasma factor activity and the clinical bleeding profile for each of the RBDs. The results are based on three different sets of data:

1. A detailed review of the available English language literature from 1990 to March 2012 (search engine: Medline PubMed), this included all reports evaluating the laboratory phenotype and clinical characteristics and having a sample size ≥ 5 patients: a total of 51 relevant original publications were retrieved;

2. Overview of data from the UKHDCO, NARBDR and Indian registries on a total of 3745 patients affected with RBDs. The laboratory phenotype severity was categorized differently by the three registries. Clinicians reporting to the UKHDCO registry are not asked to classify RBDs by severity and do not report bleeding events. For this analysis data were analysed using the criteria defined in the Table. A minority of reported patients had severe deficiency (level not always reported).

3. Overview of data from the EN-RBD, which evaluated the association between residual plasma factor activity as a continuous metric and clinical bleeding severity in 489 patients with different RBDs.

Based on the interpretation of published data and the experiences from the networks and registries, it was evident that:

- There is a heterogeneous association between coagulation factor activity level and clinical bleeding severity in different RBDs. The strongest association was observed for fibrinogen, FX and FXIII deficiencies; although different thresholds are needed to ensure patients remain asymptomatic or to prevent major spontaneous bleeding.
- These data indicate that a more detailed evaluation on each single factor deficiency is required for future planning of optimal diagnosis and management; and that it is not appropriate to use a single criterion of classification for all types of RBDs.

The results of this project were submitted as Official SSC Communications to the Journal of Thrombosis and Haemostasis with a manuscript entitled “Classification of rare bleeding disorders (RBDs) based on the association between coagulant factor activity and clinical bleeding severity”. Laboratory phenotype classifications according to the three registries, compared to the laboratory classification of haemophilia.

DISCUSSION
The audience has been asked to comment on the manuscripts reporting results of these projects available on the ISTH website. All manuscripts should be hopefully published within 2012.

Saturday, 30th June, 2012
9.00 – 10.10 CLINICAL ISSUES
Session Chairpersons: Marilyn Manco-Johnson (USA), Carmen Escuriola Ettinghausen (Germany)
Dr Escuriola introduced the session.
The Clinical Issues Session addressed the most recent insights into inhibitor incidence, its risk factors, the potentially protective effect of prophylactic treatment, treatment of patients with inhibitors, and provided information on on-going studies on the same and related topics.

09:00 The incidence of inhibitors in long term experience in prophylaxis; Erik Berntorp (Sweden)
Eric Berntorp focusses on the incidence of inhibitors in long term experience in prophylaxis. Inhibitors develop in about 30% of patients with severe hemophilia A and up to 5% of those with severe hemophilia B (1). Various issues might affect inhibitor development, including type of hemophilia, factor VIII/IX mutation type, race, immune response genes and environmental factors. These factors include type of replacement therapy i.e. plasma derived vs. recombinant, purity of products, treatment regimen and age at start of treatment. Recently the risk of inhibitor development in hemophilia A in relation to long term prophylaxis has gained great interest and some studies have indicated a protected effect of prophylaxis (2,3) although certainly more studies are needed to be convincing (4). The so called danger theory proposed by Matzinger (5,6) has shed new light on the idea that early, long-term prophylaxis may prevent inhibitor development. If the inhibitor response leading to inhibitor development is an effect of immunological danger to the organism, rather than self-versus non-self, it is appealing to believe that less risk of bleeding during prophylaxis compared to on demand treatment gives less inflammation and less danger to the immune system. Striking results along this line have been published from Germany where institution of early prophylaxis in low dose and avoidance of obvious danger signals have dramatically reduced the inhibitor incidence (7,8). This preliminary experience has prompted a larger study Early Prophylaxis Immunologic Challenge (EPIC) Study (ClinicalTrials.gov NCT01376700) where patients (FVIII%2%) up to the age of one year will start on weekly prophylaxis 25±5 FVIII IU/kg and minimization of immunological danger signals very similar to what has been reported by Auerswald and colleagues. It is hard to believe that such a regimen will abolish the risk of inhibitor development, especially in patients with high risk FVIII mutations, but even a reduction by a few % will mean a lot for patients, their families and for society.

References

09:10 Research of determinants of inhibitor development among previously untreated patients with haemophilia (RODIN study); Marijke van den Berg (The Netherlands)
Marijke van den Berg presents on behalf of the RODIN Study group (www.rodinstudy.eu) the first findings from the RODIN Study. In the RODIN study, prospective data were collected from 29 haemophilia centres. The first analysis of May 2011 concerned 606 PUPS born between 2000 and 2010 with severe haemophilia A. In total 179 patients (32.0%) developed inhibitors, of whom 118 (22.2%) with high titre inhibitors. The rather high total inhibitor incidence can be explained by the fact that in this study all eligible patients were included, which means also patients with major bleedings in the neonatal period. Prophylaxis in severe haemophilia A has been associated with a decreased risk of inhibitory antibodies. In 412 patients prophylaxis was started during the first 75 exposure days, but only a small decrease of inhibitor incidence was demonstrated. In the early phase of factor VIII treatment, inhibitor incidence was not associated with prophylaxis; after about 20 exposure days, however, prophylaxis was associated with a slightly decreased inhibitor incidence. The relative risk of prophylaxis was 1.01 after 1 to 10 exposure days, 0.95 after 11 to 20 days, 0.22 after 21 to 30 days, 0.27 after 31 to 40 days and 0.32 after 41 to 75 exposure days. An effect of prophylaxis on inhibitor incidence was only observed for patients with low risk gene mutations. Furthermore, peak treatment moments of at least 5 days were associated with a 50% increased inhibitor incidence, while dose and frequency of dosing were not associated with an increase in incidence.

09:20 Extending prophylaxis around the world – What doses can we start with?; Alok Srivastava (India)
Dr Srivastava apologises for declining to make this presentation.

09:30 Immunotolerance induction using plasma derived products; Carmen Escuriola Ettinghausen (Germany)
Carmen Escuriola Ettinghausen presented the newest insights on immune tolerance induction using plasma derived products.

Immune tolerance induction (ITI) is a powerful approach to eliminate inhibitors. Success rates of ITI may vary depending on patient variables (e.g. inhibitor titre at start of ITI, maximum inhibitor titre, F VIII genotype etc) and on factors related to the therapeutic regimen (e.g. age at start of ITI, Interval between dx and start of ITI, dosage and frequency of F VIII etc). The product chosen for ITI particularly the presence of von Willebrand Factor (VWF) may play a supportive role in inducing immune tolerance to FVIII. Reports from Germany showed stable success rates around 90% using VWF-containing concentrates in the frame of the Bonn protocol (1). But in the early 1990s a substantial decline of ITI success rates to 29% after the introduction of F VIII concentrates lacking of VWF with an unchanged use of the Bonn protocol was observed (2,3). After a switch to VWF/FVIII complex concentrates the overall success rates of ITI of around 90% have been achieved again (2).

Since then VWF/FVIII complex concentrates have been used particularly in high responders with poor prognosis or as a salvage therapy after earlier ITI failure: complete or partial success could be achieved in 50 to 100% of these patients (4-9).

In vitro data showed a lower inhibitory activity against FVIII complexed with VWF compared to pure F VIII inhibitor plasmas with anti-C2-specificity (10-14). A higher recovery was observed when the infused F VIII concentrate contained VWF during the treatment of patients with haemophilia A and inhibitors.
against the light chain of F VIII (10,15). An association between the clinical outcome of ITI with the inhibitor epitope profile and the type of concentrate used has been described in a small patient cohort: in inhibitor patients with an anti-C2 specificity treated with a VWF-containing F VIII concentrate the inhibitor could be successfully eliminated whereas those who had an anti-A2 specificity failed ITI significantly more often. These findings suggest the possibility to tailor ITI to the individual demands of the inhibitor patient by testing the inhibitor epitope specificity or the inhibitory activity against different types of F VIII concentrates.

More results from the RESIST- as well as the ObsITI- study are warranted to evaluate success of ITI and to determine patient- and therapy-related variables influencing ITI outcome particularly such as the type of F VIII product.

References


09:40 International study project; Charles Hay (UK)

He presented an administrative report and plan of future data analysis and reports. Abstract will be soon available.

DISCUSSION

Questions and comments from the audience centred on the following areas:

1. Dr Berntorp showed that the incidence of inhibitors increased during the period 2000-2007 compared to 1980-1999. It was hypothesized that this could be due to 1) higher immunogenicity of new rFVIII products or 2) general increment of the autoimmune disease in children.

2. Details on the study methodology (quality of life, countries of origin, time interval, etc) were asked to the presenters.

3. It was asked if analysis based on brand and types of products had been carried out. Speakers reported that huge sample size would be necessary and meta-analysis could be the appropriate study design.

10:10-10.50 STANDARDSATION ISSUES

Session Chairpersons: Raimondo De Cristofaro (Italy) and Anthony Hubbard (UK)

Dr Hubbard introduced the session, explaining that Dr De Cristofaro had apologized for not being present due to personal reasons.

Biological standards remain the foundation for the quantification of coagulation factors in therapeutic products. The complexity of the clotting activity of factors II, VII and X cannot be measured using physicochemical methods and their estimation relies on the relative comparison of products with the relevant WHO International Standard (WHO IS) which has an agreed assigned value in International Units (IU). The IU convention has promoted global harmonisation in the content and labelling of therapeutic concentrates for over 3 decades and the need for the replacement of dwindling stocks of WHO IS is a measure of the success of this approach. The projects presented by Drs Gray and Thelwell describe the value assignment of the replacement WHO IS for the estimation of factors II, X and VII in purified products.

The estimation of inhibitory antibodies to FVIII remains a critical aspect of haemophilia care and initiatives to improve on current methodologies through facilitation of methodology and reduction of variability are required. Dr Raut presented a modification to the current Bethesda/Nijmegen method which involves the replacement of FVIII-deficient plasma by buffered normal plasma. This approach promises reduced cost of testing and the potential of reduced variability. Initial studies have indicated equivalent results to the conventional method.

10:10 4th International Standard for FII and X, Concentrate; Elaine Gray (UK)

Twenty-eight laboratories from 14 different countries participated in a collaborative study to value
assign the proposed 4th International Standard for Blood Coagulation Factors II and X, Concentrate (11/126) by assay relative to the WHO 3rd International Standard for Blood Coagulation Factors II and X, Concentrate (98/590). Overall intra-laboratory variability was low with over 70% of the laboratories having geometric coefficients of variation (GCV) of less than 5% indicating that the participants performed assays for factors II and X reproducibly and with high precision. Inter-laboratory variability was also low for estimates of both factors with GCV below 5%. There was a small but significant difference observed for the candidate preparation (11/126) between clotting and chromogenic methods for both factors II and X. For Factor II, the overall potency from all methods was 9.44 IU/ampoule, with inter-laboratory GCV of 3%. The estimate from the Prothrombin Time based assays (9.53) was 1% higher, and the chromogenic potency (9.24) was 2% lower. For Factor X, the overall potency was 8.13 IU/ampoule, with the Prothrombin Time and chromogenic potencies being 8.29 and 7.81 IU/ampoule (2% higher and 4% lower), respectively, and inter-laboratory GCV being 5%. Taking into consideration the low inter-laboratory variation and the relatively small discrepancies between the potencies by the different methods, it was proposed to assign the overall geometric mean potency of 9.4 IU/ampoule for FII and 8.1 IU/ampoule for FX to the proposed 4th International Standard for Blood Coagulation Factors II and X, Concentrate (11/126).

10:20 A report on the collaborative study to calibrate the WHO 2nd International Standard for Factor VII concentrate; Craig Thelwell (UK)

An international collaborative study was organized to calibrate a replacement for the WHO 1st International Standard for Factor VII concentrate (97/592). The study involved 24 laboratories from 11 different countries representing manufacturers, clinical and regulatory groups. Laboratories were asked to measure the FVII content of two freeze-dried candidate materials: sample A (10/250) and B (10/252), using clotting and/or chromogenic methods. For each laboratory, potencies were calculated for A and B relative to the WHO 1st IS, and the geometric mean potency was calculated for each of the candidates independently for clotting and chromogenic methods. Statistical analysis revealed significant differences in potencies determined by clotting and chromogenic methods for both candidates. In addition there was a significant assay method bias within the clotting results for candidate A, with laboratories using recombinant thromboplastin reagent producing lower potencies compared to those using a natural purified thromboplastin. For candidate B there was no method bias observed, however the potency determined by clotting methods was significantly higher than the chromogenic potency. Clotting methods for FVII potency are sensitive to the amount of activated FVII (FVIIa) present whereas chromogenic methods are not. Candidate B was found to contain higher levels of FVIIa compared to candidate A and this may explain the relatively higher potency for candidate B determined by clotting methods. Since the difference between the potency estimates by the different methods was too large to reconcile by a mean value it was proposed that candidate B (10/252) should be established as the WHO 2nd IS for factor VII concentrate with a potency of 9.8 IU for chromogenic methods and 10.6 IU for clotting methods.

10:30 FVIII Inhibitors Assay (SMIA): A new approach in measurement; Sanj Raut (UK)

Previous studies have shown high variability between laboratories when measuring FVIII inhibitors in patient samples, with CVs ranging from 40-200%. The Nijmegen Modification is currently the gold standard inhibitor assay, in which patient’s inhibitor titres are measured relative to a "Control" mixture consisting of equal volumes of buffered normal pooled plasma and FVIII-deficient plasma (FDP). This project questioned the need for FDP, which was introduced into the assay as a “like for like” diluent for the Reference "Control" and which may actually introduce variability due to the many different FDPs now commercially available. As the inhibitor titre is based on % of FVIII in the Reference ("Control"), it was hypothesised that it may be possible to substitute the FDP with a more “like for like” diluent such as
buffered normal plasma. The unknown inhibitor titre in this modified assay (South Mimms Inhibitor Assay - SMIA) is expressed relative to 200% FVIII in the Reference “Control” (rather than 100% FVIII previously). This approach may remove the variability of FDP and significantly reduce the cost of an inhibitor assay. Results showed that, for high titre samples (5 - 40 BU/ml), equivalent inhibitor titres could be obtained using the Nijmegen method and the modified method. For low titre (1.0 - 0.15 BU/ml) samples, the Nijmegen method was able to detect inhibitor titres down to ~0.6 BU/ml, whilst SMIA was able to detect inhibitor titres down to ~0.2 BU/ml.

DISCUSSION

Dr Sanj Raut was asked to propose a new project within the FVIII&FIX SSC on the use of the SMIA assay for the evaluation of clinical outcomes.

11:00-12:45 CRITICAL ISSUES ON EVALUATION OF EFFICACY OF TREATMENT IN HEMOPHILIA

Session Chairpersons: Donna DiMichele (USA) and Steven Pipe (USA)

Dr Pipe introduced the session, communicating that Dr DiMichele had not been able to attend the meeting.

Global tests of blood coagulation were superseded by more specific tests as the mechanism of blood coagulation unfolded in the latter part of the 20th century. Specific assays used in clinical trials demand the use of national and international standards. Each assay result depends on the precise nature of the substance to be measured, the standard, assay system and reagents used and even the equipment employed. In particular, recombinant factors have special assay characteristics which can be difficult to manage, especially when results from clinical trials are extrapolated to the various assay platforms and standards used in clinical practice.

Potency assignment and biological activity of FVIII and FIX products are expected to correlate with in vivo efficacy and plasma activity measured in clinical assays. Within the constraints of current regulatory guidelines, manufacturers have limited means of assigning potency to a novel product, either by a one-stage clotting assay in the case of FIX, or, additionally, by a chromogenic substrate assay for FVIII, both of which must be calibrated against the respective WHO standard. FVIII:C quantitation with chromogenic and the classical one-stage assay may differ up to 40% and dose adjustments may be necessary depending on the region.

With the recent advent of novel, modified FVIII or FVIIa products with increased clotting efficiency and/or prolonged half-life, clinical trials are changing from replacement therapy trials to pharmacological trials with quantifiable clinical endpoints. Classical clotting or chromogenic assays to quantify the modified products may not be applicable any longer or may need assay modification (e.g. different activators). Nevertheless regulatory bodies still require objective and laboratory based evidence to support claims of efficacy and safety in clinical trials relating to the expansion of use of existing products and the development of new products. One needs to ensure that current clinical assays can accurately determine the activity of these novel products in plasma samples. This is best demonstrated through comprehensive field studies that include reagents, instruments and calibrators commonly used in clinical hemostasis laboratories.

Another solution being explored is the use of global hemostasis assays such as thrombin generation assay or thromboelastography that do not measure the infused compound directly but rather quantify and qualify the response of the hemostatic system to the therapy. This approach has the potential to not only exclude product related specific assay artifacts but to also personalize therapy by
individualization of dosing while maintaining effective factor levels in the patients to prevent bleeding. Global clotting assays may attain greater relevance in the description of the pharmacodynamic actions of the novel products and their ability to serve as surrogate markers for efficacy. However, standardization and pre-analytical challenges still need to be resolved.

An alternative approach would be to design assays that specifically measure the procoagulant activity of modified coagulation factors. In one example, an assay was developed that allows selective determination of CHO cell derived recombinant human proteins in the presence of their human plasma-derived equivalent. These examples of new assay strategies – global hemostasis assays and highly product specific assays – have the potential to substantially improve the array of laboratory endpoints used in clinical trials investigating novel molecules to treat hemophilia and related disorders.

In contrast to replacement therapy, the common clinical laboratory coagulation assays of clotting factor activity do not reflect the clinically relevant hemostatic activity of bypassing agents. Furthermore, no validated assay is available to measure the in vivo efficacy of these agents or predict individual patient responses to treatment. Recently published data from one prospective trial in haemophilia patients with inhibitors undergoing surgery have shown a correlation between in vivo clinical response to the bypassing agents and thrombin generating capacity in a TGT assay.

Novel recombinant factor VIIa molecules also introduce unique assay challenges. The design of one molecule, by example, reduces the affinity of anti-FVII commercial antibodies leading to miscalculation of antigen level as compared to the native FVII. One company has developed a product specific ELISA utilizing a product specific antibody enabling a more accurate approach for its quantitation.

The session discusses all of these approaches in turn.

**11:00 Measuring clinical efficacy and laboratory parameters in patients treated with porcine factor VIII**; **Martin Lee (Inspiration Biopharmaceuticals)**

The advent of recombinant porcine factor VIII has meant the renewed ability to treat patients with auto- and allo-antibodies to human factor VIII with a biological agent that allows for the direct measurement of the circulating levels of the infused material. However, this brings with it the challenge of assaying porcine factor VIII in human plasma (which is additionally compounded by the fact that this product is B-domain deleted), as well as the determination of anti-porcine antibodies (which, of course, requires a directed factor VIII assay). We have considered the effect of assay methodology (one-stage clotting assay versus chromogenic) as well as the standard used (plasma versus plasma-based concentrate versus recombinant-based concentrate). These comparisons shed light on the differences and similarities with the usual issues raised with assaying human recombinant factor VIII and inhibitors. Clearly, resolution of these factors is key to the appropriate and titrated use of porcine factor VIII in patients with either acquired hemophilia or congenital hemophilia A with inhibitors. We report on an inter-laboratory study of the assay and standard differences recorded and provide recommendations for clinical and laboratory use.

In addition, we will comment on a scale that is being used to evaluate clinical efficacy when recombinant porcine FVIII is used in surgery or a serious bleeding episode in these patients.

**11:10 Development of new rFVIII and rFIX molecules: clinical and laboratory evaluation of safety and efficacy**; **Stephanie Seremetis (Novo Nordisk)**

The author asked to change the tile of the presentation to: "Vatreptacog alfa: Assays in the
Vatreptacog is a rFVIIa analogue with three point mutations in the protease domain at amino acid positions 158, 296 and 298. Vatreptacog alfa has a conformation similar to that of tissue factor (TF) - bound FVIIa, even in the absence of TF. On the surface of activated platelets this translates to greater activity than rFVIIa, resulting in a fast and large thrombin burst and the rapid formation of a tight and stable clot. On the other hand, in the presence of TF similar activity is measured for vatreptacog alfa and rFVIIa. Bypassing agents do not restore the normal pathways of hemostasis in hemophilia, but rather boost thrombin generation in spite of a lack of platelet-surface FVIIIa/FIXa (“tehase”) activity. In contrast to replacement therapy, the common clinical laboratory coagulation assays of clotting factor activity do not reflect the clinically relevant hemostatic activity of bypassing agents. Furthermore, no validated assay is available to measure the in vivo efficacy of these agents or predict individual patient responses to treatment. Recently published data from one prospective trial in haemophilia patients with inhibitors undergoing surgery have shown a correlation between in vivo clinical response to the bypassing agents and thrombin generating capacity in a TGT assay.

In our phase 2 clinical trial for vatreptacog alfa we have focussed on securing robust and reliable safety assays including measures of pharmacokinetics and potential antibody formation. For the PK analysis we used the standard FVIIa clot assay.

The development of antibodies is followed closely in all clinical trial by a stepwise tiered approach using validated antibody assays. Samples are collected on a regular basis and screened for presence of antibodies binding to vatreptacog alfa using a radio-immuno assay. If any antibodies are detected these will be further characterised with respect to cross-reactivity to FVIIa and neutralising capacity. The neutralising effect of the antibodies will be investigated using two functional clot assays. One assay detects antibodies specifically neutralising vatreptacog alfa, and one assay detecting cross-reacting antibodies neutralising endogenous FVIIa.

11:20 Evolution of clinical trials in hemophilia: From simple replacement to pharmacological evidence; Rupert Sandbrink (Bayer)

Dr Prasad Mathew made this presentation on behalf of Dr Sandbrink

Until recently, clinical development in hemophilia was predominantly replacement therapy with wild-type factor VIII or IX concentrates or recombinant versions thereof. Pharmacokinetic demonstration of the pertaining factor activity in the plasma at expected levels and duration was usually sufficient to reassure pharmacodynamic action. With the emergence of the two-stage chromogenic substrate assay (CSA) to measure FVIII:C in hemophilia A and its adoption for release of FVIII products in the EU, issues arose for some FVIII products. FVIII:C quantitation with CSA and the classical aPTT based one-stage clotting assay may differ up to 40% and dose adjustments may be necessary depending on the region. The clinical impact of this assay issue is currently investigated in the clinical development of the full-length FVIII product BAY 81-8973.

With the recent advent of novel, modified FVIII or FVIIa products with increased clotting efficiency and/or prolonged half-life (e.g. BAY 94-9027; BAY 86-6150), clinical trials are changing from replacement therapy trials to pharmacological trials with quantifiable clinical endpoints. Classical clotting or chromogenic assays to quantify the modified products may not be applicable any longer or may need assay modification (e.g. different activators). Global clotting assays such as RoTEM and TGA may attain greater relevance to describe the pharmacodynamic actions of the novel products. However, standardization and pre-analytical challenges still need to be resolved.
Correlation of Potency Assignment and Clinical Performance of rFVIII-Fc and rFIX-Fc Fusion Proteins through Field Studies, Ex Vivo Coagulation and Global Hemostasis Assays; Jurg Sommer (Biogen Idec)

Potency assignment and biological activity of FVIII and FIX products are expected to correlate with in vivo efficacy and plasma activity measured in clinical assays. Within the constraints of current regulatory guidelines, manufactures have limited means of assigning potency to a novel product, either by a one-stage (OS) clotting assay in the case of FIX, or by a chromogenic substrate (CS) assay for FVIII, both of which must be calibrated against the respective WHO standard. In the case of the recombinant FVIII-Fc fusion protein (rFVIIIFc), both the OS and CS assays provide comparable results with a specific activity equivalent to that of native FVIII. Plasma FVIII level by the OS assay may thus be an appropriate surrogate marker for monitoring the activity and pharmacokinetics of this product during the clinical trials. The in vitro specific activity of rFIXFc is approximately 50% lower than that of native FIX by both the OS and the CS assays and this activity correlates with in vitro thrombin generation activity. To further verify that the potency assignments for rFVIIIFc and rFIXFc reflect their in vivo activity, global hemostasis assays (TGA and ROTEM) are being performed as part of the pharmacokinetic analysis in the clinical efficacy (Phase III) studies. These exploratory tests are expected to demonstrate comparable ex vivo thrombin generation and hemostatic activity for rFVIIIFc and Advate® (or rFIXFc and BeneFIX®) at equivalent factor FVIII/FIX levels. Ex vivo hemostatic activity of these products should also be consistent with their respective PK profiles. Lastly, in order to successfully monitor rFVIIIFc and rFIXFc therapy in patients, one needs to ensure that current clinical assays can accurately determine the activity of these novel products in plasma samples. This is best demonstrated through comprehensive field studies that include reagents, instruments and calibrators commonly used in clinical hemostasis laboratories.

Clinical Research Challenges in Developing Novel Compounds to Treat Hemophilia: The rIX-FP and rVIIa-SingleChain Experience; Debbie Bensen-Kennedy (CSL Behring)

Abstract will be soon available

Standardised specific assays or general tests of haemostatic potential? Pfizer’s view on precise science or clinical relevance; Brian Colvin (Pfizer)

Global tests of blood coagulation were superseded by more specific tests as the mechanism of blood coagulation unfolded in the latter part of the 20th century. Specific assays used in clinical trials demand the use of national and international standards. Each assay result depends on the precise nature of the substance to be measured, the standard, assay system and reagents used and even the equipment employed. In particular, recombinant factors have special assay characteristics which can be difficult to manage, especially when results from clinical trials are extrapolated to the various assay platforms and standards used in clinical practice. To address the latter challenge Pfizer has utilized two approaches for management of clinical FVIII coagulation factor assays. One BDD rFVIII product (Xyntha) has a potency assignment aligned to the one stage clotting assay that is commonly used in clinical practice. For the other BDD rFVIII product (ReFacto AF) a product specific assay standard has been introduced.

Perhaps more challenging is the utility of factor VIII and IX assays and pharmacodynamics as surrogate markers for efficacy. Pfizer is currently considering the importance and relevance of the factor IX assay in relation to the clinical efficacy and optimal use of BeneFIX in weekly prophylaxis. Preliminary results suggest that a clinical effect may extend further than the results of laboratory analysis might imply.

In the management of inhibitors, no bypassing agent so far developed has provided the level of reliable therapeutic response associated with the use of factor VIII or IX concentrates in treatment of the inhibitor-free patient. Dosage estimation and frequency of administration for aPCCs and rVIIa have
never been based on specific assays and the monitoring of treatment for inhibitor patients continues to be guided by clinical criteria. Use of pharmacodynamic markers to measure therapeutic effect, while part of the pre-clinical characterization for Pfizer’s investigational rFVIIa variant, remains experimental and, to date, has not been validated for guiding treatment of the inhibitor patient.

Nevertheless regulatory bodies still require objective and laboratory based evidence to support claims of efficacy and safety in clinical trials relating to the expansion of use of existing products and the development of new products. Pfizer will discuss these issues in the context of current clinical studies of BeneFIX and preclinical trials of a modified rFVIIa.

12:00 Novel assays for novel hemophilia therapies – Practice of clinical testing during clinical studies 2012 and beyond; Peter Turecek (Baxter)

Accurate dosing is key for treatment success with any medication. Due to the broad therapeutic range of coagulation factors in hemophilic patients safety issues in hemophilia therapies related to misdosing are mainly related to under-dosing and result in lack of efficacy. Also hemophilia therapies are costly and therefore - at least in certain geographies and with specific treatment regimens - only minimally required amount of factor is used for treatment of patients. Under these circumstances it represents a serious issue when potency assignments do not match the factor activity recovered and the one measurable in patients - as had been an issue for B-domain deleted rFVIII for more than a decade [1]. Recent recommendations therefore request post-infusion testing against a product reference composed of the same material as that which is infused. At the same time manufacturers should establish a relationship between the dose based on the labeled potency and the expected factor recovery in the patient. This can be particularly challenging as demonstrated by the example of a cysteine modified B-domain deleted FVIII that showed a severe discrepancy between the one-stage clotting and the chromogenic assay [2].

One way out of this situation would be to use global hemostasis assays such as thrombin generation assay or thromboelastography that do not measure the infused compound directly but the response of the hemostatic system to the therapy. This would not only exclude product related specific assay artifacts but would also personalize therapy by individualization of dosing while maintaining effective factor levels in the patients to prevent bleeding. An alternative approach would be to design assays that specifically measure the pro-coagulant activity of modified coagulation factors. We recently developed and validated a novel modification dependent activity assay (MDAA) to selectively measure PEGylated human recombinant factor VIII in human plasma without interference from non-PEGylated FVIII. The MDAA combines the use of an anti-PEG antibody with a FVIII activity assay in an ELISA like assay system.

A completely different challenge comes from the fact that current assays for coagulation factors do not differentiate recombinant from endogenous plasmatic proteins. This is an issue for treatment monitoring of mild forms of factor deficiency where endogenous levels of protein are existing as background. To allow measurement of a recombinant protein in the presence of the plasmatic equivalent we established a platform that allows selective determination of CHO cell derived recombinant human proteins in the presence of their human plasma-derived equivalent. This was first developed for determination of recombinant von Willebrand factor (rVWF) and was used during the preclinical drug development phase in animal studies. The assay principle is a lectin based immune assay with specific detection of VWF. Validation showed that the assays was highly specific for the CHO derived protein and could quantitatively measure rVWF derived from CHO cells in the presence of plasma-derived VWF. The use of this assay could be expanded to determination of rVWF in mild forms of human VWD with circulatory levels of endogenous VWF.
These examples of new assay strategies described here—global hemostasis assay and highly product specific assays—have the potential to substantially improve the array of laboratory endpoints used in clinical trials investigating novel molecules to treat hemophilia and related disorders.

References


12:10 A clinically validated technology for elongating the half life of coagulation factors, enabling a prolonged haemostatic activity in hemophilic animal models; Gili Hart (Prolor Biotech)

PROLOR Biotech Inc. (NYSE: PBTH) is a clinical stage public company developing biobetter long acting versions of therapeutic drugs utilizing CTP technology. CTP is a naturally occurring peptide evolved to provide long durability of hCG and has the potential to dramatically reduce injection frequency, drug load and side-effects for most therapeutic proteins. CTP is already clinically validated - Merck’s long acting FSH-CTP named Elovena® received EU marketing approval in 2010. PROLOR’s current pipeline includes long acting human growth hormone, long acting FVIIa and long acting anti obesity/type II diabetes drug. PROLOR recently announced successful completion of a Phase II trial in GH deficient adults, initiation of phase II study in GH deficient children and expects to initiate its Phase III in 2012. PROLOR has developed a long acting Factor VIIa-CTP, demonstrating a markedly enhanced pharmacokinetics, increased exposure, increased recovery and a prolonged haemostatic effect in hemophilic animals model with a comparable biological activity. PROLOR is in the initial stage of preparations for Phase I-IIa study in hemophilic patients, and due to the nature and properties of the modified, long acting FVIIa, the company is facing the following challenges in the in-vitro and in-vivo characterization of the product.

1. FVIIa-CTP quantitation: Assessment of FVIIa-CTP Ag level in -v vitro and in-vivo is performed using two methods, FVII ELISA and A280. Following the attachment of CTP the FVII content is reduced, requiring an analytical adjustment for A280 calculations. The presence of the highly O-glycosylations on the CTP portion of the molecule reduces the affinity of anti FVII commercial Abs leading to miscalculation of Ag level as compared the native FVII is. The company has developed a FVII-CTP specific ELISA, utilizing a product specific Ab enabling a more accurate approach for its quantitation.

2. Evaluation of FVIIa-CTP clotting activity: One of the major challenges in the preparation for the non-clinical and clinical studies is assessing FVII-CTP and FVIIa-CTP in vitro and in vivo activity, utilizing the well established methods. The fusion of CTP reduces the relative content of FVIIa, therefore the specific activity is lower. A conversion factor, considering the molar ratio between FVII and CTP was established and might lead to further adjustment of the injected doses in clinical setting.

3. CTP Immunogenicity paradigm: The immunogenicity of FVIIa-CTP is an important parameter in the ongoing non-clinical and planned clinical trials of the product. Fusion of CTP to FVIIa can affect the structure and properties of the molecule. Moreover, the enhanced longevity and exposure are additional unknown factors. Generation of anti-FVIIa Abs in hemophilic patients with inhibitors is a major concern, therefore a detailed immunogenicity paradigm for the
detection and characterization of anti FVIIa-CTP antibodies was developed according to FDA and EMA recommendations.

DISCUSSION
Questions and comments from the audience centred on the assay methods used to detect inhibitor and test the potency labelling of new products and the utility of the global assays. Manufacturers set up their own methods and criteria to evaluate new products. Therefore it was suggested that FVIII&FIX ISTH-SSC constitutes a committee to establish, in collaboration with manufacturers, common criteria to study new products. The FVIII/FIX SSC agreed on the idea that industry can have a mature relationship with the medical and scientific community and with bodies such as ISTH.
Factor XI and the Contact System

28 June 2012

Chairman: Thomas Renne (SE)
Co-chairs: Jonas Emsley (UK), David Gailani (USA), Christine Mannhalter (Austria), Keith McCrae (USA), Joost Meijers (The Netherlands), Ophira Salomon (Israel)

The SSC session was extremely well attended with more than 200 participants. We had 10 presentations including an overview lecture by Dr. Bolton-Maggs on clinical and diagnostic aspects of FXI deficiency. Presentations covered the three topics "Genetics and Genomics", "Activation of factor XII”, and "Novel functions of FXI and the contact system in thrombosis”. All presentations were lively and comprehensively discussed.

1. Paula Bolton-Maggs, Manchester: “Factor XI - setting the scene”

Dr. Bolton-Maggs gave a comprehensive overview on bleedings and therapeutic options in FXI-deficient patients (hemophilia C). One third of FXI-related bleeding episodes occur in women at childbirth. The majority of hemophilia C patients are CRM negative and in a significant portion of these individuals FXI antigen/clotting in analytical tests does not correlate with clinical severity of bleeding. aPTT tests are insufficient in characterizing bleeding risk in FXI deficiency. As low levels of other coagulation factors may contribute to the clinical picture of bleeding in such patients. Dr. Bolton-Maggs presented results of real time thrombin formation assays (ETP) triggered by low concentrations of tissue factor in hemophilia C patient plasmas. ETP correlated with bleeding risk in various patients and may represent a suitable diagnostic tool for assessing bleeding in FXI deficiency. Management options including rFVIIa and FXI infusions were discussed. The therapeutic use of low-dose recombinant FXI in treatment of FXI deficient patients with FXI inhibitors was also delineated.

2. Christine Mannhalter, Vienna: "Genotypic variations of complement factors and phenotypic effects”

Dr. Mannhalter presented interactions of the coagulation and complement systems. The focus of her presentation was complement factor C5. She presented an overview on C5 SNPs that correlate with C5 plasma levels and risk of thrombotic diseases. High levels of C5 represent a risk factor for stent restenosis. Dr. Mannhalter highlighted the role of platelets and platelet activation as a possible link between the coagulation and complement cascades. Platelets seem to contribute to localize thrombosis in areas of infection through both procoagulant mechanisms and activation of the classical complement pathway.

3. Gordon Lowe, Glasgow: “Epidemiology and genetics of aPTT and contact factors”

Dr. Lowe presented recently published genome-wide linkage analyses that have identified genes that affect the aPTT. About 60% of shortened aPTT is attributed to inherited factors. F12, KNG1 and HRG genes were identified to be the main regulatory genes of aPTT shortening. Five genes account for about 30% of inherited effects on aPTT. Since the FXI and contact system proteins have critical functions in thrombosis albeit having minor roles in hemostasis, the work of Dr. Lowe identifies new SNPs associated with a prothrombotic risk and novel targets for safe anticoagulation.
4. Jonas Emsley, Nottingham: "Structure of the FXII protease domain"

Dr. Emsley presented the crystal structure of the FXII enzymatic domain. He characterized kinetics and activation of recombinant FXII protease domain mutants. Key findings of the FXIIa structure are: (i) the FXII S1 substrate pocket is malformed, (ii) the FXII S1 site shares similarities with chymotrypsin, (iii) Aspartates switch to the active site. He characterized FXIIa docking onto FXI. SAXS analysis indicated an elongated FXII zymogen structure comprised of a stalk and head region.

5. Coen Maas, Utrecht: “Functional insights into FXII contact activation”

Activated FXII has the capacity to activate its substrates FXI and PK. Regulatory mechanisms and substrate specificity of FXIIa are poorly understood. Dr. Maas presented FXIIa mutants in those cleavage sites involved in activation. Specificity of the mutant proteins was characterized using chromogenic substrates. Two forms of FXII were identified which either cleave PK or FXI? depending on which cleavage takes place in the FXII activation loop. He demonstrated that the FXII protease domain may have tryptase-like activities.


Dr. Mutch presented a detailed and comprehensive overview of her work addressing FXII activation on polyphosphate polyP70. Her data indicate existence of a FXIIa single chain form, which activates PK and FXI in the presence of short chain polyP.


Dr. Phillipou showed that targeting contact system activity using CTI significantly affects the fibrin structure. Increased FXII activity thickens fibrin fibers whereas FXII inhibitors reduced the fibrin meshwork. The underlying mechanisms were discussed and potential implications for embolic disease were shown.

8. Henry Spronk, Maastricht: “Contact system activation by microparticles”

Dr. Spronk compared real time thrombin formation and phosphatidylserine (PS) exposure of microparticles from various circulating cells. Platelet-derived particles generate thrombin in a FXIIa-dependent manner. The underlying mechanism is a matter of ongoing analysis. In contrast, monocyte-derived microparticles are procoagulant due to TF exposure.

9. Judith Cosemans, Maastricht: “Targeting FXII inhibits the pathological process of thrombus formation on ruptured plaques in vivo and in vitro”

Dr. Coseman analyzed the role of FXII and FXI in thrombus formation using flow chamber systems using murine and human blood and ultrasound-driven plaque rupture in mice. She showed that FXIIa blockage increases thrombus embolization. FXII has implications in thrombus stability whereas TF determines the thrombus size.

10. Steffen Rosén, Mölndal: A chromogenic-FXIIa method with low interference for in-process and final testing of immunoglobulin preparations.
Dr. Rosén explored a new chromogenic FXIa method with a sensitivity of 0.015 pM FXIa for testing of IgG samples as an alternative to NAPTT and thrombin generation methods. There is no use of plasma in this assay. A sample dilution of 1:40 was recommended and interference from sample matrix is then claimed to be minimized as shown by recovery of added FXIa to IgG samples. Interference from contaminating proteins is also minimized with no effect of kallikrein up to 50 nM in the undiluted sample neither in the absence nor presence of FXIa and zymogen FXI. Furthermore, any contaminating zymogen FXI is not activated in the assay.
Factor XIII and Fibrinogen

30 June 2012

Chairman: Hans P. Kohler (CH)
Co-Chairmen: Moniek PM de Maat (The Netherlands), Aida Inbal (Israel), Marguerite Neerman-Arbez (Switzerland), Helen Philippou (UK), Verena Schroeder (Switzerland), John W Weisel (USA), Sanj Raut (UK)

Dr Hans Kohler (CH) opened the session.

Dr Sanj Raut (UK) gave an update on the international collaborative study for the value assignment of the 2nd International Standard for Fibrinogen, Concentrate. Due to earlier discrepancies in functional fibrinogen levels obtained with Clauss assay or CLOTr method, an additional field study was performed. Data obtained with the Clauss method showed higher interlab variability (17%) than the CLOTr method (<4%). When fibrinogen concentrates were measured against 2nd IS (09/242) there was less shift in results by the CLOTr method. From these result it has been concluded that the Clauss assay is an inappropriate method for value assignment. A proposal was made to assign a value of clottable protein based on the CLORr method. The participants supported that proposal.

Dr Marguerite Neerman-Arbez (CH) presented a new zebrafish model for fibrinogen deficiency. It is an easy-to-handle in vivo system that allows to visualise in vivo clot formation and the influence of highly conserved coagulation proteins. It may therefore be of interest to other researchers in the field.

Dr Robert Ariëns (UK), on behalf of Dr Marlien Pieters (ZA), presented an international collaborative study on the standardisation of the permeability measurements of fibrin clot structure: methodological considerations and implications for healthy control values. At the SSC meeting in Vienna in 2008, interest was expressed to standardise methods for fibrin network characterisation (incl. permeability, turbidity, and microscopy), because of high degree of variability. It was decided to start with standardisation of clot permeability. After collecting protocols from different labs, a standardised method was written. A feasibility study has been performed in five labs to 1.) investigate if standardisation of a method is possible and 2.) if lyophilised plasma can be used. Participating labs used their own method and the standardised method. Results showed an improvement in variability with the standardised method and confirmed that lyophilised and frozen plasma can be used. However, the operators experience is crucial. A position paper on this subject is currently under review at JTH.

Dr Moniek de Maat (NL) gave an overview on fibrinogen gamma’ measurements. Fibrinogen gamma’ plays a pleiotropic role in haemostasis, it binds thrombin and FXIII but not platelets, and influences clot structure and thrombotic risk. However, measurements of fibrinogen gamma’ is not standardised. Three different ELISA principles used by different groups were presented and the different ways of expressing the results (absolute values, gamma’/gamma A ratio, gamma’/total fibrinogen ratio. It was concluded that standardisation of ELISA methods is not of priority, however, for better comparability it is important to express results in all different ways (as described above) in future studies.
Dr Helen Philippou (UK) presented a method for in vivo measurement of FXIII activity and fibrin formation in real time. Two in vivo mouse models were established using ferric chloride injury, intravital microscopy, and FITC and Alexa imaging. Different software methods for image analysis (in-house macro for Image J, Imaris, and Slidebook) were compared. The in vivo models presented can be used to study mechanisms of clot formation and for future anticoagulant drug testing.

Dr Ian Jennings (UK) reviewed the current practice in investigation of FXIII deficiency showing data from UK NEQAS (Blood Coagulation). In a survey carried out in 1998, 129/137 labs performed the clot solubility test for diagnosis of FXIII deficiency. The results showed high variability and a high number of false positive and false negative results. In the latest survey carried out in 2011, this test was still used in around 50% of participating labs. The other half of labs did use quantitative assays; however, also these tests showed high variability ranging from 0.5 to 17 U/dl. Highest variability was observed for the Siemens assay (0.8-17 U/dl) depending on the automated platform used. Low variability was found for the HemosIL antigen test (0.5–4U/dl). The SSC guidelines as published as a position paper in 2011 in JTH will hopefully be implemented soon.

Dr Vytautas Ivaskevicius (DE) presented the first case of FXIII-A type II deficiency. Two cases of heterozygous FXIII deficiency due to mutations at position 37 of the FXIII A-subunit were presented. Arg37Pro leads to Type 1 deficiency with reduced antigen and activity levels, and Arg37Gln leads to Type II deficiency with reduced activity but normal antigen levels.

Dr Hans Kohler (CH) gave an update on the project on value assignment for FXIII B-subunit (total and free B) to the WHO 1st International Standard FXIII Plasma (02/206). A feasibility study is planned after additional experiments on binding of free B to fibrinogen which could interfere with ELISA measurement have been performed by Robert Ariëns, Helen Philippou and Laszlo Muszbek.

Dr Laszlo Muszbek (HU) reviewed the role of FXIII-B: just a carrier of FXIII A-subunit or a plasma protein of its own right. Dr. Muszbek presented new results showing that FXIII B-subunit binds to staphylococcus aureus protein A (SpA) which has a role in immune evasion. Binding of FXIII-B to SpA might interfere with this mechanism and promote phagocytosis by neutrophils.

Dr Akitada Ichinose (JP) presented the Japanese criterion 2012 for the diagnosis and treatment of acquired hemorrhaphilia XIII/13. A recent nationwide survey in Japan showed an unexpected high number of cases with acquired FXIII deficiency. Dr Ichinose emphasised the importance of rapid diagnosis and treatment guidelines to improve the outcome for patients.

Dr Shoichi Kawato (JP) presented a novel point-of-care test for the detection of anti-FXIII antibodies in patients with autoimmune hemorrhaphilia XIII/13 by immunochromatography. Three novel monoclonal antibodies against A2B2 were developed. Two methods (direct ICT method, spiked ICT method) were evaluated and showed very promising results compared to conventional dot blot assays for the detection of FXIII autoantibodies in patient samples.

Hans P Kohler, July 2012
Fibrinolysis

28 June 2012

Chairman: Ann Gils (BE)
Co-chairmen: Jonathan Foley (USA), Paul Kim (Canada), Osamu Matsuo (Japan), Nicola Mutch (UK), Craig Thelwell (UK), Shirley Uitte de Willige (The Netherlands), Tetsumei Urano (Japan)

Craig Thelwell: An update on WHO International Standards

Craig gave an update on three new international standard preparations.

The WHO 2nd International Standard for High Molecular Weight Urokinase

The existing WHO 1st International Standard (IS) for High Molecular Weight Urokinase, 87/594, is running out and a replacement is required. A preparation of high molecular weight urokinase was provided by a manufacturer, formulated, filled and freeze dried into 4700 sealed glass ampoules coded 11/184. An international collaborative study was organised to calibrate the candidate 2nd WHO IS for High Molecular Weight Urokinase, 11/184 against the current IS, 87/594, which has an assigned potency of 4300 IU per ampoule. A total of 15 laboratories were recruited to take part in the study and 14 laboratories from 10 different countries laboratories returned results comprising 55 independent assays. The candidate IS 11/184 was determined to have a potency of 3238 IU/ampoule with an overall geometric coefficient of variation (GCV) of 7.1%. Preparation 11/184 is therefore proposed as the WHO 2nd International Standard for High Molecular Weight Urokinase with a potency of 3200 IU per ampoule. This proposal was agreed by the study participants and an SSC expert review panel with no objections raised. The study is planned for submission to ECBS for approval October 2012.

Update on SSC Subcommittee Project on D-dimer Standard Development

A project has been endorsed by ECBS/WHO to try to develop a new International Standard for D-dimer, and an SSC Subcommittee Project has been registered. Hallamshire hospital (UK) has provided 30 pools of patient plasma containing high levels of D-dimer. A total of 280 ml was filled and freeze-dried in 0.5 ml aliquots to give 525 ampoules with 0.19% moisture. Samples have been subjected to accelerated degradation conditions at -20, 4, 20, 37 and 45 °C for 6 months to investigate stability. Stability studies on accelerated degradation samples have been performed using a variety of assay methods after 6 and 15 months and significant degradation was found at the higher storage temperatures. The loss of D-dimer antigen on storage at elevated temperatures was not anticipated and so far it has not been possible to accurately predict a shelf life for this preparation of D-dimer standard. The level of instability indicated would not be good enough to establish a WHO standard and the causes for this loss of activity and ways of improving stability are being investigated.

Program to replace the WHO 3rd IS for Plasmin

The WHO 3rd IS for Plasmin (97/536) is running out and a replacement is required. There is renewed interest in plasmin as a direct acting thrombolytic and is currently in developmental Phase II for the treatment of acute peripheral arterial occlusion, and Phase I for acute ischemic stroke. A therapeutic grade plasmin is the preferred choice for candidate material and a potential source has been identified.
An international collaborative study is planned to calibrate the new IS relative to the 3rd IS (97/536) in International Units based on chromogenic and/or fibrinolytic methods. Dual labeling with a molar concentration of active plasmin will also be investigated using active-site titration. Participants for the study are required and an invitation/questionnaire is planned for the ISTH website and to be sent to customers of the Plasmin IS directly. Information will be requested on how the IS is currently used, which assay methods are employed and on availability to participate in the study.

Jonathan Foley: Measuring the mechanical properties and fibrinolytic potential of blood clots formed via the tissue factor pathway of coagulation

Thrombelastography (TEG) is a method that is used to conduct global assays that monitor fibrin formation and fibrinolysis and platelet aggregation in whole blood. The purpose of this study was to use a well-characterized tissue factor (TF) reagent and contact pathway inhibitor (corn trypsin inhibitor, CTI) to develop a reproducible thrombelastography assay. In this study, blood was collected from 5 male subjects (three times). Clot formation was initiated in whole blood with 5 pM TF in the presence of CTI, and fibrinolysis was induced by adding tissue plasminogen activator (tPA). Changes in viscoelasticity were then monitored by TEG. In quality control assays, our TF reagent, when used at 5 pM, induced coagulation in whole blood in 3.93 ± 0.23 min and in plasma in 5.12 ± 0.23 min (n=3). In TEG assays, tPA significantly decreased clot strength (maximum amplitude, MA) in all individuals but had no effect on clot time (R time). The intraassay variability (CVa<10%) for R time, angle, and MA suggests that these parameters reliably describe the dynamics of fibrin formation and degradation in whole blood. The LY60, a fibrinolytic parameter, has a very high CVa meaning that it is not a reliable parameter for describing fibrinolysis in this particular assay. Our TF reagent reproducibly induces coagulation, making it an ideal tool to quantify the processes that contribute to mechanical clot strength in whole blood.

Sherley Uitte de Willige: Alpha-2-antiplasmin; a heterogeneous fibrinolysis inhibitor

The main function of alpha-2-antiplasmin (a2AP), the inhibition of plasmin(ogen), is influenced by the proteolytic modifications the protein undergoes in the circulation. Approximately 70% of circulating a2AP is N-terminally cleaved by AntiPlasmin Cleaving Enzyme (APCE). This results in a form of a2AP with an N-terminal Asparagine (Asn-a2AP) that is faster crosslinked to fibrin by activated FXIII, making a2AP a better fibrinolysis inhibitor as measured in a plasma clot lysis assay. Additionally, approximately 35% of circulating a2AP is cleaved at its C-terminus, resulting in a form of a2AP that has lost its ability to bind to plasmin(ogen) (NPB-a2AP, non-plasminogen binding a2AP). NPB-a2AP can still inhibit plasmin, albeit kinetically very slow.

They have set up ELISA assays to measure the antigen levels of the different forms of a2AP and show that the chromogenic-based a2AP activity assay is mainly based on C-terminally intact PB-a2AP. Furthermore they show that a2AP N-terminal cleavage has no influence on the a2AP activity as measured in the a2AP activity assay, indicating that the a2AP activity assay may not represent the full extent of a2AP heterogeneity in activity. Their next aims are to measure the various a2AP antigen levels in cohorts of thrombotic patients and controls to investigate whether a2AP heterogeneity is associated with thrombotic risk.

Paul Y. Kim and Jeffrey I. Weitz: Roles of Kringle Domains in Plasminogen Activation and Plasmin Activity
Plasminogen (Pg) contains seven major domains; an N-terminus peptide domain, followed by five kringle domains, and the protease domain. Activation of Pg to plasmin (Pn) by tissue-type plasminogen activator (tPA) requires only a single proteolytic cleavage. Fibrin (Fn), and to a lesser extent fibrinogen (Fg), serve as cofactors in this reaction. The five kringle domains of Pg possess varying affinities for Fg/Fn. Although previous work suggested that kringles 1 and 4 were the only domains essential for Fg/Fn cofactor activity, they recently reported that the kringle 5 domain also contributes to cofactor activity by interacting with Fn-bound tPA, thereby promoting Pg activation. These data explain why Fn remains a cofactor for activation of Pg derivatives that lack kringles 1 through 4, even though these truncated Pg variants have reduced affinity for Fn. What remains unclear, however, is the role of the kringles in Pn-mediated degradation of Fg or Fn. To explore this question, they generated Micro-Pn that has no kringle domains, and compared its capacity with Pn to degrade Fg and Fn. Whereas with Fn clots, lysis times are 14-fold slower with Micro-Pn than with Pn, the time courses of Fg degradation are similar with both Pn and Micro-Pn, suggesting that the kringles are essential for efficient and coordinated cleavage of Fn, but are not as important for Fg degradation. Although Pn binds Fn with 20-fold higher affinity than Fg, the removal of its kringle domains endows it with greater protection from inhibition by a2-antiplasmin, making Micro-Pn attractive for use in thrombolytic therapy. This was confirmed in an overlay experiment where Micro-Pn and Pn were both able to inhibit thrombus accretion on preformed clots. Therefore, the use of kringle-less Pn as a fibrinolytic agent may result in decreased circulating Fg levels that would 1) lower risks for cardiovascular events and 2) decrease hemostatic potential and prevent progression of thrombotic events. In summary, they have identified a novel mechanism by which kringles play on Pg activation as well as the advantage of losing the kringles on Pn that may allow for enhanced circulating Fg degradation.

Jonathan H. Foley: Evaluation of nomenclature for the CPB2 gene product (best known as TAFI)

The CPB2 gene product was discovered independently by various groups in the early to mid-1990s. As a result, this product of gene CPB2 was given various names by its discoverers the most commonly used name is TAFI.

In light of the recently established physiological role of the CPB2 product in regulating inflammation, and the lack of consensus among the fibrinolysis research community in naming the CPB2 product, Jonathan proposes that the nomenclature of the CPB2 product (best known as TAFI) be revisited as a new project. This project is NOT intended to remove TAFI (or carboxypeptidase U) from our vernacular, but to explore how and when the various names are used and if they are still relevant and appropriate in light of recent discoveries. Of course, this process may result in recommending a name other than TAFI for the CPB2 product. As an initial step, a letter describing the project was sent to members of the SSC subcommittee on fibrinolysis and to ISFP council members to gather their input. Initially, three options were suggested as a starting point for discussion:

1. Reiterate support for TAFI and/or carboxypeptidase U

2. Recommend that carboxypeptidase B2 be adopted

3. Redefine the TAFI acronym; candidate: Thrombin-activatable Attenuator of Fibrinolysis and Inflammation
All 3 suggestions garnered interest with no clear front runner. A forth option was also suggested and that was to devise a completely novel name that would harmonize currently used nomenclature. The feedback indicated that there are various opinions even within the fibrinolysis community regarding the nomenclature of the CPB2 gene product.

At this time, Jonathan recommends that the project be written up as an "SSC communication" for publication on the website of the ISTH. In this way, the scientific community at large will be able to provide input via email which will be tabulated and used to form a recommendation on the nomenclature of the CPB2 product. Once feedback from the community at large has been gathered, key opinion leaders who represent the community’s interests will be invited to participate in a decision-making process to reach consensus on the most appropriate name for the CPB2 product. This project will be updated at the 59th SSC meeting in Amsterdam.
Hemostasis and Malignancy

30 June 2012

Chairman: Agnes YY Lee (CA)
Co-chairmen: Marc Carrier (Canada), Dominique Farge (France), Alok Khorana (USA), Howard Liebman (USA), Marina Marchetti (Italy), Ingrid Pabinger (Austria), Wolfram Ruf (USA), Jeffrey Zwicker (USA)

Attendance: Wolfram Ruf (US), Dominique Farge (FR), Marina Marchetti (IT), Jeff Zwicker (US), Marc Carrier (CA), Ingrid Pabinger (AU), Howard Liebman (US)

Invited Speakers: Carla Vossen (NL), Cihan Ay (AU), Marcella DiNisio (IT), Sam Schulman, Pieter Kamphuisen

There were 11 presentations updating subcommittee activity and discussing new proposals. They were divided into 2 sessions: 1) Update on Current Projects and Clinical Trials and 2) New Proposals and Hypotheses.

Update on Current Projects and Clinical Trials

Standardization of tissue factor assays

Marina Marchetti (IT)

Dr. Marchetti provided an update of the tissue factor (TF) standardization project. Lyophilized cell lysates from MDA MB 231 breast cancer cell line have been prepared in Dr. Anna Falanga’s laboratory in Bergamo, Italy to be used as a possible TF reference material. A proposal was made to distribute and test this material amongst various laboratories involved in the TF Working Party. The proposal was accepted and the subcommittee will apply for funding from SSC to cover logistic costs.

Defining VTE in Oncology Trials

Marc Carrier (CA)

Dr. Carrier presented the rationale and development of the subcommittee’s recommendations on standardized the definition, analysis and reporting of VTE in oncology studies. As noted previously, the lack of standardization and the use of National Cancer Institute’s Common Toxicity Criteria to classify adverse events undermine the accuracy and transparency of VTE reporting. Furthermore, competitive risk analysis is a more appropriate method to account for the high incidence of death in this patient population. A position paper has been prepared and circulated to co-chairs prior to this meeting. Once approval has been received by the subcommittee co-chairs, the position paper will be submitted to JTH for publication.

International guidelines for antithrombotics in cancer patients

Dominique Farge (FR)
D Farge-Bancel presented the final draft of the International Good Clinical Practices Guidelines for Antithrombotic in Cancer Patients. Co-authors of the document include the following collaborators: P Debourdeau (co-first author), M Beckers, C Baglin, R Bauersachs, B Brenner, D Brilhante, A Falanga, G Gerotzafias, A Kakkar, A Khorana, R Lecumberri, A Lee, M Mandala, M Marty, M Montréal, S Moussa, N Haim, S Noble, I Prandoni, M Prins, M Qari, M Streiff, H Bounameaux, and H Buller. The project was initiated by the “Groupe Francophone Thrombose et Cancer” and the Academic Medical Centre (AMC), Amsterdam. The guidelines were prepared according to the GRADE methodology based on a literature review of the studies published between 1996 and 2011. Methodological support and quality control were provided by the French national cancer institute (INCa). There wasn’t time to discuss these guidelines.

2012 ACCP Guidelines on Cancer-Associated Thrombosis – Lost in Translation
Alok Khorana (US)

Dr. Lee presented on behalf of Dr. Khorana, who was unable to attend due to a family emergency. The controversial recommendations recently published in the 2012 ACCP guidelines on the prevention and treatment of cancer-associated thrombosis were reviewed. A discussion followed regarding whether a formal response from the subcommittee is warranted and in what format to express the concerns. There was general agreement that the ACCP guidelines do not reflect the opinions and standard of practice of physicians and experts in this field and a recommendation/proposal was made that the subcommittee should prepare guidance documents to outline controversial management issues.

Updates on clinical trials and registries
Agnes Lee (CA), Sam Schulman (CA), Pieter Kamphuisen (NL), Marc Carrier (CA), Jeff Zwicker (US)

A rapid review of ongoing trials in cancer-associated thrombosis was presented by various investigators. Dr. Marc Carrier summarized the activities of the SOME study. This is an open randomized study investigating the utility of aggressive screening vs. limited screening in detection of occult cancer in patients presenting with unprovoked VTE. The primary endpoint is detection of occult malignancy. 53% of the target sample size of 860 study patients has been reached. Dr. Lee presented on behalf of Dr. Simon Noble an update on the FRAGMATIC study. This is a randomized phase III study evaluating the effect of dalteparin in addition to chemotherapy on survival and disease progression in patients with primary lung cancer. Target sample size of 2203 has been reached and results are expected in 2013. Dr. Lee presented on behalf of Dr. Guy Meyer the design and activities of the TILT study. This open, randomized trial is studying the effect of adjuvant tinzaparin on survival in patients with completely resected stage I, II or IIIa non-small cell lung cancer. Total of 450 of 550 patients have been recruited. Enrollment is expected to complete in Jul 2013. Dr. Jeff Zwicker gave a brief review of the MicoTEC study. The results were presented during the “Hot Topics” session of the conference. This phase II study showed that enoxaparin showed a strong trend in lowering the incidence of DVT detected by screening ultrasound at Day 60 in patients with high levels of TF microparticles. Dr. Zwicker also presented the design of the phase II MicroSTAT study evaluating the role of rosvastatin in lowering circulating TF microparticles in women with metastatic breast cancer. Dr. Khorana’s PHACS study was presented by Dr. Lee. Patients with a high Khorana score (3 or higher) were randomized to receive
dalteparin or no treatment for primary prophylaxis. Recently the blinded data were analyzed by the FDA and the target sample size has been reduced due to higher than expected rate of VTE. The results of the study are anticipated in 1.5 years. Dr. Kamphuisen updated the activities of the LONGHEVA trial. This study is evaluating whether LMWH is superior to vitamin K antagonists in cancer patients who have already received 6 – 12 months of anticoagulant therapy for VTE. 32 patients have been enrolled and the trial is facing difficulty with recruitment. Dr. Lee presented on the CATCH trial, a multinational, phase III study comparing tinzaparinux with warfarin for treatment of cancer-associated thrombosis. The study has enrolled 1/3 of the target sample size of 900 patients and has received recommendation by the Data Monitoring and Safety Board to continue. Dr. Sam Schulman gave an update on the international registry on recurrent venous thromboembolism in anticoagulated patients with cancer. Currently 151 patients have been recruited, 200 is the target. Some baseline data were presented. New sites are still welcome to join.

**New Proposals and Hypotheses**

*Prothrombotic state and metastasis in preclinical models*
Wolfrum Ruf (US)

Dr. Ruf presented his laboratory’s work on elucidating the mechanisms of metastasis. Tumor cell tissue factor (TF)-initiated coagulation supports fibrin formation, platelet activation, and monocyte/macrophage recruitment and thereby contributes to the survival of lodged metastases in the lung vasculature. Recent studies identified host anticoagulant mechanisms as a major impediment for successful hematogenous tumor cell metastasis. We addressed the contributions of host hemostatic factors and of thrombin signaling through protease activated receptor (PAR) 1 to the markedly enhanced metastasis in hyperthrombotic thrombomodulin mutant (TM<sup>pro</sup>) mice. In this model, full-length TF- and platelet-dependent, but contact pathway-independent syngeneic breast cancer metastasis was not significantly reduced following fibrinogen depletion, pharmacological blockade of monocyte adhesion receptors for platelets, or genetic deletion of platelet glycoprotein Iba. Mice with very low levels of the endothelial protein C receptor did not phenocopy the enhanced metastasis phenotype of TM<sup>pro</sup> mice. Similarly, genetic deletion of PAR1 that is not expressed by mouse platelets did not diminish enhanced metastasis in TM<sup>pro</sup> mice. Experiments with breast cancer cells derived from PAR1-deficient mice furthermore excluded that thrombin-PAR1 signaling has major redundant prometastatic effects on tumor cells and the host. Thus, metastasis in the hyperthrombotic TM<sup>pro</sup> mouse model is largely independent of intravascular thrombin-PAR1 signaling, and primarily enhanced by increased tumor cell survival mediated by platelets.

*Role of genetic polymorphisms in thrombosis and colorectal cancer*
Carla Vossen (NL)

Dr. Vossen presented and discussed her work on the association between 6 genetic variants in coagulation- or thrombosis-related genes and colorectal cancer risk or progression. The 6 genetic variants were factor V Leiden, prothrombin G20210A, plasminogen activator inhibitor-1 (PAI-1) 4G/5G, fibrinogen gamma (FGG) 10034C>T, factor XIII Val34Leu and methylenetetra-hydrofolate reductase...
(MTHFR) 677C>T. These variants were genotyped in 1801 colorectal cancer cases and 1853 controls from the DACHS study (Darmkrebs: Chancen der Verhütung durch Screening). Follow-up information for 1322 cases on cancer progression (recurrences and mortality) demonstrated an effect on colorectal cancer risk for factor V Leiden, prothrombin G20210A and factor XIII Val34Leu, and an effect on colorectal cancer-specific mortality for PAI-1 4G/5G and FGG 10034C>T. Future plans to further investigate the role of coagulation gene variants and colorectal cancer survival were presented.

**Biomarkers in clinical practice**
Cihan Ay (AU)

Dr. Ay presented an overview and update of biomarker work in the Vienna Cancer and Thrombosis Study (CATS). In addition to previous the biomarkers which have shown an association with cancer-associated thrombosis, including platelet count, soluble P-selectin, factor VIII activity, prothrombin fragment 1+2 and D-dimer, thrombin generation has been found to be predictive of thrombotic complications. In contrast, an association between microparticle-associated tissue factor activity and VTE was not found. It was highlighted that further work is needed to re-analyze the data on previously identified markers using competitive risk methodology.

**Competing risks in oncology trials**
Jeff Zwicker (US)

Dr. Zwicker discussed the methodologies used for analyzing VTE incidence in oncology trials. It is now recognized that competing risk analysis is most appropriate statistical methodology to evaluate the probability of VTE in cancer studies. In clinical trials that include patients with advanced malignancy, death is considered a competing risk for thrombosis due to its high incidence. Because VTE and death are unlikely to be independent events in these patients, the Kaplan and Meier approach to assessing the cumulative incidence of VTE is inappropriate because it ignores the effect of death as competing risk and thus overestimates the incidence of VTE. Gray’s test should be utilized to compare cumulative incidence rates of VTE between two or more groups when competing risks are present. This recommendation has been incorporated into the position paper on standardizing the definition, analysis reporting, of VTE in oncology trials.

**Registry on incidental PE**
Marcello DiNisio (IT)

Dr. DiNisio presented on the rationale and design of a multicenter, international, prospective registry on the treatment of incidental pulmonary embolism (PE). Clinically unsuspected PE represents a common finding in cancer patients. Current guidelines suggest the same initial and long-term anticoagulation as for patients with symptomatic PE which in most cases implies indefinite treatment. These recommendations rely, however, on expert opinion in the absence of solid studies. The aim of this registry is to evaluate the current treatment approaches for unsuspected PE and to assess their efficacy and safety in a large prospective cohort of ambulatory or hospitalized cancer patients. The main outcomes are recurrent (symptomatic) PE or deep vein thrombosis, bleeding, and mortality. Follow-up visits are scheduled at 3 and 6 months after inclusion with a visit or phone contact at 12 months. A total
of about 500 patients will be recruited in approximately 40 centers. Outcomes will be adjudicated by a central adjudication committee. An electronic case report form will be used for data collection. Dr. DiNisio also presented a cohort study to evaluate the efficacy and safety of a prophylactic dose of LMWH in preventing recurrent VTE in cancer patients with isolated subsegmental PE. There was strong support for both of these studies by the subcommittee.

**New Oral Anticoagulants in CAT**

Agnes Lee (CA)

Dr. Lee presented a brief overview of the phase III data on the efficacy and safety of the new oral anticoagulants for the treatment of acute VTE. Limitations and concerns regarding the use of these new agents in cancer patients were outlined, including the paucity of clinical trial evidence, the potential for unpredictable bioavailability because of mucosal breakdown of the GI tract, the higher risk of GI bleeding reported in phase III studies, drug interaction with some chemotherapeutic agents, and the lack of an antidote. Further research with phase III/IV clinical trials focusing on cancer patients was strongly encouraged and received unanimous support. The challenges of trial designs and the necessity of industry support were discussed. A recommendation was made for a position paper by the subcommittee to highlight these issues.

The meeting was adjourned at 1300.
Lupus Anticoagulant/Phospholipid-Dependent Antibodies

29 July 2012

Chairman: Thomas Ortel (USA),
Co-Chairman: Katrien Devreese (Belgium), Armando Tripodi (Italy), Silvia Pierangeli (USA), Bas de Laat (The Netherlands), Tatsuya Atsumi (Japan), Guido Reber (Switzerland), Vittorio Pengo (Italy)

The Lupus Anticoagulant/Phospholipid-Dependent Antibodies Subcommittee meeting covered four major topics during the session. A primary objective of this meeting was to identify important topics that needed to be actively addressed by the subcommittee, and individual members were assigned specific tasks to cover during the coming year.

Anticardiolipin, anti-b2-glycoprotein I, and antiprothrombin antibodies. Dr. Silvia Pierangeli provided an update from the criteria antiphospholipid task force from the 13th International Congress on Antiphospholipid Antibodies, which was held in 2010 in Galveston, TX. Dr. Bas de Laat’s colleague presented an update on the importance of the conformation of b2-glycoprotein I in the assays used to detect these antibodies. Dr. Tatsuya Atsumi discussed the clinical significance and international standardization of an assay to detect phosphatidylserine-dependent antiprothrombin antibodies. Dr. Katrien Devreese and Dr. Thomas Ortel provided the final presentation in this section, which reviewed the contentious topic of standardization of antiphospholipid antibody assays and ended with the question "What can the SSC do?"

Important issues that were discussed during these presentations included consideration of the cut-off values for anticardiolipin and anti-b2-glycoprotein I antibodies, the units that should be used to quantify the data, better definitions of low, medium, and high positive results, comparison of titers across different assays, and the recommended test(s) that should be used. An inquiry directed to the attendees of the session revealed a strong desire for the Subcommittee to draft an official communication addressing important issues in the detection of anticardiolipin and anti-b2-glycoprotein I antibodies, similar to prior communications on the detection of lupus anticoagulants. Drs. Devreese, Pierangeli, and Ortel will spearhead this effort with the objective being an official communication from the Lupus Anticoagulant/Phospholipid-Dependent Antibodies Subcommittee on these important immunoassays.

Lupus Anticoagulants. Two presentations were given on the topic of lupus anticoagulants. First, Dr. Philip de Groot discussed approaches to the diagnosis of lupus anticoagulants in the presence of new anticoagulant agents. The second presentation was by Dr. Guido Reber, who discussed problems and possible solutions in the detection of antiphospholipid antibodies, with an emphasis on laboratory performance with the "weak" lupus anticoagulant. This presentation built on last year’s presentation by Dr. Miejer reviewing the ECAT survey results, which demonstrated that many laboratories were struggling with consistent detection of "weak" lupus anticoagulants.

These two presentations led to discussion about several issues that needed to be addressed since the last official communication from the Lupus Anticoagulant/Phospholipid-Dependent Antibodies Subcommittee in 2009. First, there was concern raised about the criteria for defining a "weak" lupus anticoagulant. This led to the second issue, which concerned the optimal definition of how the cutoff for the normal ranges should be defined, and how laboratories should proceed with establishing this cutoff. It was felt that some clinical laboratories were not using the 99th percentile as recommended in our 2009 report due to logistical concerns about the number of normal donors that would be required.
to establish this cutoff. To address these two issues, Dr. Reber will put forward a document addressing the issue of "weak" lupus anticoagulants, and Dr. Armando Tripodi will organize and conduct a study to investigate the optimal approach for defining the 99\textsuperscript{th} percentile cutoff for normal ranges in these assays.

**New Assays for Antiphospholipid Antibodies.** This section included one presentation from Dr. Jacob Rand, concerning the Annexin A5 resistance assay that he has developed and has been investigating in different patient populations. Dr. Rand proposed an international, multi-center study investigating samples positive for one or more of the standard assays used for the detection of antiphospholipid antibodies (lupus anticoagulant, anticardiolipin antibodies, and anti-\(\beta_2\)-glycoprotein I antibodies) for the presence of annexin A5 resistance. In general, the subcommittee felt that this was an important next step in the analysis of this assay, but that optimally it would be coordinated with efforts to characterize domain 1-specific anti-\(\beta_2\)-glycoprotein I antibodies and phosphatidylerine-dependent anti-prothrombin antibodies, in order to obtain maximal information about antibody specificity.

**Guidelines for the Diagnosis of Antiphospholipid Syndrome.** The final section of our subcommittee session switched from a discussion of the laboratory studies used in the diagnosis of antiphospholipid antibodies, to a recommendation for an update of the laboratory criteria for the diagnosis of antiphospholipid syndrome. Dr. Vittorio Pengo presented his data on patients who are positive for all three assays (lupus anticoagulant, anticardiolipin antibodies, and anti-\(\beta_2\)-glycoprotein I antibodies, or the "triple-positive" patient) and the risk for developing thromboembolic complications and/or pregnancy morbidity, compared to patients who are positive for only one or two of these assays. It was proposed that all patients should have all three tests performed on two (or more) occasions at least 12 weeks apart, and that the results from these analyses would be used to classify patients as having "definite", "possible", or "unlikely" thrombotic and/or obstetric antiphospholipid syndrome depending on the number of tests returning positive results (3 tests, 2 tests, or 1 test, respectively).

This proposal generated considerable discussion, particularly with concerns raised about patients with a single positive test for a lupus anticoagulant falling into the "unlikely" category. It was felt that "uncertain" might be a better classification for this group of patients. Overall, it was felt that this was an important issue, and the subcommittee has requested Dr. Pengo to submit his recommendations as a draft manuscript for review by the subcommittee (the first draft is currently under review by Dr. Ortel).

Following the session, the members of the Subcommittee met to review the day’s events and come up with a plan for the upcoming year. The following tasks were chosen for the next year, and individual subcommittee members were assigned to each task:

- Preparation of an official communication from the SSC on recommendations for the use of immunoassays (anticardiolipin and anti-\(\beta_2\)-glycoprotein I antibodies) in the detection of antiphospholipid antibodies. Primary subcommittee members working on this task include Drs. Devreese, Pierangeli, and Ortel.
- Organization of a study analyzing the recommended approach to defining the cutoff for each of the assays. Dr. Tripodi will lead this effort.
- An analysis and potential official communication from the SSC on the phenomenon of the "weak" lupus anticoagulant and the best approach to detecting these antibodies. Dr. Reber will lead this effort.
Preparation of an official communication from the SSC on recommendations concerning the laboratory criteria used for diagnosis of patients with antiphospholipid syndrome. This effort will be led by Dr. Pengo.

Implementation of a strategy to harmonize efforts to evaluate new assays that may be useful in the identification of clinically-relevant antiphospholipid antibodies. Drs. de Laat and Atsumi are working on studies to characterize domain 1-specific anti-b2-glycoprotein I antibodies and phosphatidylserine-dependent antiprothrombin antibodies, respectively, and they will lead this effort. They will also review the proposal from Dr. Rand concerning a study to look at the clinical relevance of annexin A5 resistance in patients with antiphospholipid antibodies. Strategies promoting collaborative efforts when analyzing samples will be developed.

The Subcommittee members also considered two additional items during this session. First, Dr. Pengo has received support from the SSC for his proposal entitled "Centralized testing for antiphospholipid antibodies in Europe". This proposal will provide partial support for the shipping of bio-specimens from patients with antiphospholipid syndrome from across Europe to Dr. Pengo’s laboratory for centralized testing. This activity will compare testing from multiple sites to the results obtained at a single center with extensive experience with performing the assays. The results from this study will be submitted as a publication from the SSC. The second item considered the possibility of a specific reagent being provided from a single laboratory in an effort to improve standardization. In particular, Dr. de Laat wished to offer purified domain 1 as a standard for use in other laboratories that work on antiphospholipid antibodies. It was decided that the Subcommittee would investigate the possibility of supporting this activity.

Following these discussions, the Subcommittee adjourned with the plan being to follow-up on the assigned tasks through teleconferences and email.
Perinatal/Pediatric Hemostasis

29 July 2012

Chairman: Paul Monagle (AU)
Co-chairmen: Mariana Bonduel (Argentina), Elizabeth Chalmers (UK), Anthony Chan (Canada), Janna Journeycake (USA), Christoph Male (Austria), Paolo Simioni (Italy), Guy Young (USA)

The paediatric/ perinatal subcommittee focus over the last 12 months has been to:

1. Increase the active participation of co-chairs by making each of them responsible for delivery of at least one project outcome.

2. Involve a larger number of junior colleagues in the project work

3. Develop a series of position papers and active projects that would contribute significantly to the field.

This has been achieved through:

1. Clear delegation of responsibilities to co-chairs with timelines defined

2. Formal meetings (usually over dinner) of the subcommittee at each SSC meeting to discuss and plan work for the subsequent 12 months.

3. Semi regular teleconferences via ISTH mechanisms to monitor progress during the year.

4. Opportunistic and directed involvement of junior faculty in the work of the SSC. Of note 3 people have come via the ISTH website, who were previously unknown to the SSC members and are now actively working on projects.

This report will outline that the SSC has been successful in achieving these three goals over the last 12 months.

1. Education Program

The Education program in Liverpool was very successful. Topics were well received and over 100 people attended the session. In particular the debates were very well received and use of this format could be further enhanced in the future. The contribution from Trevor Baglin (Control of anticoagulation SSC) and from non haematologists was most helpful.

ISTH remains the only major congress/meeting that enables a significant focus on paediatric haemostasis/thrombosis and hence it is very important that the education focus and the involvement of paediatrics in the congress scientific and education program continues to be enhanced. This draws most paediatric haematologists to the meeting, and also creates an opportunity for predominantly adult based haematologists who seem some children to update themselves.

2. SSC business
The agenda of the SSC has focussed on thrombosis and bleeding in unwell patients so as to not overlap with other SSC. We are involved in the collaborative work promoting the use of the bleeding score (BAT standing committee).

Following discussion amongst the subcommittee, and with the chair of the women’s health SSC, we recommend that the name of the subcommittee be changed to the PAEDIATRIC and NEONATAL SSC in recognition of the fact that our focus is children, and that any consideration of perinatal issues is perhaps more relevant to women’s health or preferably joint sessions between the two subcommittees.

There are a number of other opportunities for joint work with other SSC, including platelet immunology (FMAIT), control of anticoagulation (heparinoid monitoring, point of care monitoring etc) and definition of APLA in children. We are actively discussing with those SSC chairs to promote this crossover.

In the last 12 months:

Four position papers have been published:


Please note that every paper has been led by junior staff who are not formally SSC members, and supervised by an SSC co chair.

Position papers are particularly important in paediatrics. Our field lags a long way behind our adult colleagues in many aspects. Often research projects very relevant to paediatrics are rejected by funding organisations who apply adult criteria to the project. Position papers which clearly explain the current status in paediatrics, and highlight the current research needs are incredibly useful in enabling paediatricians to justify much needed research. Further they are very important in enabling clinicians to provide clinical practice within a paediatric perspective.

A further five position papers are currently in advanced development.


4. Standard approach to introducing new anticoagulant drugs in children Young, G, Male C.


In addition,

Two Clinical guidance papers are in advanced development and will be forwarded to clinical guidance writing committee within the next few months,

1. Thrombophilia testing in children,

2. vena cava interruption in children

Working groups have been assembled and initial meetings held to progress a number of further position or clinical guidance papers:

1. HIT in children

2. Antiplatelet therapy in children

3. Investigation of bleeding child

4. Paediatric anticoagulation clinics

5. AT replacement in artificial circuits

6. TEG in children

7. APLA definition and management in children

8. FFP use in children

9. Coagulopathy in paediatric liver disease

10. Liver and renal biopsies in coagulopathic children

Once again, every project has a designated responsible co chair, and then a number of people external (often younger colleagues) to the formal SSC involved in the preparation of the paper. Further, where relevant non haematologists (radiologists, neurologists, renal physicians etc ) have been included in the
working parties to provide a balanced and clinically relevant perspective. Most groups have found the conference call facilities provided by ISTH to be very useful.

The paediatric SSC is working towards establishing a registry to follow up children with homozygous protein C or S deficiency who present with neonatal purpure fulminans. A working party has been established, and a number of meetings held to progress the project, and a funding proposal for start up funds will be put to SSC over the coming months.

Finally, as my term as chair of the paediatric/perinatal finishes I would like to thank my co-chairs and colleagues for their support and encouragement. I would like to thank the SSC for giving me the opportunity to contribute to the care of children through this mechanism and I look forward to finding other avenues to continue to work with ISTH to support the improved care of children with bleeding and clotting disorders.

Paul Monagle
Plasma Coagulation Inhibitors

28 June 2012

Chairman: Steve Kitchen (UK)
Co-chairmen: Elisabetta Castoldi (The Netherlands), Tilman Hackeng (The Netherlands), Richard Marlar (USA), Piet Meijer (The Netherlands), Laurent Mosnier (USA)

Update on Subcommittee projects. S Kitchen (UK)

Two projects are now approved and are beginning. One is led by Elisabetta Castoldi (e.castodli@maastrichtuniversity.nl) and will update the existing mutation databases of Antithrombin, Protein C, and Protein S and will create the infrastructure for the indefinite maintenance/update of these databases. A ‘task-force’ of 4-5 people led by an expert in the field will be set up for each gene. New mutations reported since the last update of the respective database will be identified by searching public resources (HGMD, PubMed) and entered in the existing databases manually. Meanwhile, an electronic interface for the submission of all newly identified mutations directly by the discoverer(s) will be created and advertised. Submitted information will be checked by the competent task-force before being officially posted.

The second approved project is the Investigation into discrepancies in Protein S activity assay results. This aims to elucidate the cause of marked differences between PS activity results obtained with different manufacturers kits (as reported by several external quality assessment programmes). This is being led by Ian Jennings (ian@coageqa.org.uk) with Piet Meijer and Richard Marlar. Frozen and lyophilised samples from normal and PS deficient subjects will be sent to participating centres employing different PS activity methods together with a common calibrator.

Session 1. Effects of New Oral anticoagulants on plasma coagulation inhibitor testing: Chairs Steve Kitchen (UK), Piet Meijer (The Netherlands)

1.1 The Effect of Rivaroxaban on laboratory testing for coagulation inhibitors: experience from ECAT surveys Piet Meijer (The Netherlands)

Rivaroxaban is an oral anticoagulant acting as a direct factor Xa inhibitor. It is used for the prevention of venous thromboembolism (VTE) in patients who have undergone total hip replacement or total knee replacement surgery as well as for stroke prophylaxis in patients with non-valvular atrial fibrillation.

Rivaroxaban may affect haemostasis assays because of the effect on Factor Xa. This was investigated in a number of different studies [1-5]. These studies have clearly shown that samples with rivaroxaban taken from volunteers as well as in-vitro spiked samples affect both global and specific haemostasis assays.

In haemostasis laboratories samples could be presented from patients under treatment with Rivaroxaban without any information available about this treatment. It is therefore important that technical personnel as well as clinical chemists are aware of the potential effect of Rivaroxaban on several haemostasis assays. For that reason the ECAT has distributed in several of their surveys a normal
pooled plasma spiked with approx. 200 ng/mL Rivaroxaban. This concentration is within the therapeutic range and shown to have impact on several haemostasis assays [1-5].

In the 2011-2 survey such a sample was used in the thrombophilia module. The effect of Rivaroxaban is expressed as the relative change in activity in comparison to a similar normal pooled plasma, except for the APC Resistance ratio results. The activities of haemostasis factors investigated in the spiked plasma were corrected for the dilution factor of the plasma as a result of the addition of Rivaroxaban.

**Antithrombin, protein C and protein S activity assays**. It was observed that there is no effect of Rivaroxaban on the anti-IIa Antithrombin assay and the chromogenic protein C assay. A significant effect of Rivaroxaban can be observed for the anti-Xa antithrombin assay (+20%), the protein C clotting activity assay (+30%) and the protein S activity assays (+100%). Because both the protein C clotting activity assay and the protein S activity assay are APTT-based assays an effect could be expected.

**APC Resistance**. Table 1 shows the effect of Rivaroxaban on APC Resistance testing for the most frequently used methods. It can be observed that for all methods, except the Chromogenix Coatest APC Resistance test, an increase in the APC ratio occurs in the presence of Rivaroxaban. Such an effect was also observed in the study of Hillarp and co-workers [5]. They show a concentration-dependent increase of the APC Resistance ratio. This implies that in principal a heterozygous Factor V Leiden patient under the treatment with Rivaroxaban could have a ratio close to normal.

**Conclusion**: Data in the literature as well as observations from ECAT surveys using a sample enriched with Rivaroxaban show a significant effect on several haemostasis assays. Laboratories should be aware of this phenomenon to interpret appropriately results from samples of a patient under treatment with Rivaroxaban.

Table 1: The effect of Rivaroxaban (= 200 ng/mL) on the measurement of APC Resistance.

<table>
<thead>
<tr>
<th>Method</th>
<th>APC ratio - Rivaroxaban</th>
<th>APC ratio + Rivaroxaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromogenix Coatest APC Resistance (global test)</td>
<td>3.58</td>
<td>3.15</td>
</tr>
<tr>
<td>Chromogenix APCR-V / I.L. HemosIL FVL (specific test)</td>
<td>2.58</td>
<td>3.03</td>
</tr>
<tr>
<td>Siemens ProC AcR (global test)</td>
<td>2.29</td>
<td>2.66</td>
</tr>
<tr>
<td>Siemens PC Global/FV (specific test)</td>
<td>2.14</td>
<td>2.74</td>
</tr>
<tr>
<td>Pentapharm Pefakit APC-R FVL (specific test)</td>
<td>3.83</td>
<td>5.17</td>
</tr>
</tbody>
</table>

References


1.2 Effects of dabigatran on PC PS and AT assays Bob Gosselin1, Dot Adcock2, Steve Kitchen3, Denis Dwyer1 1UC Davis Health System, Sacramento CA, 2Esoterix Coagulation Englewood, CO, 3Royal Hallamshire Hospital, Sheffield, UK.

Dabigatran etexilate, an inactive precursor, is rapidly converted in the blood and liver by esterase-catalyzed hydrolysis to its active form, dabigatran. Dabigatran is a rapidly acting, non-peptidic inhibitor of both free and bound thrombin used to anticoagulate patients with atrial fibrillation, hip and knee arthroplasty, or venous thromboembolism. We sought to assess the effect of dabigatran on various coagulation assays using plasma was spiked with this drug. Laboratories in United States, Canada and United Kingdom with expertise in coagulation laboratory testing volunteered to participate in our blinded study. Four laboratories performed antithrombin (AT), three performed protein C (PC), and three performed protein S (PS) testing. Thrombin-based AT assays overestimated AT concentrations in the presence of dabigatran at ~125ng/mL resulting in about 10% increase in concentration compared to baseline, with ~ 20-30% fictitious increase of AT with drug concentrations of ~400-500ng/mL. Falsely increased PC activity was observed in clot-based methods at dabigatran concentrations of ≥150 ng/mL, and results fell outside test linearity at drug concentration of 400-500ng/mL. Chromogenic PC methods demonstrated no significant effect to any concentration of dabigatran. Clot-based PS activity assays, whether APTT or RVV based, were sensitive to dabigatran with significantly elevated results at 25ng/mL of dabigatran. Free PS antigen by LIA methods, did not show any significant effect to any concentration of dabigatran.

Rivaroxaban, an oral direct factor Xa inhibitor, and dabigatran, an oral direct thrombin inhibitor, are developed for prophylactic treatment of thromboembolic disorders. Laboratory monitoring is not needed but the effects on coagulation assays, including tests for natural plasma inhibitor, are incompletely known.

The objectives were to investigate the effect of rivaroxaban and dabigatran on coagulation assays used to evaluate natural plasma inhibitors. Rivaroxaban and dabigatran were added to normal plasma in the concentration range 0–1000 μg/L and analysed using different reagents for antithrombin, protein C, protein S and activated protein C (APC) resistance. The results were highly variable, depending on the assay type and drug. Not surprisingly, antithrombin assays based on Xa was sensitive for rivaroxaban whereas thrombin-based antithrombin assays over-estimate the antithrombin in samples containing dabigatran. Chromogenic protein C assays and latex enhanced immunoassays for protein S were not affected but coagulation-based functional assays for protein C or protein S were compromised by both drugs. The APC resistance assay based on the activated partial thromboplastin time is unreliable during treatment with the new anticoagulants. However, the APC resistance test that is based on the activation of coagulation at the level of prothrombinase are unaffected by rivaroxaban. Different assays, and even different reagents within an assay group, display variable effects by therapeutic concentrations of rivaroxaban and dabigatran. Without detailed knowledge about the specific effects on locally used assays for plasma inhibitors it is advised against to perform these assays during treatment with the new anticoagulant drugs.

Session 2 Chairs: Richard Marlar, Steve Kitchen

2.1 The effect of CRP and factor VIII on clot based protein C and protein S assays.

Jun Teruya, MD, DSc, Kim Nguyen, MLS(ASCP), Vadim Kostousov, MD, Sridevi Devaraj, PhD, DABCC, Texas Children’s Hospital and Baylor College of Medicine, Houston, TX, USA

PTT based protein C (PC) and protein S (PS) assays are known to be affected by heparin of >1 unit/mL and factor VIII (FVIII) of >250%. PTT is used to monitor heparin therapy, but is known to be affected by the presence of C-reactive protein (CRP) in the presence of heparin. CRP binds phosphocholine, thus the phospholipids in a PTT reagent may be partially neutralized by CRP. It is not known if an increased CRP level affects PTT-based PC and PS assays. This study investigated if CRP affects PC and PS assays in the presence of heparin. We also studied if PC and PS assays are not affected by FVIII up to 250%. Normal plasma was spiked with CRP purified from ascites fluid (endotoxin and azide-free) in the absence and presence of heparin (0, 0.6, and 1.2 units/mL). CRP did not clearly affect PC or PS level up to 25 mg/dl. Since the plasma specimens for PC and PS assay are diluted by Owren-Koller buffer, we assume that 10 times diluted CRP may not be affecting both assays. Higher heparin of 1.2 units/mL affected PC assay but not PS assay. Increased FVIII activity from 115 to 428% lowered both PC and PS levels in a linear manner that accompanied by ~5.5% decrease PC and PS activity with each +100% FVIII increment.
2.2 Stability of Antithrombin, Protein C and Activated Protein C Resistance in Whole Blood. Kieron Hickey, Peter Cooper and Steve Kitchen. Royal Hallamshire hospital, Sheffield UK.

Whole blood samples were drawn from healthy donors (0.109M, BD Vacutainer). One sample was processed (centrifuged 2000g for 10 minutes, frozen at -80°C) within 1 hour of venepuncture and the remaining samples stored at room temperature for 24, 48, or 72 hours prior to processing. Antithrombin, tested by FIIa and FXa assays demonstrated stability in normal donors (n=24) for up to 72 hours, with a mean 2% loss in activity for both FIIa and FXa methods. Protein C was assayed using an in house venom-chromogenic (n=23) and clot-based functional assay (n=13). Samples demonstrated stability for 24 hours (mean loss -3%, range +3% to -11%) and less than 24 hours (mean loss -5%, range +5 to -15%) for the chromogenic and functional assay respectively. Samples tested using an activated protein C resistance assay (n=10) showed stability for up to 72 hours with a mean loss in activity of 9%.

2.3 Total protein S assay system: Clinical significance and pre-analytical quality control. Hiroko Tsuda¹, Takao Kobayashi², Tomohide Tsuda³

¹Nakamura Gakuen University, ²Hamamatsu Medical Center, ³Shiono-Test Corporation, Japan.

Activated protein C (APC) cofactor activity of protein S (PS) has been measured on free PS using clotting assays. However, marked variations in results obtained with different kits make PS activity assays to be problematic for diagnosing type II PS deficiency. Recently, we developed a novel chromogenic total PS activity assay for automated analyzers, enabling a simultaneous measurement of total PS antigen using latex-based assay (Blood Coagul Fibrinolysis 2012, 23:56-63). By using our new total PS assay system, the specific activity of PS in the plasma samples of five out of twelve Japanese patients suffering from venous thromboembolism were found to be less than 0.70, and thus supposing type II PS deficiency.

The storage of whole blood samples at room temperature for less than 4 hours resulted in a 3 to 5% reduction of total PS activity, the reduction rising to 20-40% after 24 hours. However, total PS antigen was unchanged after 24 hours sample storage. Western blotting analysis of the plasma with reduced total PS activity revealed the presence of an additional PS band with a slightly faster electrophoretic mobility, probably being the PS molecule cleaved at thrombin-sensitive region.

2.4 Genotype and laboratory and clinical phenotypes of protein S deficiency. Michael Spannagl, Munich, Germany, Hemostasis Outpatient Clinics, Munich University Hospital

The diagnosis of hereditary thrombophilia caused by protein S deficiency remains laborious. From 2005 to 2010, we documented 135 patients with suspected hereditary protein S deficiency due to low PS activity and/or antigen plasma levels for whom mutational analysis of the PROS1 gene had been performed by direct double-stranded sequencing of the amplified 15 exons including splice sites. Multiplex ligation-dependent probe amplification was performed on 12 of 15 exons in cases with no mutation found but a large deletion in the PROS1 gene was suspected. Mutations were identified in 49 patients, 9 by familial screening. Altogether, 17 new and 11 previously described mutations of PROS1 were identified among the 49 patients. Lower protein S plasma levels have been confirmed in women in
the hormonally active age. After the exclusion of acquired protein S deficiency due to pregnancy or hormonal contraceptives, there remained only 1 case with protein S activity levels less than 40% that could not be explained by sequence variations or deletions in the examined regions of the PROS1 gene.

In conclusion after the exclusion of conditions associated with acquired protein S deficiency, persistently low protein S activity levels are highly indicative of a genetic alteration in PROS1. We observed a clear correlation between the laboratory phenotype and the type of mutation. This could not be demonstrated comparing clinical presentation and laboratory phenotype, compatible with the multifactorial etiology of venous thrombosis.

Session3 Chairs : Laurent Mosnier (USA), Tilman Hackeng (The Netherlands)

3.1 Novel assays for EPCR encryption and cellular APC resistance

Eveline A. Bouwens and Laurent O. Mosnier (USA). Scripps Research Institute, La Jolla, USA.

Activated protein C (APC) is a plasma coagulation inhibitor with potent anticoagulant and cytoprotective activities. Activation of protein C zymogen to generate APC occurs on the endothelial cell surface by the thrombin-thrombomodulin complex and is greatly accelerated in the presence of the endothelial protein C receptor (EPCR). After activation, APC bound to negatively charged phospholipids mediates anticoagulant activity via the proteolytic inactivation of coagulation factors Va and VIIIa. Alternatively, APC in complex with EPCR mediates direct anti-apoptotic and anti-inflammatory cytoprotective effects on cells via EPCR-assisted activation of protease activated receptor 1 (PAR1). Thus, EPCR plays a central role in both APC’s anticoagulant and cytoprotective activities. During inflammation, inflammatory mediators and various cytokines severely reduce EPCR-dependent binding of (A)PC to cells, thereby negatively affecting protein C activation and APC’s cytoprotective effects. Depletion of EPCR-dependent binding of (A)PC to cells conceptualizes a novel phenomenon of “cellular APC resistance” similar to the well-known “anticoagulant APC resistance” associated with an increased risk for venous thrombosis. According to the current paradigm, inflammatory mediators induce EPCR shedding by the induction of the metalloproteinase TACE/ADAM17. Recently a second mechanism was proposed according to which a phospholipase can modify or "encrypt" EPCR to lose its ability to bind APC, thereby rendering EPCR dysfunctional. These observations may have important implications for thrombotic and inflammatory vascular disease since EPCR inactivation in vivo, either genetically or induced by blocking antibodies, aggravates and increases susceptibility to thrombotic and inflammatory disease.

3.2 Thrombin generation as an intermediate phenotype for genetic studies on venous thrombosis Elisabetta. Castoldi (Maastricht University, The Netherlands)

Venous thrombosis is a multifactorial disease with a strong genetic component. Although several genetic risk factors for venous thrombosis have been identified in the last 50 years, recent progress has been slow. This may be due, at least in part, to the fact that classifying study subjects as cases (=individuals with the disease) and controls (=individuals without the disease) does not discriminate well between carriers and non-carriers of the risk alleles, leading to a dramatic loss of statistical power, especially for low-risk variants. This problem can be circumvented by taking an intermediate phenotype,
i.e. a measurable quantitative trait that closely correlates with the disease risk, instead of disease status itself, as the end-point of linkage or association studies. In fact, measuring an appropriate intermediate phenotype makes it possible to place every study subject on a continuous scale of disease risk, thereby improving the correlation between genotype (=carriership of risk alleles) and phenotype (=position on the risk scale). Intermediate phenotypes are already successfully exploited in the genetics of several complex disorders, from type II diabetes to asthma. In this lecture, I present evidence that thrombin generation, which measures the overall tendency of a plasma sample to form thrombin, may be a suitable intermediate phenotype for genetic studies on venous thrombosis.
Platelet Immunology

30 June 2012

Chairman: Yves Gruel (France)
Co-Chairmen: Donald Arnold (Canada), Beng Chong (Australia), Andreas Greinacher (Germany), Hartmut Kroll (Germany), Yoshiaki Tomiyama (Japan)

Introduction

Yves Gruel (Chairman, Tours, France) welcomed all participants, recalled the missions of SSC and briefly presented the agenda. He also informed the participants that new human platelet antigens (HPA) have been identified recently approved by the ISBT and the chairman and cochairmen of the SSC Platelet Immunology also approved these new HPA and the EBI/IPD database will be updated (http://www.ebi.ac.uk/ipd/hpa/).

Part 1. Drug-induced and autoimmune thrombocytopenia

Standardization of methods/ evaluation of drug-induced ITP testing

Donald Arnold (Hamilton, Canada) presented a brief review of drug-induced immune thrombocytopenia. This is a common diagnosis that is often missed resulting in the administration of inappropriate therapies. Reliance on clinical diagnosis is inadequate. Challenges with laboratory testing for drug-dependent platelet antibodies are that it is technically demanding, a wide array of tests has been used and their is lack of standardization. Dr. Arnold presented a new grading system developed to evaluate the validity and reproducibility of test methods and results, called the ‘DITP’ criteria: 1. Drug or drug metabolite-dependence; 2. Immunoglobulin binding; 3. Two separate positive test results obtained by two or more laboratories; 4. Platelet-specificity. If drugs met all criteria then the laboratory diagnosis of DITP was considered definite; if only one lab reported result, then the diagnosis was considered probable; and if any validity criterion was not met the diagnosis was unlikely. Dr. Arnold presented new data on how this grading system was applied to published reported of DITP testing. Using this strategy 15 drugs were identified that met all DITP laboratory criteria: abciximab, carbamazepine, ceftriaxone, eptifibatide, heparin, ibuprofen, mirtazapine, penicillin, quinidine, quinine, rifampin, suramin, tirofiban, trimethoprim/sulfamethoxazole and vancomycin.

Dr. Arnold proposed a new SSC project to refine validity criteria for DITP testing. For example, a number of test methods may be used, but it is not clear which method is most suitable; testing for drug-dependence requires drugs in solution, but it is uncertain how this should be accomplished for many pills; and certain drugs react to a metabolite of the parent compound and it is the metabolite that must be used in the assay, but it is uncertain how this should be performed. An initial step might be to survey laboratories, summarize current test methods and identify the key questions regarding DITP testing.
Based on the discussion, the organization of a workshop focusing on the most frequently used techniques for detecting drug-dependent antibodies i.e. flow cytometry and ELISA has been proposed and will be animated by D Arnold.

**Animal model of drug-induced ITP**

**Beng Chong (Sydney, Australia)**

Drug-induced immune thrombocytopenia (DITP) is an adverse drug effect mediated by drug-dependent antibodies. Although there are good animal models for primary immune thrombocytopenia (ITP) and heparin-induced thrombocytopenia (HIT), there was none for DITP until recently when we established a DITP non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse model. This murine model is useful for investigating the in vivo pathophysiology of DITP and also to study the potential efficacy of current and new drugs used in the treatment of DITP such as intravenous immunoglobulin (IVIG). The mechanisms explaining the effects of IVIG remain unclear and their efficacy is uncertain. In the DITP NOD-SCID murine model, human platelets are injected intravenously; they survive for more than 24 hours, allowing platelet clearance by DITP/ITP antibodies to be studied. Rapid human platelet clearance was uniformly observed with all quinine-induced thrombocytopenia (QITP) patient sera studied (mean platelet lifespan: QITP 1.5 ± 0.3 hours vs. controls 16.5 ± 4.3 hours), consistent with the clinical presentation of DITP. In the control experiments, clearance rates with ITP antibodies were slower and more variable. In addition, B Chong and his team also use a mathematical modeling to calculate mean platelet survivals and this approach gives more precise results than the customary platelet half lives. They found IVIG treatment partially prevented platelet clearance by DITP and ITP antibodies. Their results suggest that the NOD/SCID mouse model is useful for investigating the efficacy of DITP therapies, an area in which there is little experimental evidence to guide treatment. With regards to pathophysiology, the role of complement activation in platelet clearance in DITP remains controversial. We propose three experimental approaches in which the murine model can be used to investigate this research question. In conclusion, this NOD/SCID mouse model of DITP would be useful for the investigations of in vivo mechanisms of DITP and the potential efficacy of current and novel therapies.

During the discussion, it was proposed to form a working group aiming to define recommendations for the use of NOD/SCID mice models for immune thrombocytopenia.

**In vivo animal model of drug-induced thrombocytopenia: the clinical relevance of anti-protamine sulfate antibodies.**

**Tamam Bakchoul (Greifswald, Germany)** Thrombocytopenia is often seen in patients after extracorporeal circulation (ECC) for heart surgery. Protamine sulphate (PS) is given for heparin reversal and is associated with thrombocytopenia for unknown reasons. The aim of the following study was to investigate the clinical relevance of antibodies recognizing PS in patients with cardiac surgery. In this study, sera from patients after cardiac surgery were tested by ELISA to identify a possible target antigen. Heparin-induced platelet aggregation assay (HIPA) was modified in order to identify the antibodies’ capability to activate platelets in vitro. Finally, IgG fractions prepared from these sera were explored for their ability to remove human platelets in an in vivo NOD/SCID mouse model of immune
thrombocytopenia. **Major findings:** 1) Workup of the target antigen revealed specific binding to PS in 9/20 sera. 2) In a modified HIPA, 7/9 sera induced platelet activation in the presence of PS but not in the presence of heparin. 3) In a murine model of immune thrombocytopenia, human platelets were cleared from the circulation with an elimination rate of 18% per hour in the presence of IgG and PS plus heparin, whereas platelet clearance was not above baseline in the presence of IgG and PS alone (elimination rate, 4% per hour). 4) Platelet destruction was inhibited by blocking FcγRIIa. **Conclusion:** Antibodies against PS induce thrombocytopenia via FcγRIIa-induced platelet activation. Ongoing study is will investigate the clinical relevance in patients undergoing cardiac surgery with PS reversal of heparin.

**Drug-induced antibody effects on megakaryocytes**

**Beng Chong (Sydney, Australia)** presented a study on the effects of drug-dependent antibodies on megakaryocyte (Mk) biology. They analyzed the effects of sera from several quinine-induced thrombocytopenia (QITP) patients on Mks derived from human CD34+ cells cultured in vitro for 9 – 12 days with human thrombopoietin (TPO). They demonstrated by flow cytometry and confocal microscopy that QITP IgGs bind Mks and platelets efficiently in the presence of quinine but poorly in its absence. Incubation of Day 4 Mks with QITP sera or purified IgG and then cultured for further 5 -8 days, resulted in induction of apoptosis (increased caspase 3 activation and annexin V binding), with a significant decrease in cell viability, and an increase in cell death. Furthermore, QITP sera preferentially reduced the number of late GPIX+/GPIIb+ Mks and the number of receptors per cell in the surviving population. Ploidy distribution and average cell size of Mks remained unchanged after treatment. In addition, treated Mks showed a marked decrease in their proplatelet production capacity, suggesting that drug-dependent antibodies inhibit platelet production.

In summary, this study showed that QITP antibodies significantly reduced in vitro cultured MK numbers, induced MK apoptosis and strongly inhibited proplatelet production of Mks despite undetectable effects on DNA content and cell size.

**Application of PLT antibody testing in ITP trials: The effect of Rituximab on PLT antibodies in ITP**

**Donald Arnold (Hamilton, Canada)** This presentation represents a continuation of work done by the Platelet Immunology SSC on the implementation of PLT antibody testing in ITP clinical trials. The initial project resulted in an official SSC communication (Recommendations for the implementation of platelet autoantibody testing in clinical trials of immune thrombocytopenia. Arnold DM, Santosso S, Greinacher A; on behalf of the Platelet Immunology Scientific Subcommittee of ISTH. J Thromb Haemost. 2012). Dr. Arnold presented a follow up study demonstrating the implementation of the SSC recommendations into practice: In an RCT of rituximab vs. placebo for non-splenectomized ITP patients led by investigators in Canada (Canadian R-ITP trial, Arnold et al, Blood 2012), PLT antibody testing was systematically incorporated into the trial design at 4 time points: baseline, 1 month, 3 months and 6 months and measured using the direct antigen capture method. Dr. Arnold presented new data on the results of platelet antibody testing from the Canadian R-ITP trial. The proportions of patients with anti-GPIIbIIa, anti-GPIbIX or either antibody at baseline were 34%, 28% and 43% respectively. Baseline antibody positivity appeared to be associated with a lack of response to rituximab; however a response to
therapy (in either treatment arm) was associated with a loss of the autoantibody. Titre of anti-GPIibIIa and anti-GPIbIX autoantibodies dropped below positive cut-off values among rituximab responders more often than non-responders, although the sample size was small. The conclusion is that the implementation of PLT autoantibodies in ITP trials is feasible and should continue to be endorsed. The low frequency of ITP autoantibodies will require large samples to determine their predictive value.

Part 2. : Alloimmune and isoimmune thrombocytopenia

Usefulness and biological aspects of a national registry of donors for the treatment and prevention of alloimmune thrombocytopenia

Françoise Boehlen (Geneva, Switzerland).

In 2005, a pilot study was conducted in Geneva to establish a registry of 500 platelet donors HPA-genotyped. This study was completed by the search of platelet specific alloantibodies, which were found in 2.5% of women with at least one pregnancy. These antibodies were anti-HPA-5b in 2/98 homozygous women for HPA-5a and anti-HPA-5a in 1/2 homozygous women for HPA-5b. The retrospective analysis of the medical charts of the 37 recipients of 55 blood components from these 3 women showed no case of passive alloimmune thrombocytopenia.

After this pilot study, the decision was made to extend the HPA-registry to establish a Swiss registry of HLA- and HPA-genotyped platelet donors, the goal being to have 2'000 platelet donors. Donors were genotyped for HPA-1, -2, -3 and -5 and search for antiplatelet antibodies was performed by MAIPA assay. 2'142HPA-genotyped donors are actually available in the registry. Frequency of HPA-1bb, -5aa and -5bb donors is comparable to the frequency described in the Caucasian population. None of the 19 HPA-1bb women had anti-HPA-1a alloantibodies but 9/454 HPA-5aa women had anti-HPA-5bb alloantibodies and 1/6 HPA-5bb women had anti-HPA-5a alloantibodies.

F Boehlen raised several questions concerning such registries. Are such national or loco-regional registries available in most countries? Which is the real usefulness of such registries? There is a need for prevention and treatment of NAIT. However random platelets or maternal platelets can also be transfused in case of NAIT. Furthermore, due to treatment with IVlg and steroids during pregnancy, there is perhaps no real need of such HPA-compatible platelet transfusion.

Another question concerns donors with rare genotypes. Do we have to test homozygous women for platelet alloantibodies who had a previous pregnancy? If yes, what about women with anti-HPA antibodies? Do they have to be excluded from further donation? Do they have to be followed in case of new pregnancy? What about HPA-1bb women never pregnant? Do they have to be retested after pregnancy? Do we need to obtain a donor informed consent?

In summary, this registry confirms some data already available (genotyping) and adds some new information (alloantibody detection) and has been proven to be useful in some selected cases. However, many questions have to be resolved.

Usefulness of surface plasmon resonance technology for monitoring pregnancies with expected NAIT
T Bakchoul (Greifswald, Germany) presented a study during which sequential sera from pregnant women with expected fetal/neonatal alloimmune thrombocytopenia (FNAIT) were assessed for HPA-1a binding antibodies by surface plasmon resonance (SPR). Group I (n=6) was treated with IVIG and steroids from the 19th week of pregnancy, group II (n=4) received intrauterine platelet transfusions (IUT) from week 24. Maternal antibodies were quantified using a HPA-1a monoclonal antibody as standard. Antibody avidity was determined as ratio of B700 (end of the dissociation phase) to B350 (end of the association phase); the area under the curve (AUC) was calculated to determine the overall antibody binding. Major findings: 1) After gestational week 22, antibody characteristics remained stable in both groups, while there was a steep decrease in B700 and B350 values between week 16 and 22 (assessed only in group I). Similar results were observed when the AUC was analyzed. 2) Interestingly, the AUC measured at weeks correlated with fetal/neonatal PLT-count (spearman r: 0.79; p=0.001). This first study applying SPR for assessment of serial serum samples during pregnancy of women at high risk for FNAIT supported that SPR provides a precise and accurate method for quantifying anti-HPA-1a antibodies in maternal sera. 3) Furthermore, the AUC values developed differently according to treatment and correlated with the fetal platelet count. This supports that SPR could be an interesting tool for further clinical studies in FNAIT.

Development of antibodies to αIIbβ3 in a French cohort of Glanzmann thrombasthenia patients

M Fiore (Bordeaux, France) addressed the question related to the development of antibodies directed against the αIIbβ3 integrin in glanzamnn thrombasthenia patients. Despite many reports, there is no consensus pertaining to their frequency, their long-term evolution in the circulation, or their formation in relation to either (i) the extent of the αIIbβ3 deficiency in the patient’s platelets or (ii) the nature of the genetic defect (ITGA2B or ITGB3 genes). Antibody screening was performed on a series of 24 GT patients in South-West France: (i) 16 patients with the French gypsy mutation (c.1544 + 1G>A) within ITGA2B that gives platelets totally lacking αIIbβ3 and (ii) 8 patients carrying other defects of ITGA2B or ITGB3 with different expression levels of αIIbβ3. Their results confirmed that patients with premature termination mutations resulting in platelets lacking αIIbβ3 are the most susceptible to form antibodies, a finding that may be useful in deciding the choice of therapy between PLT and the use of rFVIIa.

Part 3. : Heparin-induced thrombocytopenia

Pathogenesis of HIT

Binding study of HIT antibodies to PF4 modified by enoxaparin oligosaccharides using SPR.

Y. Gruel (Tours, France) presented a study that evaluated the ability of 20 well-characterized enoxaparin-derived oligosaccharides (OS) to promote the exposure of PF4 epitopes recognized by antibodies developed in heparin-induced thrombocytopenia (HIT) by surface plasmon resonance (SPR) and serotonin release assay (SRA). HIT plasmas induced platelet activation with decasaccharides having at least 11 sulphate groups, but a release >80% without full inhibition at 100 mg/ml, was achieved with decasaccharides having 14 or 15 sulfate groups, 2 dodecasaccharides and 2 tetradecasacharides. The SPR method was developed with purified PF4 immobilized on carboxymethylated dextran. Antibodies of all HIT plasmas tested bound to PF4 modified by UFH with significant increase of resonance units.
Therefore, the “RU ratio” i.e. (RU 10 sec after the end of plasma injection / RU 10 sec before) x 100, was always above 100 (range: 109-173 vs 88-93 with control plasmas). RU ratios >100 were also measured with HIT plasmas when PF4 had interacted with OS having at least 10 saccharide units and one octasaccharide with 10 sulphate groups. However, the highest RU ratios (> 140 and similar to those measured with UFH and enoxaparin) were obtained with the dodeca and tetradecasaccharides. Moreover, RU values were highly correlated with the number of sulfate groups present in the decasaccharides tested (r = 0.93, p = 0.02). These data support that LMWHs with fragments longer than 10 saccharides and a large number of sulphate groups are likely associated with higher risk of HIT. In addition, these structure-activity relationships were independent from the oligosaccharide’s ability to bind antithrombin.

**Gene variations and modulation of FcgRIIA-dependent platelet activation**

**Jérôme Rollin (Tours, France).** Heparin-induced thrombocytopenia (HIT) is due primarily to IgG antibodies specific to platelet factor 4/heparin complexes (PF4/H), which activate platelets via FcyRIIA. CD148 is a protein tyrosine phosphatase that regulates Src kinases and FcyRIIA-induced platelet activation. Three polymorphisms affecting CD148 (Q276P, R326Q, D872E) were studied in HIT patients and 2 control groups, with or without antibodies to PF4/H. Heterozygote status for CD148 276P or 326Q alleles was less frequent in HIT patients, suggesting a protective effect of these polymorphisms. Aggregation tests performed with collagen, HIT plasma, and monoclonal antibodies cross-linking FcyRIIA showed consistent hyporesponsiveness of platelets expressing the 276P/326Q alleles. In addition, platelets expressing the 276P/326Q alleles exhibited a greater sensitivity to the Src Family Kinases (SFK) inhibitor, dasatinib, in response to collagen or ALB6 cross-linking FcgRIIA receptors. Moreover, the activatory phosphorylation of SFK was considerably delayed as well as the phosphorylation of LAT and PLCγ2, two major signaling proteins downstream from FcgRIIA. In conclusion, this study showed that CD148 polymorphisms impact platelet activation and probably exert a protective effect on the risk of HIT in patients with antibodies to PF4/H.

**Diagnosis of HIT**

**Validation of the Multiplate assay for HIT diagnosis**

**Gregor Hron (Greifswald, Germany)** presented a study that evaluated whether whole blood from unselected donors could be used to detect platelet activating antibodies by the multiplate-assay in 277 consecutive patients in whom HIT was suspected. Area under the curve (AUC) was used as readout-parameter. Platelet activation was induced in 300 μl of citrate-anticoagulated whole blood from unselected healthy blood donors by adding 150 μl of heat inactivated patient serum and 150 μl of Reviparin (final concentration 0.2 U/ml). The pretest probability for HIT was determined using the 4T’s score. The diagnosis of HIT was determined by a positive result in HIPA supported by intermediate to high probability of HIT. The results obtained showed that both HIPA-positive and HIPA-negative patients had a high pretest-probability of HIT (median 4T’s score 5 and 4, respectively). Although the median AUC was significantly higher in HIPA-positive than in HIPA-negative patients (116vs.0, p<0.001), the range (minimum-maximum) of test results showed a large overlap (0?527 vs. 0?278) between HIPA-positive
and HIPA-negative patients, respectively. By applying a cut-off of 84 AUC in the multiplate-assay, which was determined by testing sera from 25 healthy donors, platelet activating antibodies were detected in 66 of 107 HIPA-positive patients. Thus, 41 HIPA-positive patients had false negative results in the multiplate-assay. Furthermore, false positive results were obtained in 45 of 170 HIPA-negative patients.

In summary, due to the large overlap between HIPA-positive and HIPA-negative groups, the rate of false positives and false negative results in the multiplate-assay was high. The frequent false negative results in HIPA-positive patients indicated that using unselected whole blood donors with unknown platelet reactivity impairs the performance characteristic of the multiplate-assay. Standardization of whole blood donor selection and the reagents are essential to enable the comparison between different studies.

**Functional assays for HIT in Australia: where do they fit in current algorithms?**

**Chris Ward (Sydney, Australia)** presented his experience on functional HIT assays that can confirm the diagnosis and provide useful information in patients where a switch to alternate antiocoagulants is difficult.

Functional assays can either be centralized in a single reference laboratory, or performed in multiple laboratories and the Australian/New Zealand pathology system is better suited to the latter approach.

According to his experience, whole blood impedance aggregometry is a practical and sensitive assay, but selection of a responsive donor is important to detect weak heparin-PF4 antibodies.

Plasma samples are preferable to serum samples and heat inactivation is not required.

Patients with low 4T scores or ELISA titers below 1.0 can have positive functional HITS results and should not be excluded from testing.

**Rapid exclusion or confirmation of heparin-induced thrombocytopenia: a single-center experience with 1,291 patients**

**L. Alberio (Bern, Switzerland)** presented the results of a study that aimed to assess the ability of rapid immunoassays to predict the presence of functionally relevant anti-platelet factor 4/heparin-antibodies.

The results showed that among 1291 patients evaluated for suspected HIT, 96 (7.4%) had a positive heparin-induced platelet aggregation-test: 7/859 (0.8%) with a low, 50/358 (14.0%) with an intermediate, and 39/74 (52.7%) with a high 4T-score. Receiver operating characteristics analysis indicated that best immunoassays’ thresholds for predicting a positive platelet aggregation-test were: Titer ≥4 (ID-H/PF4-PaGIA), optical density >0.943 (Asserachrom-HPIA) and >1.367 (GTI-PF4). A 100% negative predictive value was observed at following thresholds: Titer ≤1 (ID-H/PF4-PaGIA), optical density <0.300 (Asserachrom-HPIA) and <0.870 (GTI-PF4). A 100% positive predictive value was reached only by ID-H/PF4-PaGIA, at titers ≥32. Positive and negative likelihood ratios were calculated for results between the thresholds with 100% negative or positive predictive value.
His conclusions were: 1. Negative and weak positive results of immunoassays detecting anti-platelet factor 4/heparin-antibodies exclude heparin-induced thrombocytopenia; 2. Anti-platelet factor 4/heparin-antibody titres ≥32 (ID-H/PF4-PaGIA) have a 100% positive predictive value for functionally relevant antibodies; 3. Combining the pre-test probability with the likelihood ratio of intermediate immunoassay results allows to assess post-test probability for heparin-induced thrombocytopenia in individual patients. Reference: Nellen V et al. Haematologica 2012;97:89-97.

**Prospective evaluation of a lateral flow immunoassay**

Dorothée Leroux (Tours, France) presented a study that evaluated a recently developed rapid lateral flow immunoassay (Stic Expert HIT*, Stago Asnières France) which is based on the use of PF4/polyanion complexes linked to biotin as antigens and of gold nanoparticles coated with antibodies specific to biotin. This test is IgG specific since these particles are immobilized on the nitrocellulose strip by anti-human IgG, and become visible as a colored line. **Patients and methods:** Two hundred sixteen consecutive patients were enrolled from February to June 2012 in 10 French centers. The pretest probability of HIT was evaluated using the 4T’s score blind to antibody test results. The Stic Expert HIT was performed in each center on plasma and serum. IgG specific ELISA (Assrechrom HPIA IgG*) and serotonin release assay were performed in the principal investigator center and HIT was confirmed only when both SRA and Ig specific H/PF4 ELISA were positive. **Results:** Definite HIT was diagnosed in 24 patients and therefore the incidence of the disease was 11.1% in the cohort. The risk of HIT was evaluated by the 4T’s as low (LowR), intermediate (IR) or high (HR) in 27.8%, 63.2% and 9.0% respectively. The negative predictive value of (NPV) of the 4T’s was 96.6% since definite HIT was diagnosed in 2 of these patients. The interpretation of Stic Expert results is visual but the inter reader reproducibility was excellent (Kappa test ratio higher than 0.9) whether the test was performed with plasma or serum. When plasmas were tested, the Stic Expert HIT was negative in 159 patients without HIT (99.4%, NPV) and the negative likelihood ratio (LR-) was 0.05. Results obtained with serum samples were similar, with a NPV of 100% and LR- lower than 0.01. Alternatively, Stic Expert HIT was positive in 54 plasma samples, including 23 from HIT patients (positive predictive value = 42.6%), with positive LR of 5.87. The pretest probabilities were defined according the frequencies of HIT in our 3 groups of patients (i.e. LowR, IR or HR). A negative Stic Expert HIT decreased the probability of HIT in IR patients from 9.7% before assay to 0.4%, whereas a positive result did not substantially increase the likelihood for HIT. **Conclusions:** The Stic Expert HIT can be performed both on serum and plasma samples and the diagnosis of HIT can be confidently excluded in a patient with Low or Intermediate Risk of HIT if the rapid assay is negative.

**Evaluation of LFI in ICU patients suspected of HIT**

Grigoris Gerotziafas (Paris, France) presented the results of a prospective study including only ICU patients suspected for HIT (n=72; 40 males; January-June 2012). The 4T scoring was supplied by referring providers and HIT testing was based on the following assays : Stic Expert HIT (Stago) = LFI Milenia (Germany), an ELISA screening (Zymutest Hyphen Biomed, France), Isotype determination (IgG, A, M) in case of positive ELISA and Serotonin Release Assay. HIT positive definition was based on a 4T’s Score ≥4 (Clinical/Platelet evolution) and positive SRA. Various associated clinical contexts were reported (Sepsis...
(n=31, Extra Corporeal Circulation (n=27), Hemodialysis (n=11), Chemotherapy (n=3)) and patients had received mainly UFH (n=61). ELISA was negative in 40 cases in accordance with both SRA and STIC Expert HIT assays. Ten patients were confirmed as having developed HIT but 9 false positive HIT were obtained with a positive STIC Expert HIT and negative SRA. STIC Expert HIT had a very good sensitivity (100%) with a high NPV (100%) and better specificity (85%) than ELISA screening (35%) and ELISA IgG (71%). These tests were in agreement i.e. negative or positive in 88% of cases allowing reinforcing HIT diagnosis or exclusion. These tests were discrepant in only 12% underlying the difficulty for HIT diagnosis. The authors concluded that the new LFI (STIC Expert HIT) is rapid, ready-to-use reliable, to rule out HIT.

**AcuStar HIT-IgG and HIMEA for rapid diagnosis of type-II HIT**

HemosIL® AcuStar HIT and heparin-induced multiple electrode aggregometry (HIMEA) were recently proposed as new rapid methods for the diagnosis of HIT.

The primary objective of the study presented by François Mullier (Namur, Belgium) was to assess the performances of AcuStar HIT-IgG(PF4-H), AcuStar HIT-Ab(PF4-H) and HIMEA. The secondary objective was to compare the performances of these assays with: PF4-Enhanced®, Light Transmission Aggregometry (LTA), 14C-Serotonin Release Assay (SRA), and clinical outcomes. Sera HIT-suspected patients (n=106) were studied retrospectively by AcuStar HIT-IgG(PF4-H), AcuStar HIT-Ab(PF4-H). HIMEA was performed on 81 patients. These tests were compared with ELISA (PF4-Enhanced®), LTA, SRA and clinical outcomes data by Chi-Square tests and ROC Curves.

The cut-off recommended by the manufacturer for HIT-IgG(PF4-H) and HIT-Ab(PF4-H) (i.e. 1 AU) showed positive predictive value (PPV) of only 64.3% and 45.0%, respectively. When clinical outcome was considered as the reference, negative predictive values of HIT-IgG(PF4-H), HIT-Ab(PF4-H) and HIMEA were 100%. The PPV reached 75.0%, 81.8% and 80.0%, respectively. Seventy-nine patients presented a medium-high pretest probability requiring biological testing. HIT-IgG(PF4-H) allowed to exclude the diagnosis of HIT in 67 of these patients. Among the 12 positive HIT-IgG(PF4-H) using our cut-off, 9 patients were confirmed HIT and HIMEA allowed to exclude the diagnosis of HIT in 2 out of 3 non-HIT patients.

F Mullier concluded that the combination of AcuStar HIT-IgG and HIMEA with optimized cut-offs is useful for rapid and accurate diagnosis of type-II HIT.

**Differences between enzyme immunoassays in the high heparin step are independent of the source of PF4** Andreas Greinacher et al (Greifswald, Germany) reported a follow up study on the issue of inhibition of PF4/heparin antigen assays by coincubation with high doses of heparin to increase diagnostic specificity of PF4/heparin EIAs. They compared different lots of PF4 prepared in Greifswald and PF4 supplied by GTI, which was complexed with heparin and coated on a microtiter plate by the same protocol. A commercial PF4/heparin kit from GTI was used as comparator. Most samples gave comparable/very similar results (negative, positive, inhibition by high dose of heparin) with all PF4 lots and the GTI EIA. However, a subgroup of samples was not inhibitable in the EIA as performed in Greifswald, whether PF4 from Greifswald or GTI was used, while they were inhibited by >50% when tested in the commercial GTI kit. The authors conclude that the discrepancies between different
PF4/heparin EIAs in regard to the high heparin step do not depend on the PF4 preparation but on other yet not identified factors, e.g. source of heparin/polyvinylsulfonate, method of coating, material of the micro titer plate, etc. This indicates that each manufacturer needs to validate their EIA for the high heparin inhibition step, even if the same PF4 source is used. As the details of the GTI coating method are proprietary information, it is currently not possible to further narrow down the factor causing the discrepancies in results obtained.

**Final discussion and perspectives**

Based on the presentations and the discussion of this SSC meeting on “Platelet Immunology”, it was concluded that 3 projects have to be planned for the next year.

The first is related to drug-induced thrombocytopenia (DITP) and the aim is to define updated and practical recommendations for the laboratory testing in suspected patients, and therefore a group lead by Donald Arnold (Hamilton, Canada) will work on this issue.

The second project is about ITP and DITP and it was proposed to form a working group led by Tamam Bakchoul (Greifswald, Germany) to define recommendations on the use of NOD/SCID mouse as a model for studying the pathogenesis and treatment of these immune thrombocytopenias.

The third project will focus on HIT and aims under the coordination of Chris Ward (Sydney, Australia) to evaluate and standardize the whole blood impedance aggregometry (using the Multiplate analyzer) for the diagnosis of HIT.
Platelet Physiology

29 June 2012

Chairman: Paul Harrison (UK)
Co-chairmen: Marco Cattaneo (Italy), Christian Gachet (France), Paolo Gresele (Italy), Dermot Kenny (Republic of Ireland), Diego Mezzano (Chile), Andrew David Mumford (UK), Alan T Nurden (France)

Paul Harrison briefly outlined the mission statement of the SSC for the benefit of new/young members of the audience and followed this with an update of current ongoing projects and a number of possible new projects.

The LTA guideline that was finished and agreed upon in Cairo (available on the SSC website) in 2010 is now in draft form and will be edited by the participating authors/experts for final submission to JTH as soon as possible.

Paolo Gresele presented the final data analysis of the detailed online survey of laboratory practice for the diagnosis of inherited platelet disorders that was collected and presented in 2011 and further refined in 2012. The data was collected from over 200 laboratories worldwide and will be published as soon as possible in JTH.

Paolo Gresele then followed this with the first presentation of the working party for the diagnosis of inherited platelet disorders including a detailed diagnostic algorithm. The proposed diagnostic algorithm was generated taking into account the responses to the world wide survey on current practice for the diagnosis of platelet disorders and based on intense discussion among working party members and includes a step-wise approach which allows even to not specialized laboratories to obtain a clear characterization of their patients and, when required, to refer to more specialized laboratories for definitive diagnosis of the most complex cases. After some discussion with the audience this will be further refined and edited for publication as a new guideline in JTH as soon as possible. This also included the bleeding score.

Maha Othman then presented an overview of Platelet Type VWD as suggested by the VWF SSC. Data from over 100 patients was presented and this enhanced the awareness of the audience of the problems with diagnosis of Type 2B VWD and platelet type VWD. Maha also presented the online database and put out a call for more patient information and samples. A surprising high number of samples (18%) remain misdiagnosed at the molecular level even though presenting with a gain of function phenotype to low dose ristocetin. These patients may well have another defect or there remain problems with the platelet function tests. Mixing studies are also recommended to help confirm diagnosis.

Dirk Sibbing presented an excellent and up to date overview of the monitoring of P2Y12 inhibition. We aim to produce a writing panel to produce a position statement similar to the position statement on aspirin resistance by Alan Michelson published a few years ago. At present, different assays are available for P2Y12 directed platelet function testing. Whereas the laboratory-based methods (light transmission aggregometry, VASP) are excellent assays for research purposes, point-of-care devices such as the VerifyNow P2Y12 assay, the Multiplate and the PFA-100 are most promising for use in clinical practice. Of note, the inter-assay correlation is modest at best, meaning that the assays and obtained results are
not interchangeable. The predictive value of P2Y12 directed platelet function testing in general is clearly established. Studies in more than 20,000 patients have shown an association of high on-treatment platelet reactivity (HPR) and a higher risk for ischemic events including stent thrombosis. However, it may well be that the value of testing as a risk marker for patient’s outcome might be restricted to patients with an acute coronary syndrome. Randomized trials in stable patients that have investigated the value of an individualized treatment based on testing results (TRIGGER-PCI, GRANITAS) showed negative results and are not supportive for routine testing. However, based on available evidence from observational studies, current guidelines (ESC, AHA/ACC) leave room for using platelet function testing in clinical routine by declaring a class IIb indication. Further studies are surely needed before a widespread use of testing (outside research institutions) can be recommended.

As a precursor to the first Thrombogenomics SSC session in Amsterdam next year Kathleen Freson and Graham Kiddle gave two talks on the platelet transcriptome. In platelet disorders caused by a reduction in platelet number (thrombocytopenia) and/or platelet function (thrombocytopenias), bleeding occurs immediately after injury, primarily in skin, mucous membranes, nose and gastrointestinal tracts. Classical thrombopathies/thrombocytopenias can be associated with some other phenotypes including neurological, endocrinological and metabolic diseases. Platelets are easy accessible cells and different techniques are possible to study platelet function under basal and activated conditions. Defects in platelet adhesion, G protein signaling and secretion can result from mutations in platelet-specific genes leading to isolated thrombopathies or from mutations in widely expressed genes leading to broader clinical phenotype including a platelet defect. Therefore, it is important to recognize how platelet research becomes an important tool to improve our knowledge in broad phenotype mendelian disorders of which some were discussed in further detail. Recently, novel methods in sequencing, epigenetics, proteomics, functional genetics in animals (including zebrafish) and transcriptomics, plus unprecedented large-scale cooperative efforts, led to the generation of novel insights in the complexity of inherited platelet disorders of which the pathology was known long before finding the responsible underlying genetic causative factor. Especially medical DNA sequencing is expected to give clinicians important information regarding the genetic phenotype of the inherited platelet disorder in their patients to improve early diagnosis and prognostication. For inherited bleeding and platelet defects the initiatives for candidate gene screening (thrombogenomics; www.thrombogenomics.org.uk) and exome sequencing (Bridge study consortium; www.bridgestudy.org) will be essential for diagnosis of patients with unknown defects that result in bleeding.

The aim of the thrombogenomics project is to reduce the time to diagnosis of rare platelet and bleeding disorders through the provision of next generation sequencing technologies (NGS) capable of the simultaneous sequencing of all such disorders. There are 2 main areas of activity, namely, to curate relevant genes into a stable and sustainable reference database and then to use this alongside a diagnostic sequencing platform. Tools have been developed to assist with the curation and ISTH experts have already been and will continue to be recruited by the four co-chairs of thrombogenomics. The ISTH experts have the crucial role to assist with the curation and to act as final approvers of what will be uploaded to the Locus Reference Genomic (LRG) database that is maintained by the European Bioinformatics Institute (EBI). The advancements in NGS were summarized alongside the possibilities presented by selective sequence capture. The combination of low cost high output sequencing with the selective capture of the areas of the genome specific to the causes of rare platelet and bleeding disorders makes feasible the sequencing of all disorders simultaneously. Due to the indexing of DNA samples, a single sequencing experiment can also be used to sequence multiple patient samples for all disorders which keeps the potential cost per patient at a low level. It is expected to have preliminary
data by May 2013 with the development of the platform, recruitment of DNA samples and curation of the genes already well under way.

Johan Heemskerk then gave an excellent overview of the history of in vitro devices including microfluidics for studying thrombus formation in real time.
Predictive Variables in Cardiovascular Disease

28 June 2012

Chairman: James Douketis (CA)
Co-chairmen: Shinya Goto (Japan), Karel Moons (The Netherlands), Frederick Spencer (Canada), Alex Spyropoulos (USA), Alberto Tosetto (Italy), Richard White (USA)

1.0 Predictive Variables in Cardiovascular Disease Subcommittee Session

Part A: Educational Session Topics

Managing patients on new oral anticoagulants who need surgery: how to minimize risk for thrombosis and bleeding? (Marc Samama, Fr)

Managing patients on new oral anticoagulants who need surgery: how to use and interpret coagulation tests? (Jerrold Levy, USA, replacing Beverley Hunt, UK)

Managing patients on antiplatelet drugs who need surgery: can platelet function assays predict bleeding? (Jerrold Levy, USA)

Weighting of thrombosis and bleeding risk in clinical trials: application to the perioperative setting (Alex Spyropoulos, USA)

Part B: Update of SSC-Related Activities Topics

The Austrian Study on Recurrent Venous Thromboembolism (AUREC): an update (Paul Kyrle, Aus)

Assessing patients’ risk for stroke and bleeding: how this informs decisions about which anticoagulant to use (Shinya Goto, Jp) replaced by: Update of new proposals by `D-dimer and other predictors of recurrent VTE` Collaborative Group (M. Marcucci)

Patient risk scores: how to assess their usefulness in clinical practice? (Carl Moons, Neth)

CHADS\textsubscript{2} and CHADS\textsubscript{2}VASc to determine stroke risk: one or both? (Scott Kaatz, USA)

Summary: The meeting was very well attended (larger venue could be considered for future years) with engagement by audience. Two of the original speakers could not attend due to illness and family reasons (B. Hunt, S. Goto).

2.0 SCC Project: D-dimer and other predictors of recurrent VTE

A meeting was convened of a collaborative research groups studying pooled data from several studies assessing patients with VTE and risk for recurrence.

This is the 4\textsuperscript{th} meeting of this group, which was formed after the 2008 SSC meeting in Vienna.
During this meeting, ongoing and future collaborative initiatives relating to determinants of recurrent VTE were discussed. Plans were made for future research collaborations involving the existing pooled database and involving other independent studies with common aims to those of our group.

To date, our group has 5 publications in: Ann Intern Med, BMJ, J Thrombo Haemost (2), J Clin Epidemiol

The attendees of this meeting included: J. Douketis (Can - Chair), P. Kyrle (Aut), S. Eichinger (Aut), M. Marcucci (It), K. Moons (Neth), G. Palareti (It), D. Poli (It), C. Tait (UK), A. Tosetto (It), G. Lowe (UK).

3.0 SSC Project: Perioperative Determinants of Thromboembolism and Bleeding

A collaborative group was formed (see: attached document) with an overall aim to study patients who are receiving dual antiplatelet therapy and new oral anticoagulants and require surgery or procedures. We will coordinate studies aimed at addressed gaps in practice in this clinical domain, for example, managing patients with coronary stents who require urgent surgery or those receiving a NOAC who require elective surgery.

This is the 1st meeting of this group.

The attendees of this meeting included: J. Douketis (Can - Chair), C. Samama (Fr), G. Levy (USA), A. Greinacher (Ger), A. Spyropoulos (USA), 3 accompanying clinical research fellows.

This collaborative group plans to meet on an annual basis (SSC meetings) and, when possible, at other scientific meetings.
Vascular Biology
29 June 2012

Chairman: Françoise Dignat George (FR)
Co-chairmen: Elizabeth Gardiner (Australia), John Griffin (USA), Nigel Key (USA), Peter Newman (USA), Rienk Nieuwland (The Netherlands), Florence Toti-Orfanoudakis (France)

This year, the SSC VB was divided into three parts addressing respectively: Part A, "Shed proteins/receptors". Part B "Detection and Characterization of circulating endothelial cells and their progenitors", and Part C "Detection and enumeration of circulating microparticles." The success of this SSC VB was attested by the presence of an average of 80 to100 participants during the whole session.

Part A of the session, chaired by Elizabeth Gardiner and Peter Newman, covered the mechanism of protein shedding and their relevance as clinical biomarkers of vascular disease.

Elizabeth Gardiner (Australia) gave a talk on shear-induced release of platelet receptors by metalloproteases based on previous results showing that ADAM10 is the key platelet surface metalloprotease activated by coagulation or platelet collagen receptor GPVI engagement by ligand. Absence of GPVI from the surface of platelets either through genetic disposition or presence of anti-platelet autoantibodies causes a marked bleeding defect indicating that functional GPVI is essential for normal haemostasis. Loss of GPVI from the surface of a circulating platelet by shedding leads to changes in platelet responsiveness and reduced ability of platelets to activate. Elizabeth Gardiner examined whether shear force, in the absence of GPVI ligand, was sufficient to induce shedding of GPVI. She examined how chronic exposure to elevated and changing shear stresses differentially regulates metalloproteolysis of GPVI, a key platelet adhesion-signalling receptor. Human citrated PRP or washed platelets were subjected to increasing shear rates in a cone-plate viscometer and levels of intact and cleaved GPVI were examined by western blot and ELISA. Patho-physiological shear rates (3,000-10,000 s⁻¹) induced platelet aggregation and metalloproteinase-dependent appearance of soluble GPVI ectodomain, and GPVI platelet remnant. Shedding of GPVI continued after transient exposure to shear. Blockade of allbb3, GPIba or intracellular signalling inhibited shear-induced platelet aggregation but minimally affected shear-induced shedding of GPVI. Shear-induced GPVI shedding also occurred in PRP or washed platelets isolated from a von Willebrand Disease Type 3 patient with no detectable VWF, implying that shear-induced activation of platelet metalloproteinases can occur in the absence of GPVI and GPIba ligands. Finally, Elizabeth Gardiner showed that significantly elevated levels of sGPVI were observed in 10 patients with stable angina pectoris, with well-defined single vessel coronary artery disease. These data suggest that loss of GPVI in platelets exposed to shear could have potential implications for the stability of a forming thrombus at arterial shear rates.

Elaine W. Raines (USA) presented data showing that proteolytic shedding by ADAM 17 functions as a gatekeeper for leukocyte accumulation at inflammatory sites and for resolution of inflammation. Leukocyte recruitment to inflammatory sites involves sequential adhesive and de-adhesive interactions associated with dynamic changes in expression and function of cell surface proteins. Elaine W. Raines hypothesizes that proteolysis of cell surface proteins allows instantaneous uncoupling of adhesive
and signaling events, and thus could be a key regulator of the inflammatory response. ADAM17 (also known as tumor necrosis factor-a-converting enzyme or TACE) proteolytically sheds multiple cell surface proteins, but the physiological importance of cleavage of specific substrates is incompletely understood. She presented in vivo analyses of leukocyte trafficking demonstrating that substrate-, context-, and myeloid cell-type-specificity of ADAM17-mediated cleavage of its substrates. For neutrophils, but not monocytes, ADAM17 targets their initial interaction with activated endothelium through cleavage of L-selectin, and thus limits neutrophil rolling and subsequent downstream adhesion events required for their transendothelial emigration into tissues. In contrast to neutrophils, ADAM17 regulates monocyte accumulation within inflammatory sites by apparent proteolytic release of its mitogen, macrophage colony-stimulating factor. Finally, spontaneous exiting of inflammatory macrophages, critical for resolution of inflammation, is controlled by ADAM17 through its cleavage of the integrin aMb2 (Mac-1). In the absence of leukocyte ADAM17, the number of macrophages remaining at the inflammatory site is two-fold elevated relative to wildtype, a cell-intrinsic characteristic. Taken together Elaine W. Raines concludes that ADAM17 functions are critical for restriction of neutrophil infiltration, promotion of monocyte proliferation and resolution of the inflammatory response.

Andreas Ludwig (Germany), gave an overview of the pro-inflammatory activities of vascular ADAMS proteins. The metalloproteinasises ADAM10 and ADAM17 are responsible for several shedding events leading to increased levels of soluble vascular surface molecules in the course of vascular inflammation. Among the shed proteins there are proinflammatory cytokines, adhesion molecules that are involved in endothelial barrier function or transendothelial transmigration of leukocytes and growth factors that have been implicated in transactivation of vascular cells and vascular remodelling. Inhibition as well as knock-down experiments have demonstrated that both ADAM10 and ADAM17 are critically involved in leukocyte transendothelial migration or detachment and also mediate increased vascular permeability. Andreas Ludwig presented recent data that identity ADAM10 and ADAM17 as sheddases for transmembrane chemokines CX3CL1 and CXCL16, junctional adhesion molecule JAM-A, syndecan-1 and -4 and VE-cadherin in vitro and in vivo. He recently showed that intranasal application of ADAM10/ADAM17 inhibitors reduces the development of acute lung injury induced by endotoxin. Conditional knock out of ADAM10 or ADAM17 in endothelial reduces LPS-induced permeability changes and protects mice from acute lung inflammation (5). Conditional knock out of ADAM17 in smooth muscle cells also protects mice by suppression of growth factor-mediated vascular transactivation leading to reduced production of cytokines and chemokines. All together, these data show that ADAM17 activation in endothelial and smooth muscle cells promotes acute pulmonary inflammation in response to endotoxin by multiple shedding events leading to enhanced vascular permeability, proinflammatory mediator production and leukocyte recruitment.

François Lanza (France) presented an overview of GPV function and its utility as a marker of thrombosis in vascular disorders. Despite its identification more than 30 years ago the real function of GPV is still subject to question. This 82 KDa platelet specific glycoprotein and late marker of thrombopoiesis non-covalently associates with the GPIb-IX complex, a process required for its efficient surface exposure. GPV-deficient mice do not exhibit a Bernard-Soulier phenotype, their platelets normally express GPIb-IX, but they exhibit decreased and increased responses to low concentrations of collagen and thrombin,
respectively. These subtle defects could explain the variable anti- or prothrombotic tendencies reported in different models of thrombosis, but have no consequence on haemostatic responses. GPV’s sensitivity to a series of proteases (thrombin, elastase, ADAM10, -17), with the production of 69 to 80 kDa soluble fragments, is conserved in other species, suggesting a role of the released forms in thrombotic and inflammatory situations. These hypotheses could be tested in models expressing GPV mutated at these cleavage sites. Based on GPV expression on platelets coupled to its thrombin sensitivity, François Lanza discussed the utility of GPV as a marker of thrombosis in cardiovascular disorders. Increased levels of soluble GPV were recorded in arterial (myocardial infarction, stroke, and peripheral arterial disease), as well as in venous thrombotic manifestations (deep vein thrombosis, pulmonary embolism). A similar increase in diabetic patients indicated thrombin-independent GPV release mechanisms in certain cardiovascular diseases. GPV has also demonstrated its utility in the quality control of platelet manufacturing processes in transfusion. In summary, despite having demonstrated its utility as a biomarker, GPV has yet to reveal its exact function in fields other than haemostasis.

The Part B of the session, chaired by Francoise Dignat George, covered the detection and measurement of circulating endothelial cells and their progenitors.

Gian Paolo Fadini (Italy), presented an overview on the clinical relevance of circulating endothelial progenitors (EPCs) for assessment of cardiovascular risk. Indeed, a significant proportion of cardiovascular events (CV) occur in subjects categorized into intermediate risk categories. Therefore, discovery of new CV risk biomarkers is important to aid a better CV risk stratification. Endothelial progenitor cells (EPCs) are involved in endothelial repair and angiogenesis. EPC level is reduced in the presence of CV risk factors compared to healthy subject and further decline when CV disease develops and worsens. Gian Paolo Fadini gives a review of the main clinical studies that identify EPCs as a new candidate mechanistic biomarker to assess CV risk. A few longitudinal studies have assessed the ability of EPCs and other progenitor cell phenotypes to predict CV outcomes in different categories of patients. These studies consistently show that a low baseline progenitor cell level is associated with a poor outcome at follow-up. However, a meta-analysis of patients’ level data of such studies has shown that the incremental information provided by progenitor cell level over and beyond traditional risk assessment is limited. Intriguingly, however, EPC level is amenable to pharmacologic modulation by several mechanisms. Therefore, EPC-based intervention may represent a new avenue for the prevention and treatment of CV disease. Whether restoring EPC levels improves long term cardiovascular outcomes will need to be ascertained in future studies. Therefore, methodological standardisation is needed for a better evaluation of their clinical relevance. Hovewer, methodological standardisation is needed for a better evaluation of their clinical relevance.

Jamie Case (USA) presented data on the potential of polychromatic flow cytometry to identify immature and mature circulating endothelial cells. Jamie Case, (USA) identifies and characterize two distinct populations of bona fide circulating endothelial cells, including the endothelial colony forming cell (ECFC), by electron microscopy, colony assays, immunomagnetic selection and polychromatic flow cytometry (PFC). A population of cells containing both ECFCs and mature circulating endothelial cells (CECs) were determined by varying expressions of CD34, CD31 and CD146, but not AC133 and CD45. HE showed that after immunomagnetic separation, these cells failed to form hematopoietic colonies.
Clonogenic endothelial colonies with proliferative potential were obtained, thus verifying their identity as ECFCs. The frequency of ECFCs were increased in human umbilical cord blood and were extremely rare in the peripheral blood of healthy adults. In addition, Jamie Case, also detected another mature endothelial cell population in the circulation that was apoptotic. Finally, when comparing this new PFC protocol to a prior method, he determined that the present protocol identifies circulating endothelial cells while the earlier protocol identified extracellular vesicles. Jamie Case concluded that two populations of circulating endothelial cells including the functionally characterized ECFC are now identifiable in human umbilical cord blood and peripheral blood by PFC. Therefore, methodological standardisation is needed for a better evaluation of their clinical relevance.

Florence Sabatier (France) presented data showing that changes in circulating endothelial cells and their progenitors during percutaneous coronary angioplasty (PCI) reflect endothelial response to injury. Percutaneous coronary intervention (PCI) became the most commonly used revascularization strategy in coronary artery disease patients due to the advents of stents and improvement of anti-platelet therapy. However, the main pitfall of this procedure remains the development of in-stent restenosis (ISR) and the need for subsequent target lesion revascularization (TLR). Disruption of endothelial integrity is recognized as the primary event triggering inflammatory and proliferating signals leading to intimal hyperplasia. This endothelial injury results from mechanical trauma but also inflammatory processes involving platelet activation and interactions with the vessel wall. Animals studies indicate that early regeneration of a functional endothelial layer is critical for preventing pathological remodeling of the arterial wall and may involve bone marrow derived endothelial progenitor cells.

Florence Sabatier investigated the usefulness of enumeration of circulating endothelial cells (CEC) reflecting endothelial injury and endothelial progenitor cells (EPC) indicators of repair capacity. EPC and CEC were enumerated to monitor the endothelial response to PCI and the prediction of cardiovascular outcomes, in patients with stable CAD undergoing PCI with bare metal stent implantation enrolled in a prospective longitudinal study. CEC were enumerated using the standardize CD146 based immunomagnetic separation assay. Enumeration of circulating CD34+ progenitor cells and CD34+KDR+ EPC was performed using multicolor flow cytometry protocols adapted from the standardized ISHAGE strategy. Measurements were performed before PCI and at 6h and 24 hours after the procedure to analyze dynamic changes of these markers.

She showed that PCI induced transient rise in CEC levels attesting significant endothelial lesion. Changes in CEC levels were more pronounced in patients presenting with high on treatment platelet reactivity (VASP index >50) after a loading dose of 75mg clopidogrel compared to good responder patients (VASP <50%). In multivariate analysis, VASP group, the number of disease vessels and the number of implanted stents independently predicted endothelial injury. PCI also induce the mobilization of CD34+ PC and CD34+KDR+ EPC as indicated by increased levels at H6 and H24 compared to baseline levels. Interestingly, Changes in CD34+KDR+ cells independently predicted the occurrence and Major acute cardiovascular events at 6 months follow up.

These data indicate that efficient inhibition of platelet reactivity in response to clopidogrel therapy exert protective effect on endothelial cells during PCI. In addition, the mobilization of cells with a capacity for
endothelial differentiation and repair determines the risk for ISR in CAD patients undergoing PCI and BMS implantation. These data provide new clues of the usefulness of endothelial biomarkers in the identification of patients at risk of poor outcome after PCI and to design and evaluate preventive therapeutics.

The Part C of the session, chaired by Francoise Nigel Key and Florence Toti covered the detection and measurement of circulating cell-derived miro particles

John. Nolan (San Diego), as actual President of ISAC (Int. Soc. for Advancement of Cytometry) works to encourage merging ISTH expertise in Hemostasis and technological expertise in FCM present among ISAC members. In order to help communication in the field of MP/MV studies, an Interest Development Group (IDG) on microvesicles has been launched that can be reached on the ISAC website. Due to its multi-parametric analysis capability FCM remains the most widely used technology for the evaluation of MP/MV. However, their small size challenges FCM sensitivity limits for both sizing and immunological detection. Among the many technological issues that are to be considered (and will be in the context of ISAC), emphasis is made on 1/ choosing the best triggering approach, with a growing interest towards fluorescence-based triggering, 2/ avoiding artefacts generated by coincidence (when more than one particle at a time is present in the laser beam, a phenomenon improperly named as "swarming") by doing dilution experiments by doing dilution experiments, 3/ the relevance of calibrating fluorescence axis in addition to scatter axis for the sake of standardization 4/ developing new reference standards such as for example fluorescent liposomes.

Alain R. Brisson (France) presented a characterization of microparticles from blood and activated platelets by cryo-electronmicroscopy. He provides a comprehensive structural description of circulating MP/MV found in various body fluids in physiological and pathological situations. In this first study, he focused plasma from healthy donors and on microparticles derived from activated platelets, with the objectives of characterizing the morphology and size distribution of the whole microparticle population using two probes: annexin 5 that bind to phosphatidylinerine and an antibody to GP-1b platelet protein.

He used cryo-Electron Microscopy and specific probes were made of Annexin-5 covalently coupled to gold nanoparticles. Cryo-EM provides the unique advantage to reveal the genuine structure of microparticles in pure PPP (Plasma-Poor-Platelet) samples, in the absence of any treatment, like fixation, staining or drying. Microparticles could be classified into several morphological families, namely isolated microparticles, microparticles made of clusters of vesicles and aggregate-like particles. Plasmatic microparticles range in size from 50 nm to 3 µm. About 75% of them are smaller than 500 nm. Microparticles derived from activated blood platelets present the same morphologies and the same size range. Size histograms were determined for the whole microparticle population and for the sub-population of PS-exposing microparticles.

The data presented by Alain R. Brisson provide novel structural information on plasmatic microparticles and open avenues for characterizing circulating microparticles present in various physio-pathological situations.
Jovan Antovic, (Sweden) proposed a strategy to study microparticles in hemophilia.

He analyzed samples of patients with hemophilia A and determined microparticles (MP) on a Beckman Coulter Gallios flow cytometer using the 5-color protocol he used: 1/phalloidine as a quality control – MP were defined as phalloidine negative particles < 1µm. 2/lactadherin positive, which binds PS in a Ca-independent manner 3/ CD42a for the determination of platelet MP (PMP), CD45 for leukocyte MP (LMP) and CD144 for endothelial MP (EMP).

He reported a paradoxical decrease of circulating TMP, PMP and EMP after on-demand treatment with FVIII concentrate while TMP and PMP inversely correlated with hemostatic activation and fibrin gel tightness. A plausible explanation may be that MP are incorporated in the hemostatic plug formed after FVIII substitution on the site of injury (injury and bleeding usually precede the concentrate injection in patients treated on-demand). He proposed that MP may provide a negatively charged surface for the activation of coagulation in situ and also promote coagulation via the expression of TF and P-selectin on their surface.

Micah Mooberry (USA) presented the effect of different techniques on microparticle analysis by flow cytometry. Flow cytometry remains the most commonly used methodology for the detection, enumeration and characterization of microparticles (MPs). Despite this fact, there are inherent limitations with flow cytometry that impede the detection of MPs, most of which are technologically driven. Of these, the intrinsic resolution of the cytometer and the level of background noise have significant impact. To determine the effect of different triggering techniques on these limitations, and thus the sensitivity for MP detection, Micah Mooberry evaluated several newer generation flow cytometers. For each cytometer, 2 sets of biological samples were analyzed using SSC, FSC +/- SSC, and FITC triggers. Biological samples included PFP obtained from whole blood incubated with LPS and the supernatant of washed platelets treated with ionophore, both of which were analyzed for PMPs (AnnexinV+, CD41+). Overall, sensitivity for PMP detection using FSC trigger was roughly equivalent amongst all cytometers on both sets of biological samples and included a portion of the smaller subset of MPs < 0.5 µm. SSC trigger data were more variable across machines, however compared to FSC, it offered a better sensitivity for PMP detection, when performed on a cytometer with high sensitivity SSC PMT, which detected > 2x the number of PMPs compared to FSC trigger. Fluorescence triggering provided the overall highest sensitivity for PMP detection, although the reliability of these results are questionable due to potential problems with specificity and nonspecific fluorescent signal. Micah Mooberry concluded that the optimal trigger technique may vary with cytometer, based upon the individual strengths of the machine. Fluorescence triggering may also offer the greatest sensitivity for MP detection, but possibly at the expense of specificity.

Philippe Poncelet (France) presented forward versus side scatter strategies to standardize microparticle count by flow cytometry. Reliable measurement of cell-derived microparticles (MP) by flow cytometry (FCM) is hampered by their low size range (0.1–1µm). He showed that extending a 1st standardized protocol for MP counts in the limited size range of 0.5 to 1µm bead-eq., expanded this range down-to 0.3µm on Beckman-Coulter (BC) Gallios, thus detecting previously undetectable “small” MP. VB SSC studies showed that this strategy, optimized on BC FCMrs measuring Forward Scatter (FSC)
at high solid angle (1-19°), did not totally fit with FCMrs using low angle (1-8°) collection, including Becton-Dickinson (BD) FACS and LSRs. Side Scatter (SSC) was a more robust size-related triggering signal on such FCMrs but different bead-based reference systems (“Megamix-Plus SSC” rather than “Megamix-Plus FSC”) were needed when using this alternative parameter. Philippe Poncelet aimed to provide scatter-based reference levels for threshold and/or gating permitting general standardization of MP counts across platforms.

Comparisons of PMP counts were operated on the same plasma samples on FCMrs of each group (FSC- and SSC-optimized). Delineation of small PMP was made in FSC on BD Gallios using 0.3µm and 0.5µm beads; large PMP were found between 0.5µm and 0.9µm beads. The boundaries of the same two subsets were found on the SSC scale of BD FCMrs between 0.17 and 0.22 µm bead-eq (small PMP) and between 0.22 and over 0.5 µm bead-eq (large PMP). Similar counts were obtained on these size-defined PMP subsets using different platforms, demonstrating the ability of this standardization strategy to monitor inter-instrument reproducibility.

On the behalf of Rienk Nieuwland, (The Netherlands), Edwin van der Pol (NL) presented a new project, granted within the “European Metrology Research Programme” of EURAMET, and based on metrological characterization of microvesicles from body fluids as non-invasive diagnostic biomarkers.

In this project, which is collaboration between a university hospital (AMC, Amsterdam) and four European metrological institutes (Netherlands, Germany, Belgium, and Switzerland), Rienk Nieuwland had two major aims: 1/ Detailed characterisation of extracellular vesicles (size, distribution, concentration, morphology, (bio) chemical composition) using several techniques in parallel such as SAXS, ASAXS + XRF, AFM, NTA, RPS, etc. 2/ Develop standardized methods, protocols & reference materials to enable comparison of MV data between laboratories.

With regard to point 2, the aim is to develop MV reference standards which are stable, provide repeatable measurements, and have properties similar to MV (such as size, morphology, refractive index). In his presentation he explained the background and aims(s) of the project, with emphasis on point 2, explaining his approach. As one of the last steps in this project, was inter-clinical laboratory comparison of selected MV reference materials (at least 10 different European clinical laboratories from at least 5 different countries). Via the SSC of the ISTH, he asked for participants in this survey.

Closing remarks

Finally, Francoise Dignat George presented the update and perspectives of the ISTH Vascular Biology Working Group on the Measurement of microparticles by flow cytometry. Important questions were raised during previous SSC VB, namely: 1/ the possibility of standardizing MP enumeration by flow cytometry; 2/ the need for new generation flow cytometers or alternative technologies that would allow enumeration and characterization of particles of smaller sizes; 3/ study the impact of pre-analytic parameters on MP determination. Some of these questions have been partially been addressed.

During the ISTH SSC that took place in Vienna in 2008, a first collaborative workshop on the standardization of microparticle counts was set up to define the inter-laboratory reproducibility of PMP
counts using flow cytometry. Presented in Boston in 2009, the results of this workshop have been published in the Journal of Thrombosis and Haemostasis. J Thromb Haemost. 2010 Nov;8(11):2571-4 "Standardization of platelet-derived microparticle enumeration by FCM using calibrated beads: results of ISTH SSC. Coll workshop": R., S. Robert, P. Poncelet, S. Glover, N.S. Key, F. Dignat-George, and all SSC participant. We thank all the investigators who helped to set up this workshop as well as those who actively participated. However some questions remained unsolved because this strategy was not suitable to all types of FCM. Future directions are: to test new synthetic reference standards to enlarge the compatibility to all types of FCM.

In 2010, the pre-analytical phase was identified as a critical target for future standardization studies, and the Cairo SSC in VB was the opportunity to propose a new collaborative workshop on the impact of pre-analytic variables on MP determination. The first conclusions of this ongoing study have been presented in Kyoto. The results demonstrated that a standardized protocol results in a significant reduction of the inter-laboratory variability in PMP analysis that was more marked for flow cytometry. The future steps are now to evaluate the impact of this improvement of preanalytics on other MP subsets.

In addition, important advances were made during the Kyoto meeting indicating that: 1/ recent technological improvements maintain flow cytometry as a competitive analytical method to measure MP of smaller size; 2/ alternative technologies (NTA, DLS, ISADE, AFM, ...) open new options to enumerate MP of small size.

During Liverpool meeting, the most critical new questions were: 1/ Propose homogeneous preparations of biological and non biological particles as standards within the community (See proposals of new subcommittees projects) 2/ Propose new reagents (probes, antibodies…) specifically dedicated to MP determination.

These questions point directions for two novel collaborative working parties in the VB SSC that were proposed during Liverpool:

- **New project 1**

**Name of the project:** Collaborative SSC project on standardization of MP counting using calibrated bead standards and high sensitivity-Flow Cytometric platforms

**Person responsible:** Francoise Dignat-George

**Methodology:** candidate material (Frozen Platelet Free Plasma with defined levels of MP and calibrated beads used as standard) will be send to participating laboratories to 1/ check the adequacy of their high sensitivity-Flow Cytometric platform 2/ evaluate the sensitivity and reproducibility of MP counts.

**Year of starting:** 2012 (Liverpool)

**Annual report:** 2013 (Amsterdam)

**Year of expected completion:** 2014 (Milwaukee)
New project 2

Name of the project: Collaborative SSC project on development and distribution of traceable reference materials

Person responsible: Rienk Nieuwland

Methodology: validate developed protocols using:

- traceable reference materials (synthetic particles and biological particles)
- various detection methods available for particle determination (FCM, NTA, DLS, ISADE, AFM, Raman spectroscopy "")

Year of starting: 2012 (Liverpool)

Distribution of reference material: 2014

Expected completion and Publication as SSC report: 2016

Instructions and documents to download will be available from the ISTH / SSC website www.isth.org

If you are interested to participate, Send an e-mail to:

Project 1: francoise.dignat-george@univmed.fr before October 2012

Project 2: r.nieuwland@amc.uva.nl, before October 2012
von Willebrand Factor

Part I: 29 June 2012

Part II: 30 June 2012

Chairman: Jeroen Eikenboom (NL)
Co-chairmen: Thomas Abshire (US), Imre Bodo (HU), Jorge DiPaola (US), Yoshihiro Fujimura (JP), Daniel Hampshire (UK), Paula D. James (CA), Johanna Hovinga (Switzerland)

Audience: approximately 80-100

Summary of VWF Subcommittee Approvals and Projects

· Project Standardization of VWF propeptide estimation: assignment of VWFpp value (since October 2011) to WHO 6th IS FVIII/VWF Plasma (07/316) and (since June 2012) to the SSC/ISTH Secondary Coagulation Standard Lot #4. SCC Official communication has been published JTH 2012;10:959-960.

· New Project initiated: Prepare detailed methods document for VWF multimer analysis and minimal requirements of quality of multimer analysis for diagnostic purposes (in collaboration with CLSI)


· Project Comparison of VWF activity assays has started. Uniform nomenclature is to be decided on for the new activity assays.

· ISTH-SSC VWF Online Database (http://www.vwf.group.shef.ac.uk/): database moved to new platform (Leiden Open Variation Database (LOVD) format). Ongoing.

· Registry on Acquired Von Willebrand Syndrome (www.intreavws.com): new website will be operative within September 2012. Ongoing.

· Registry on platelet-type VWD (www.pt-vwd.org). Ongoing.

· Project "Desmopressin in the management of Von Willebrand disease: biological response versus clinical efficacy", enrollment closed, data analyzed, manuscript in preparation, submission anticipated August 2012.

Friday, June 29, 2012

Joint session VWF and Factor VIII and IX subcommittees

Bleeding scores

Session Chairpersons: Flora Peyvandi (IT)/Paula James (CA)

- Update from the standing committee on Bleeding Assessment Tool (Francesco Rodeghiero, IT)
The Scientific and Standardization Executive Committee of ISTH has established in October 2011 the ISTH-BAT Standing Committee, which stems from a joint agreement between ISTH and the Rockefeller University, that recently deployed an electronic version of the ISTH-BAT (Rodeghiero et al, J Thromb Hemost 2010: 8: 2063–5). The tool is now available online at https://bh.rockefeller.edu/bat/ and is aimed at:

- providing a web-accessible platform, encouraging uniformity in the standardization and collection of bleeding histories;

- maintaining a web-accessible database of bleeding symptoms that can be used by individual investigators for cooperative studies.

Members of the Standing Committee for the first 5-year term are F. Rodeghiero (Chairman); A. Tosetto (Secretary); B. Coller (representative of the Rockefeller University); A. Falanga (SSC Chairman); D. Lillicrap (SSC Chairman-elect); J. Eikenboom, S. Eichinger and P. Monagle as current chairmen of the SSC Subcommittees on Von Willebrand Factor, Women's Health Issues in Thrombosis and Hemostasis and Perinatal/Pediatric Hemostasis. The rules for the use of this common database, settled by the S.C., have been presented.

The ISTH-BAT Standing Committee is open to receive any proposal for collaborative scientific studies from all SSC Subcommittees willing to investigate the clinical phenotype of inherited bleeding disorders in a wide range of contexts (write to rodeghiero@hemato.ven.it).

- Web based Bleeding Assessment Tool (BAT) (Barry Coller, USA)

Dr. Coller described the elements of the ISTH-BAT infrastructure, including the bleeding history ontology and the electronic questionnaire. At present, Rockefeller University will host the system, including the database. He also described the measures to insure subject confidentiality and data security. The system is available to all investigators, but requires an IRB-approved protocol, participants’ informed consent, and compliance with the governance rules developed by the ISTH/SSC. Individuals can opt to have access to their own data exclusively or to aggregate data, but the latter requires approval for others to view the deidentified data from the investigator’s center under the auspices of the ISTH/SSC. Several investigators are currently using the system.

- What type of scoring system is necessary in hemophilia (Alberto Tosetto, IT)

The use of standardized bleeding assessment tools (BAT) has been proposed for the diagnosis and the clinical description of mild hemorrhagic disorders such as von Willebrand disease. There is no experience in patients with severe bleeding disorders, with most evidence coming from type 3 VWD. For hemophilia, the use of the recently proposed ISTH-BAT could improve the ability to more accurately describe the clinical picture and offer a more reliable estimate of the incidence of severe bleeding symptoms in these patients. Presently used BATs, however, lack some contents that could be useful to define the amount of disability in hemophiliac patients (e.g., degree of arthropathy). Such measures would be extremely important to generate a standardized measure of disease outcome, that would be important for prognostication.

- Bleeding score in rare bleeding disorders (Roberta Palla, IT)
Rare bleeding disorders (RBDs) represent 3% to 5% of all inherited coagulation deficiencies and include fibrinogen, factor (F) II, FV, FVII, FX, FXI, FXIII, and combined FV and FVIII deficiencies (FV+VIII). Due to their rarity, diagnostic and management challenges persist within RBDs. A tool was presented that could help distinguish patients with RBDs from normal controls based on their bleeding history, to aid in planning of subsequent diagnostic workup after an initial encounter. The tool is based on a bleeding score constructed by retrieving data related to previous bleeding types, frequency, spontaneity, extent, localization, and relationship with prophylaxis and treatment. A multivariate model was constructed, with a RBD diagnosis as the outcome, and a formula that generates the probability of having a RBD based on demographics and the bleeding score was established.

**VWF subcommittee, part I**

**Standardization of assays**

Session Chairperson: Thomas Abshire (USA)

- Review of the available WHO IS for all VWF-related analytes, and VWFpp calibration (Tony Hubbard, UK)

Quantification of the diverse properties of von Willebrand factor is achieved by the assay of test samples relative to reference standards calibrated in International Units (IU). Two WHO International Standards (IS) define the IU for VWF, namely the WHO 6th IS Factor VIII/VWF Plasma (07/316) and the WHO 2nd IS VWF Concentrate (09/182). Inter-laboratory variability is greatly reduced when test samples are assayed relative to reference standards composed of similar material (like vs like). For VWF it appears that the multimer profile is one of the most important determinants of similarity with regard to agreement between methods and laboratories. Hence plasma test samples should be assayed relative to plasma reference standards calibrated, in IU, against the WHO IS Plasma and therapeutic concentrates should be assayed either directly against the WHO IS Concentrate or relative to concentrate reference standards calibrated against the WHO IS Concentrate. The WHO IS Plasma has assigned values for VWF:Ag, VWF:RCo, VWF:CB and (since October 2011) VWF propeptide (VWFpp). The WHO IS Concentrate has assigned values for VWF:Ag, VWF:RCo and VWF:CB. A secondary plasma standard, the SSC/ISTH Secondary Coagulation Standard Lot #4 (SSC Lot #4), is available to manufacturers for the purpose of harmonising the calibration of commercial reference plasmas and diagnostic kits. SSC Lot #4 has assigned values for VWF:Ag, VWF:RCo, VWF:CB and (since June 2012) VWFpp.

- Standardization of the high quality multimer assay: a Hamburg-Budapest project (Imre Bodo, HU)

The goal of this project was to replicate the high quality multimer technique of Ulrich Budde’s lab in another lab. First, a PhD student studied the method in Hamburg, which improved the multimer quality but it was still unsatisfactory. Then, the entire laboratory equipment was moved to Hamburg, and a lab technician from Budapest worked side by side in Budde’s lab. Many minor technical details were identified with this approach which all seem to impact the multimer quality. The current data is a small sample-exchange exercise to check on the quality of the technique in Budapest: approximately 20 samples from each center were analyzed in each center, „blinded”. A total of 40 patient samples along with one normal pooled plasma in each gel were analyzed. Overall quality, densitometric assessment (percent >10 multimers), sensitivity, and interpretation were assessed. Very similar results were obtained at the two study sites.
It is possible, but labor-intensive to replicate high quality multimer analysis. The current technique in Budapest provides basically identical results, but minor technical improvements are still possible/necessary.

It was proposed to prepare a detailed methods document for multimer analysis including also the minimal requirements of quality of multimer analysis for diagnostic purposes (in collaboration with CLSI –Clinical and Laboratory Standards Institute).

- International reference preparation for ADAMTS13 activity (Johanna Kremer Hovinga, CH)

Development of the WHO 1st International Standard for ADAMTS13 in Plasma: The project has been started, trial fills are tested currently to establish optimal formulation by the end of 2012. The definitive fill is scheduled for Q1/2013, followed by a primary evaluation in Q2/2013; dispatch of samples for collaborative study to interested labs for testing is planned for Q4/2013; statistical analysis and report to participants by end Q1/2014; endorsement of project at SSC Business Meeting (Milwaukee, 2014) and establishment by WHO in October 2014. Labs interested to participate in the collaborative study should send their contact details, a short description of ADAMTS13 assays performed (including normal range data and if available literature reference) to Tony Hubbard (Anthony.Hubbard@nibsc.hpa.org.uk)

- Gene mutation analysis for Upshaw-Schulman syndrome (Koichi Kokame, JP)

The autosomal recessive form of thrombotic thrombocytopenic purpura (TTP) is called hereditary TTP or Upshaw-Schulman syndrome (USS), caused by congenital deficiency of ADAMTS13. Over 100 causative mutations of ADAMTS13 have been identified in the patients with USS. A sequencing method was introduced using modified PCR primers to save time. The universal M13 forward or reverse sequence is attached to the 5'-end of each ADAMTS13 exon-specific forward or reverse primer, respectively. This modification makes the sequencing reaction step simple, because all exons can be analyzed using only M13 forward and reverse primers instead of exon-specific primers. Although the cost of PCR primers are about double, we can recommend the modified method for gene mutation analysis.

- Comparison of VWF activity assays (Imre Bodo, HU)

One of the problems in VWD diagnosis relates to the Ristocetin cofactor assay, which is insensitive (sensitivity > 10-20 IU/dl) and imprecise (inter- and intra-assay coefficient of variation up to 20-30 %). Over the past several years, a number of new methods appeared with higher sensitivity and precision. However, it is currently not known how they exactly relate to each other and to the original VWF:RCo assay in measuring VWF function.

At the 2011 SSC von Willebrand Factor Subcommittee meeting it was decided to design a collaborative study to compare all the available tests with each other and with the Ristocetin Cofactor assay. Eight laboratories joined the project, and eight new methods were selected for the comparison along with traditional VWF:Ag and VWF:RCo analysis. Each study site selected its own set of techniques. The most difficult problem turned out to be the identification of well characterized samples. The most likely solution will be prospective collection of samples, based on the survey probably at two centers (Vicenza and Budapest). Inclusion of the D1472H and P1764S polymorphisms would only be possible with using recombinant proteins reconstituted in type 3 patient plasma. At this point, this is where the project
stands, we have the support of three companies (Siemens, IL and GTI), who will provide reagents. There is no other financial support for the study.

Uniform nomenclature should be implemented for the new activity assays.

**VWF, VWD, and TTP registries**

Session Chairperson: Jorge DiPaola (USA)

- **VWF database** ([www.vwf.group.shef.ac.uk/](http://www.vwf.group.shef.ac.uk/)) (Dan Hampshire, UK)

A brief overview was presented concerning the current information contained in the ISTH-SSC on VWF database (VWFdb) and improvements made to VWFdb over the past year, including moving the variant registry over to the Leiden Open Variation Database (LOVD) system. Two initiatives were described that VWFdb has begun working with over the past year; a potential combined variant submission process for multiple clotting factors and the Human Variome Project ([http://www.humanvariomeproject.org/](http://www.humanvariomeproject.org/)).

- **Platelet Type–VWD registry/database** ([www.pt-vwd.org/](http://www.pt-vwd.org/)) (Maha Othman, CA)

The search for more PT-VWD cases and collecting information about the disease worldwide is maintained and is facilitated through the website ([www.pt-vwd.org/](http://www.pt-vwd.org/)) as well as the international collaborations. Questions to be addressed: bleeding phenotype variability, development of platelet antibodies, management during pregnancy and changes of haemostatic status in response to infection. An international study using an electronic bleeding questionnaire was initiated. Electronic format helps data collection, automatically calculates bleeding scores and facilitates worldwide participation. A novel c.793G>T was reported in the GP1BA gene that led to Asp235Tyr and a mechanism for the gain-of-function through was proposed. The PT-VWD mouse model was acquired and phenotype assessment during pregnancy, in response to infection as well as testing novel treatment modalities are currently in progress. PT-VWD work and registry update are shared with SSC-Platelet subcommittee.

- **International registry on acquired von Willebrand syndrome** ([www.intreavws.com](http://www.intreavws.com)) (Augusto Federici, IT)

An updated version of the previous International Registry on Acquired von Willebrand syndrome is now ready to be reactivated online by the same webmaster who prepared the previous version of the database. This time the clinician and/or investigator who would like to introduce clinical and laboratory data on each novel patient with AVWS will be guided by more detailed questionnaire. The information collected into the database will be sent for evaluation and comments by the webmaster to one of the expert listed in a special part of the website. In cases where diagnosis of AVWS must be confirmed with more specific assays plasma samples will be sent to the expert lab. The best therapeutic approach will be discussed online with the clinician who follows the patient. This website will be operative within **September 2012** and the costs of this updated version will be covered by residual amount of money available at the ISTH-SSC VWF account. Letter of intent for describing the objective of this project (2012-2015) and requesting the money to cover the costs of this Interactive Registry on AVWS will be sent to Pharmaceutical Companies **within September 2012**.

- **Hereditary TTP registry** (Johanna Kremer Hovinga, CH)
The hereditary TTP registry as established in 2006, over the years the questionnaire has been adapted to meet suggestions and input from many physicians and researcher treating hereditary TTP cases. The hereditary TTP registry (clinicaltrial.gov registration NCT01257269; www.ttpregistry.net) is open for enrollment of interested patients. It has IRB approval in several countries including the US and will apply for approval in further countries depending on the origin of patients and local requirements.

Saturday, June 30, 2012

VWF subcommittee, part II

Multicenter studies on VWD

Session Chairpersons: Dan Hampshire (UK)/Imre Bodo (HU)

- European Project on type 3 VWD (Augusto Federici, IT)

All the milestones scheduled for the first year of the project have been achieved. The detailed Work Packages have been defined, the WEBSITE including the online DATABASE for including clinical and lab data from the enrolled patients at the 23 different sites, 16 European and 7 Iranian is now available and will be operative within the first week of July 2012. Three Centers received approval at their local ethical committees and most sites have submitted the complete documentation to their ethical committees. The general management of the project has been defined by the Scientific Coordinator with the help of the CRO representatives who are working to activate the activities in all the 23 sites. Six expert laboratory centers have been identified in Europe and 3 in Iran to perform the confirmation of phenotypic and genotypic diagnosis of VWD3. We are planning to enroll the first 150 patients during the second year of the study and we will discuss next report at the Amsterdam Congress.

- EUVWD Cooperative Group (Anne Goodeve, UK)

The EU VWD co-operative group was formed in 2008 to enable continuation of projects run by the Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease study (MCMDM-1VWD) and to encourage and facilitate development of further European projects on VWD. The group meets at ISTH and EAHAD conferences. The MCMDM-1VWD group continues to investigate phenotypic and genetic markers that provide further insight into type 1 VWD. The 3WINTERS-IPS study on type 3 has developed from group members and progress of this project along with those of the Willebrand in Netherlands (Win) and Spanish VWD Registry are discussed. Principles of care for the diagnosis and treatment of von Willebrand disease are in preparation.

- Zimmerman Project (ZPMCB-VWD) (Bob Montgomery, USA)

The Zimmerman Program Project has enrolled 621 families from HTC who carry the diagnosis of VWD. While those with moderately severe type 1 VWD, type 2 VWD, and type 3 VWD have a high prevalence of VWF mutation, those with VWF levels of >30 IU/dL have lower prevalence of molecular alteration. The Zimmerman PPG was recently successfully refunded for another 5 year grant cycle to continue follow-up of those previously enrolled and to complete enrolment to achieve defined recruitment targets.
- VWD International Prophylaxis (VIP) Study (Tom Abshire, USA)

Dr Abshire reviewed current status of the VIP study. The prospective study is closed to enrollment and both the retrospective prophylaxis and GI bleeding natural history studies will close to enrollment on 30 June. The initial manuscript describing the results of the retrospective study comprising 59 subjects from 20 Centers in 10 countries has been accepted by Haemophilia. There was a significant reduction in bleeding pre and post prophylaxis for the overall group (from 50-90% reduction) and for the epistaxis, joint bleeding and GI bleeding indications. This effect was similar for both children and adults. Over the next 6 – 12 months, additional analysis will take place for both the retrospective and prospective studies, as well as finalization of the Natural History of GI Bleeding manuscript.

- WIN (Willebrand in Netherlands) (Frank Leebeek, NL)

The Willebrand in the Netherlands (WIN) study is a nation-wide study in patients with moderate or severe VWD, defined as VWF:Ag or VWF:Act < 30%. A total of 806 patients have been included of whom clinical characteristics, including bleeding phenotype, plasma and DNA has been collected. In the last year we have focused on variability of bleeding symptoms and the use of the Tosetto bleeding score as assessment tool of bleeding phenotype. We are currently investigating the specific bleeding problems encountered in children with VWD. In addition we have investigated co-morbidity in VWD. It seems that VWD patients are protected against arterial thrombosis. In the presentation some recent findings and plans for future research in the WIN study will be discussed.

- Canadian Type 3 VWD Study (Paula James, CA)

Dr James summarized the findings of the national Canadian study evaluating the genetic basis of VWD in 34 Type 3 VWD families (98 individuals). The unique features of the Canadian population were highlighted in comparison to other publications (more missense mutations, many families with both Type 1 and Type 3 VWD). Furthermore, their cellular expression studies on the Exon 4/5 deletion including BOEC work on a heterozygote and homozygote were reported.

- Belgian-Czech cooperation in the Brno-VWD study (Alain Gadisseur, BE)

In a collaboration between the University Hospital Brno (Czech Republic) and the Antwerp University Hospital (Belgium) a family-based analysis of VWD in the Moravia area of the Czech Republic was performed. Blood samples were collected from VWD patients (proband) together with at least one affected sibling or parent. Blood was collected from 205 patients from 95 families with suspected VWD. The distribution of different subtypes of VWD was as follows: VWD type 1 in 60/95 families (incl. 1 type 1 Vicenza), and type 2 in 29/95 families (type 2A in 14/95, type 2B in 4/95, and type 2M in 8/95), with 6/95 still unconfirmed/unclassified. Molecular analysis is still ongoing with currently 34/95 families fully analysed and the remainder partially. Sofar, 25 Mutations in the VWF gene have been found in 51/95 families, of which 2 are new to the ISTH VWD database and awaiting gene expression studies.

Genetic modifiers of VWF levels

Session Chairpersons: Johanna Kremer Hovinga (CH)/Jeroen Eikenboom (NL)

Human studies:
- Potential role of novel VWF clearance pathways (David Lillicrap, CA)

Results from four recent genetic studies of Type 1 VWD indicate that ~35% of the index cases have no identifiable candidate mutations in the coding region, promoter and splice sites of the VWF gene. A recent GWAS meta-analysis (the CHARGE study) has now documented associations with several novel loci including three genes that encode scavenger and lectin receptors (Stabilin-2, C-type lectin family 4 member M and Scavenger receptor type 1 member S). Genetic variability at these loci may well play a role as modifiers to the variances in plasma VWF levels.

- Genetic variability as a determinant of in vivo release of VWF (Frank Leebeek, NL)

VWF is synthesized by endothelial cells and megakaryocytes and two distinctive pathways are known, a constitutive pathway and an acute release by Weibel Palade Bodies in response to stress or several agonists. In a recent GWA analysis by the CHARGE consortium novel candidate genes have been identified that are possibly involved in the secretion of VWF, i.e. syntaxin-binding protein5 (STXBP5) and syntaxin 2 (STX2). Their encoding proteins interact with SNARE complex proteins, such as SNAP23 and syntaxin-04, which have been shown to be involved in WPB exocytosis. We have investigated whether genetic variations in STXBP5 and STX2 affect the release of VWF after heavy physical exercise, a model to provoke acute release of VWF from WPB, in 105 healthy subjects. The relative increase of VWF:Ag was 47% (median, range 25-73) after exercise. This increase was not associated with genetic variations in STXB2 and STX5. The release seemed to be most dependent upon physical fitness and intensity of the exercise performed.

- Genetic modifiers of VWF antigen levels in healthy young adults (Karl Desch, USA)

Genetic factors are thought to account for 60 - 70% of the variance in VWF antigen levels with ~30% of this effect due to ABO blood type. In order to identify additional genetic determinants of VWF antigen levels, we performed genome-wide association studies and linkage analysis in a large cohort of young healthy siblings, the Genes and Blood Clotting Study and a cohort of young healthy individuals, the Trinity Student Study. The heritability of VWF antigen levels was 64.5 - 66.3%. A meta-analysis revealed SNPs at the ABO (rs678289, p-value 7.9E-139) and VWF (rs1063856, p-value 5.5E-16) loci to be the major common variants associated with VWF levels. Linkage analysis demonstrated significant signals at chromosome 2q12 – 2p13 (LOD score 5.27) and at the ABO locus on chromosome 9q34 (LOD score 2.87) that explained 19.2% and 24.5% of the variance in VWF levels respectively. A more complete understanding of the genetic determinants of VWF variation should lead to new insights on the regulation of plasma VWF levels and facilitate the development of more accurate diagnostic tests for VWD and prognostic tests of thrombo-embolic disease risk.

- Genetic modifiers of VWD in large pedigrees: association and linkage (Jorge DiPaola, USA)

While linkage has been used in the past for the discovery of rare variants that cause mostly Mendelian disorders, more recently genome wide association studies have been utilized for the discovery of common traits that influence phenotypes. We have studied a large Amish pedigree with more than 3000 individuals and a unifying mutation in VWF (C4120T) that is present in 132 of the family members. On search for modifying genes that affect not only VWF levels but also bleeding, we have performed genome wide linkage analysis, fine mapping and association studies. We found novel loci and confirm
others described before, that influence VWF levels. Interestingly in this Amish family rare and common variants appear to contribute the complex VWD phenotype.

Animal studies:

- Mouse modifiers of VWF (Jordan Shavit, USA)

Animal models provide a powerful alternative method for the identification of modifiers of VWF. Large pedigrees can be generated and the genome interrogated in an unbiased fashion, enabling a search for modifiers affecting transcription through plasma clearance. Mouse strains express highly variable levels of VWF and have been intercrossed to identify genomic loci associated with VWF regulation. This has led to the identification of a glycosyltransferase that alters clearance, likely similar to ABO modification of human VWF, as well as distinct VWF allelic variants.

- Modifier screens in zebrafish and possible application to VWF (Jordan Shavit, USA)

The zebrafish is an alternative vertebrate model with a high degree of genetic conservation with mammals, including the coagulation cascade. Functional conservation has been demonstrated with the production of thrombi in response to laser injury and injection of activated thrombin. Zebrafish Vwf displays conservation of domain structure, multimerization and intracellular storage. We anticipate that the ability of zebrafish to form large pedigrees will enable a significant increase in the throughput of identification of modifiers of VWF.

Jeroen Eikenboom, Chair VWF subcommittee
Welcome and Introduction of co-chairpersons

Sabine Eichinger welcomed all participants also on behalf of the co-chairpersons. She provided an overview of the program and reasons for change in the chair of the menorrhagia working group presentations as Claire Philipp could not attend the meeting.

Educational activities

Sabine Eichinger summarized the educational activities of the SSC.

This year, women’s health issues in thrombosis and haemostasis covered a large part of the Educational day of the meeting. The topics were chosen under the consideration of providing most practical aspects in the field of thrombosis and haemostasis related aspects in women’s health. The session was very well attended and well perceived.

The SSC will continue and try to expand its educational activities.

Benjamin Brenner summarized activities regarding the organization of the 5th International Symposium on Women’s Health Issues in Thrombosis and Haemostasis, which will be held in February 2013 in Vienna. He gave an overview on the development of the meeting since its first gathering. Registered attendees at the last meeting 2011 have been around 650.

Women’s Health Issues in Thrombosis

At the beginning of 2012 the new edition of the ACCP Guidelines on "Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy" have been published. We have respectively had two of the panelists of the current chapter among our co-chairpersons. They provided an update and an overview of the most relevant changes related to:

1. Thromboprophylaxis after caesarean section (Ian Greer)

2. Prevention of VTE in pregnant women at increased risk (Saskia Middeldorp)

3. Thrombophilia and pregnancy complications (Ian Greer)
In addition, the process of developing ACCP guidelines and the rationale for suggestions and recommendations were extensively discussed between the audience and the two panelists.

Saskia Middeldorp also gave an update on recent advances of some of the investigator initiated trials in women’s issues. The Highlow study comparing 2 different doses of MWH during pregnancy and the ALIFE II investigating LMWH prophylaxis in women with thrombophilia and recurrent pregnancy loss have now gained funding and will start recruitment by the end of 2012. International participants are welcome and should contact S. Middeldorp.

Astrid van Hylckama-Vlieg presented results of two recent studies related to hormone use and the risk of venous thrombosis. She evaluated the risk of hormone contraceptives in women older than 50 years. This is highly interesting information as there are no data thus far.

Benjamin Brenner presented on behalf of Yona Nadir data on a new assay, the heparanase procoagulant assay in women on hormonal therapy.

This year we again featured a Spotlight Session to highlight special issues related to women’s health with the intention to raise attention and awareness. Benjamin Brenner provided an overview of “Pregnancy and Malignancy” with special consideration of thrombosis and haemostasis. Data on almost all of the clinical aspects are scarce. A need for more studies has been expressed. Because of the rarity of cases international collaboration is required. There was consensus that a registry would be important and our SSC will serve as a platform to foster this research activity.

Menorrhagia Working Group

The menorrhagia projects are numerous due to the activities of several of our co-chairpersons has (Claire Philipp, Rezan Kadir, Claire McIntock, Takao Kobayashi, Andra James).

Thus far, members of the group not only deal with menorrhagia-related issues but also with other bleeding complications in women.

Takao Kobayashi reported on a multicenter case series in Japan of the use of recombinant activated factor VII (rFVIIa/NovoSeven®) in the management of severe postpartum hemorrhage. Harmonization of the management of postpartum bleeding is needed. A task force recruited from among our co-chairpersons and others interested our SSC and this topic would be helpful to achieve this goal.

Report from the Bleeding Assessment Tool Standing Committee – what is relevant for women and girls

Sabine Eichinger reported from the business meting and the first public meeting of the Bleeding Assessment Tool Standing Committee. The chairman of the SSC on Women’s Health Issues is part of the steering committee. She provided an overview of how the project developed and how it works. Some data on the usefulness of the bleeding assessment tool in women with menorrhagia have been already provided and have been briefly summarized by S. Eichinger. The bleeding assessment tool is available
online and free to use for every researcher with an appropriate scientific projects and who provides the necessary documents (e.g. IRB approval of the study).

Closing Remarks (S. Eichinger)
Working Group on Coagulation Standards

30 June 2012

Chairman: Anthony Hubbard (UK)

Final review of Lot #3 (A Hubbard)

Despatch of Lot #3 commenced in June 2006 with an initial batch size of 54,800 vials. Orders for Lot #3 have been fairly consistent over the last 6 years with an average of approximately 7,000 vials despatched per year. During the year July 2011 – June 2012 6,180 vials were ordered and less than 300 vials remain in stock. Stability studies conducted over 6 years indicated that Lot #3 was extremely stable when stored at the bulk storage temperature of -20°C with mean predicted loss of labile factors V and VIII less than 0.1% per year.

Introduction of Lot #4 (A Hubbard)

Lot #4 comprises 100,000 vials and was manufactured in 2009. Calibration for 20 coagulation-related analytes was completed in 2011 and the assigned values were endorsed at the SSC Business meeting in Kyoto (Table 1). Stability testing has been initiated using an accelerated degradation protocol similar to that used with Lot #3. Four factors have been included in the stability study – factor V, factor VII, factor VIII and antithrombin. Testing has been completed at two time-points by NIBSC and the Royal Hallamshire Hospital, Sheffield. Data from the latest time-point (December 2011) after storage of samples at elevated temperatures for 2 years was used to generate predicted degradation rates for vials stored at -20°C. Predicted degradation rates were 0.1% per year or less for all analytes except for FVII using the data generated by NIBSC where a degradation rate of 0.22% per year was estimated. This value reflects the variability of the test results and it is expected that the addition of future test results will produce a lower predicted degradation rate in line with the other analytes. Estimation of shelf-life followed the approach used for Lot #3 and was based on the most labile predicted degradation rate. A proposed shelf-life of 9 years with an expiry date of "End December 2020" was accepted by the Executive Board; this date may be revised as more stability data is accumulated.

Despatch of Lot #4 commenced in May 2012 at a cost of £4.00 (GBP) / $6.22 per vial. Up to 26 June 2012 4,870 vials have been ordered.

Calibration of Lot #4 for von Willebrand factor propeptide (A Hubbard)

Eight laboratories have participated in a collaborative study to assign a value for VWFpp to Lot #4 by direct assay against the WHO 6th IS Factor VIII/VWF Plasma (07/316). Results from the 8 laboratories were in very good agreement (inter-laboratory variability, GCV 2.5%) and a consensus mean value of 0.97 IU per ml was accepted by the participants and the Executive Board. Endorsement for this value assignment will be requested at the SSC Business meeting on 30 June.

Experience of EQA schemes with Lot #3 and Lot #4:

UK NEQAS (S Kitchen)
An update on the use of Lot #3 for the resolution of discrepant results from individual laboratories (trouble-shooting) was presented. An example from one laboratory was presented where the use of Lot #3 confirmed the cause of discrepant results as the inaccurate calibration of a reference plasma. In the last 12 months 10 vials of Lot #3 have been used to investigate test results for Protein C, Protein S, Antithrombin, Factor VIII and Factor IX.

Lot #4 was distributed in May 2012 as an anonymous sample in an EQA survey involving 360 participants. The overall median value for FVIII (0.89 IU/ml; n=354) agreed extremely well with the assigned value of 0.88 IU/ml. The overall median value for FIX (1.106 IU/ml; n=325) was 5% higher than the assigned value. Most individual commercial reference plasmas produced median results within 5% of the assigned potency. However, two reference plasmas were associated with large differences between the assigned values and the survey median values (10% for FVIII and 18% for FIX).

**College of American Pathologists (D (Adcock) Funk)**

Lot #3 was included in the VWF Survey (CGS3-A) and the Thrombophilia Survey (CGS2-A) in 2011. Mean estimates for FVIII deviated by less than 5% from the assigned value for 4 out of 5 assay systems; one system produced a deviation of 10.6%. Mean estimates for VWF:ristocetin cofactor and antigen deviated by 7% or less from the assigned value. Mean estimates for antithrombin activity and antigen deviated by 5% or less from the assigned value. Mean estimates for Protein C antigen deviated by 4.6% or less from the assigned value, however, two methods for Protein C activity were associated with mean deviations of 10% (chromogenic) and 16.5% (clotting) respectively. Mean estimates for Protein S activity and total antigen were associated with deviations up to 18% from the assigned values. Mean estimates for Protein S free antigen deviated by less than 10% from the assigned value. Some methods for Protein C activity and Protein S activity have consistently shown large deviations from the assigned values in CAP surveys over the last 3 years.

**Lot #4 and the JCTLM database (Elaine Gray)**

Lot #4 will need to be submitted to the JCTLM database for internationally recognised reference materials and this will require demonstration of commutability. It is planned to present the data from the calibration exercise, which included a comparison of Lot #4 relative to Lot #3, to fulfil this requirement.

**Any other business**

Dr Kitchen indicated that it is necessary to renew the use of the SSC standard for trouble-shooting purposes. A proposal will be submitted to the Executive Board to enable the use of Lot #4 for this purpose.

**Table 1 Values assigned to SSC Lot #4**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Assigned value (IU/vial)</th>
<th>Inter-lab variability (GCV%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II function</td>
<td>0.91</td>
<td>2.6</td>
<td>28</td>
</tr>
<tr>
<td>Factor V function</td>
<td>0.89</td>
<td>5.7</td>
<td>22</td>
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<tr>
<td>Factor VII</td>
<td>0.97</td>
<td>4.1</td>
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<tr>
<td>Factor VIII</td>
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<tr>
<td>Factor IX</td>
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<td>3.4</td>
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<td>Factor X</td>
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<tr>
<td>Factor XI</td>
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<td>Factor XIII</td>
<td>0.76</td>
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<td>von Willebrand Factor</td>
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**Fibrinogen**