57th Annual Scientific and Standardization Committee Meeting

Kyoto, Japan
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Animal, Cellular, and Molecular Models of Thrombosis

23 July 2011

Chairman: Timothy Nichols (US)
Co-chairmen: Edward M. Conway (CA), Shaun R. Coughlin (US), Jay L. Degen (US), Cecile Denis (FR), Nigel Mackman (US), Toshiyuki Miyata (JP), Eva-Maria Muchitsch (AT), Susan S. Smyth (US), Hugo ten Cate (NL), Hartmut Weiler (US)

Educational Session

1. Cecil Denis - Rapid in vivo evaluation of haemostatic agents using hydrodynamic injection

Dr. Denis discussed the important methodological issues, strengths and limitations of using hydrodynamic injection to evaluate wild type and mutant hemostatic agents in the circulation (ADAMTS13, VWF, FVIII, FIX, alpha1 antitrypsin), transmembrane proteins (e.g., Siglec-5 in the life cycle of the FVIII-VWF complex), as well as SiRNA applications. This powerful tool is being broadly applied to achieve short-term expression (or reduced expression) of relevant proteins for in vivo characterization and mechanistic studies.

2. Susan Smyth - Models of vascular inflammation. Dr. Smyth discussed the aortic arch ligation model of inducing left ventricular hypertrophy and the associated inflammatory-mediated coronary artery remodeling used in various strains of mice. The important methodological issues were reviewed (e.g. standardization of extent of ligation, inclusion of a sham-operated control). Remarkably, after placement of the ligature, platelets accumulate along the coronary endothelial surface at sites of subendothelial macrophage deposition. Mechanisms mediating these processes are being identified by infusion of anti-platelet Gp1b antibodies and utilization of relevant knock out mice (e.g., Jnx mice with decreased granule secretion in NK, CTL, platelets, and neutrophils). This ongoing work addresses the role of inflammation and coagulation in a very common and debilitating disorder (LVH, diastolic heart failure) that is associated with a significantly increased mortality rate in humans.

3. David Motto - Scanning electron microscopy studies of endothelial injury and thrombus formation in mouse models of thrombosis." Dr. Motto presented an elegant series of SEM taken over the first few minutes following application of ferric chloride (0.37 M, 10%) to murine carotid arteries. Remarkably, endothelium has not sloughed off during these early timepoints and red blood cells appear to be one of the first adherent cells by unknown mechanisms. These RBCs then elongate in the direction of blood flow and form a ruffled border at the attaching edge and eventually slough off leaving the ruffled-edge fragment behind. In addition, amorphous (possibly proteinaceous) material begins to envelop the collection of adherent cells and cell fragments. Then, platelets begin to attach to this growing clump of adherent cellular and amorphous debris. This work focuses on understanding the mechanisms that mediate these events and determining their relevance to clinical arterial thrombotic disorders.
Focused talks

1. **Tom Knudsen** – Characterization of canine coagulation factor VII and its complex formation with tissue factor: canine–human cross-species compatibility. Dr. Knudsen presented the very recent data that validate using canine models to study human FVIIa in vivo. The essential point is that canine TF binds human and canine FVIIa in a comparable manner however human tissue factor does not bind canine FVIIa efficiently. Thus, infusing human or the recently produced canine FVIIa into dogs is a valid approach for the study of TF-FVIIa coagulation events in vivo. The recent and important reports of coating of platelets by canine FVIIa and canine FVII clot and antigen assays were discussed. The role of canine TF in signaling and its comparison to human TF signaling is a future avenue of research.

2. **David Lillicrap** - Mouse models of FVIII immunogenicity. Dr. Lillicrap presented the rationale for developing these models and exploring the varying immunogenicity noted among FVIII products. Notably, humans with hemophilia A have different prevalence of inhibitors when treatment consists of single donor, pooled plasma, and recombinant FVIII. The goals are to develop an animal model for identifying mechanisms that mediate immunogenicity, to identify mechanisms that mediate tolerance, and to test the immunogenicity of new formulations of FVIII. The relative immunogenicity of FVIII with and without VWF was studied, an array of ~1100 immune response genes has been utilized to characterize this response, and mice with varying degrees of humanization of the immune system have been created to facilitate this work. These tools are acquiring broad utilization in research focused on the development, treatment, and prevention of inhibitor formation in hemophilia A.

3. **Nigel Mackman** - Measurement of a procoagulant state in mice. Dr. Mackman discussed the role of procoagulant states in mice induced by endotoxin, sepsis, colitis, sickle cell disease, antiphospholipid antibodies, heparin induced/associated thrombocytopenia, and cancer. The importance of standardized animal models and detection assays between labs was emphasized. Six assays currently available for detecting and characterizing mechanisms that mediate procoagulant states were reviewed: 1. Microparticle-TF activity, 2. The Zymuphen MP activity kit, 3. The calibrated automatic thrombogram (CAT) assay, 4. Enzygnost Thrombin-antithrombin (TAT) assay, 5. Fibrin western blot, and 6. D Dimer (Asserochrome). The method and quality of obtaining blood samples from the animals cannot be overemphasized.

4. **Hugo ten Cate** - Thrombin and atherosclerosis: effects on plaque morphology. Dr. ten Cate reviewed the essential role of thrombin generation in hemostasis and the pathophysiology in thrombosis. Refinements of mouse models of atherosclerosis are yielding insights into mechanisms that produce a vessel wall localized hypercoagulable state and mediate thromboatherosclerosis, angiogenesis in atherosclerotic plaques, and the role of inflammation and plaque instability in thrombosis complicating atherosclerosis. These refinements will guide future clinical translational research.

5. **Fumiaki Banno** - Genetic mouse models for evaluating pathophysiological roles of ADAMTS13. Dr. Banno reviewed mouse models of polymorphisms and mutations in ADAMTS13 and their role in thrombosis and TTP. Differential effects of polymorphisms of ADAMTS13 alter the outcome of middle cerebral artery occlusion and reperfusion injury. These
models provide the tools to test the hypothesis that hyperactive VWF is present with the different forms of ADAMTS13 and mediate thrombosis.

6. Eva-Maria Muchitsch - Animal models for TTP: Development of a mouse model of TTP for preclinical efficacy testing of rADAMTS13. Dr. Muchitsch reviewed the history of botrocetin, antibody, and sepsis–induced animal models of TTP. The current mouse models with ADAMTS13 knocked out and infused with human recombinant VWF have reproduced the constellation of findings in TTP (save possibly fever). One somewhat unusual finding in mice was heart hemorrhage, a finding reported in some human cases. Current work is focusing on validating the clinical scenarios that recombinant ADAMTS13 will prevent or mollify induction of TTP and will likely provide justification for human clinical trials.

7. Jianglin Fang - Transgenic rabbit models for the study of atherosclerosis. Dr. Fang reviewed the rationale for using transgenic rabbits in atherosclerosis studies: sensitivity to high cholesterol diets, rapid development of atherosclerosis, development of plaques with many human-like features, and the seminal development of the Wantanabe heritable hyperlipidemic (WHHL) rabbit by Dr. Yoshio Watanabe. Transgenic rabbits producing Lp(a) and CRP have been developed by Dr. Fang and provided important tools for determining whether these molecules are causative or consequence of atherogenesis. The rabbit remains an important and powerful tool in atherosclerosis research.

8. Hartmut Weiler - In vivo function of human aPC in mouse models of thrombosis and inflammation. The role of aPC in placental development, sepsis outcome, and preventing thrombosis was reviewed. Dr. Weiler raised the question of whether or not all these effects were sole due to aPC or were other factors involved. He presented data on hyperactivatable aPC and the action of aPC on histones that will be presented later at this meeting.

9. Timothy C. Nichols – Animal Models Committee Business Meeting. Progress in publishing proposed short papers and the development of projects was discussed. Two of three papers proposed at the 56th annual SSC meeting have been published (or accepted for publication) and a third will be ready for submission soon.1-3 The proposed project that would provide a standardized mouse tail bleeding time method was suggested by the manuscript below and the Poncz and Muchitsch laboratories are discussing an approach to this project.

Publications


Project being planned: Validation of a standardized mouse tail bleeding time method.
Chairman: Michael R. King (US)
Co-chairmen: Lawrence Brass (US), Thomas Diacovo (US), Johan Heemskerk (NL), Shaun Jackson (AU), Armin Reininger (DE), J. Zwaginga (NL)

The session was well attended, with 180 attendees in Part I and 130 attendees in Part II.

14:00-14:10 Michael King (Cornell Univ., USA): Welcome and Opening Comments

King welcomed attendees and briefly summarized the activity of the Biorheology subcommittee over the previous year, including three JTH publications. He mentioned that a major activity over the next year will be to consider flow dependent thrombin and fibrin generation. He introduced the two-part session to follow, and reminded speakers to keep formal presentations under 15 minutes in length to allow for discussion.

Part 1: In vitro thrombosis assays and standardization (Moderators: T. Diacovo and M. King)

14:10-14:30 Judith Cosemans (Maastricht Univ., NL): Standardization of the use of flow devices to measure thrombus formation.

Dr. Cosemans showed movies of platelet aggregation on collagen under flow, and categorized the different systems used in the parallel-plate geometry. Advantages of parallel plate geometry were presented. A microgrid robot machine is used to create different spots of protein, separated by a distance of 1 mm, which is a sufficient distance to avoid “cross-over” effects between spots. Readout from these experiments is automated on computer, and a morphological score from 0 to 5 assigned. New disposable flow chambers were presented, fabricated in PDMS in the form of 4 parallel flow channels. A multicenter study using this device will start in September 2011, with the goal to look at variability between subjects and labs. Dr. Diacovo asked about the negative control experiments to demonstrate the specific molecular interactions. Dr. Turrito questioned whether there is an upstream vs. downstream dependence on different stripes. King asked whether the stripe order is randomized. Cosemans answered that reversing the order gives the same result. Dr. Brass asked about the potential clinical use of the device. Cosemans expanded on the application for diabetic patients vs. normal as an example.

14:30-14:50 Keith Neeves (Colorado School of Mines, USA): Clinical evaluation of microfluidic flow assays.

Dr. Neeves described a clinical study at University of Colorado Denver and Colorado School of Mines involving over 1000 subjects (500 healthy; Hemo A,B; other rare disorders). All consenting adults >18 yrs of age were included. 100x500um PDMS channels were used with type I collagen surface, following SSC recommendations. Raw data videos were shown alongside the binary analysis videos. Lag time, slope, and final surface coverage were measured.
as a function of time. Donor variability as well as day-to-day variability of the same donor were examined. Area fraction was not found to correlate with platelet count or hematocrit, but did correlate with bleeding score. Sufficient overlap between normal and diseased samples prompted nanoscale examination of different collagen thin films (fibrillar vs. non). Microcontact stamping of different collagen spots has been used. Coagulopathy has been examined with blood from hemophilia A, thrombus height correlated with FVIII level. Alisa Wolberg from UNC Chapel Hill asked about the lipid combinations and concentrations of tissue factor in experiments. Willem Ouwehand from Cambridge asked about whether enrollment numbers of the diseased population will be increased from 75. Neeves hopes to eventually get up to 1000. The audience member advised increasing to 500-1000. Dr. Heemskerk asked about fibrin production with or without CTI (corn trypsin inhibitor). Neeves uses CTI, they find that it yields more reproducible results. Turrito asked about the differences between normals and diseased, and whether this depends on collagen density. Neeves briefly discussed pros and cons of “increasing the gain” on these experiments.


Dr. McCarty briefly reviewed typical blood cell counts and coag factor concentrations in blood. He showed high resolution movies of platelet spreading, and showed thrombus formation movies in re-calcified blood over collagen fibers. McCarty showed his system that uses gravity driven flow at constant pressure gradient. Flow rate is monitored by eye or using optical methods. Occlusion is formed somewhere in the tube, as visualized by loss of flow. The dependence of time to occlusion was measured as a function of tissue factor concentration, with TF introduced on microspheres. McCarty also presented the initiation of thrombosis by breast epithelial cell lines, motivated by the study of metastatic cancer. He hopes to scale down the assay volume in the future, to enable use of mouse blood. Neeves asked about variation along the tube length, and where coagulation occurs. In future experiments some optical methods will be used to monitor clot location. King asked about whether blood is added during the experiment to maintain the fluid height (it is, added manually by the experimentalist).

15:10-15:30 Attie Tuinenburg (Univ. Med Center Utrecht, NL): Perfusion chamber with static mixer: New possibilities to study the interplay between platelets and coagulation under conditions of flow.

The general in vitro model for studying primary hemostasis was briefly reviewed, along with previous ex vivo experiments directly from the donor. Challenges in re-calcifying blood (improper mixing) were presented. The new microfluidic device with static mixer has no moving parts, fluids are mixed by flowing through the device passing a certain repeating geometry. The static mixer splits the flow stream in two, rotates the flow, and then combines the two streams. With every cycle (split, rotate, combine) the number of layers increases exponentially. Between the layers molecular diffusion occurs. The injection molding manufacture of the disposable device was presented. A coated coverslip is held in contact with the multi-layer device using vacuum. The two parts of the device are pressed together using an aluminum frame. Video results in the prototype chamber were shown (calcium (end concentration after mixing) at 8 mM; shear 1600 1/s) and robust thrombus formation over 15-20 minutes. At lower shear (500
1/s), more fibrin in a dense network was observed. Validation of the final model is ongoing, as is optimization of the experiments. A more accurate in vitro analysis will be possible, that includes coagulation with well mixed calcium. Turrito asked about the shear conditions upstream within the mixing elements (e.g., is there higher shear than in the main channel?). Dr. Tuinenburg will investigate this. Shear rate in the static mixer is lower than in the channel. The dimensions of the static mixer are 1mmx1mm, whereas the height of the parallel perfusion channel is 125 micrometer and the width is 1 mm. Brass asked about potential clinical questions to address with this model, and next steps. Tuinenburg answered that first, they will look at normal blood, and then move on to different disease states.

15:30-15:50 Johan Heemskerk (Maastricht Univ., NL) and Peter Lenting (Univ. Paris-Sud, France): Questionnaires and literature review on mouse models and genes in arterial thrombus formation in vivo and in vitro.

Dr. Lenting presented the questionnaire distribution and number of responses asking about in vivo thrombosis models used by various research labs. Different models: laser, mechanical injury, photochemical, FeCl3 are used, and most labs are satisfied with their own models. A large range in protocols (e.g., conc. Of ferric-chloride or Rose-Bengal) exists between labs. Laser wavelength also varies, as well as optical configuration. After reviewing the inventory, new recommendations were presented, related to the mouse, vessels, and measurement parameters, that are model independent. Then, standardization recommendations for different models were given: ferric-chloride, photochemical, and laser. Some parameters are difficult to standardize. An audience member from France asked a good question about which model is best for which scientific questions. Lenting agreed that this is a good question. Heemskerk then presented the standardization paper and questionnaire results for in vitro assays. 25 questionnaires were returned, 27 questions with more than one answer possible for each question. Strengths and weaknesses of the different custom made vs. micro-capillary vs. commercial chambers were presented. Most labs utilize human blood, but also mouse blood or cell lines. Different coatings and anticoagulants are used. Also, differences in blood storage, inhibitors, imaging and quantification are used. Finally, recommendations of the Biorheology SSC were presented, related to the flow chamber, surface coating, blood collection and storage, image recording. An inter-laboratory study comparing the various chambers is proposed.

15:50-16:00 Break

Part 2: In vivo thrombosis models (Moderators: K. Neeves and J. Heemskerk)

16:00-16:20 Shaun Jackson (Monash, Univ., AU): New approaches to investigate biomechanical platelet activation.

Dr. Jackson introduced the different zones of flow disturbance in arterial stenosis. Discoid platelet aggregation and membrane tethers as biomechanical sensors were the main topics of this talk. He showed evidence that changes in vessel geometry induce platelet aggregation, and that the flow shows shear micro-gradients, shear acceleration and deceleration. A microfluidic device with fixed stenosis was created to mimic the needle manipulation in mouse. Movies were shown that demonstrate that platelet aggregation begins right after the tip of the stenosis. There are three
key variables regulating discoid platelet aggregation: rate of flow acceleration, peak shear, and rate of flow deceleration. Video evidence of membrane tethers forming in vivo was presented, to introduce the corresponding microfluidic experiments. TIRF (total internal reflection fluorescence) microscopy was used in vitro to image membrane tethers very close to the surface. Calcium spikes in spreading platelets has been visualized. Tethers restructure and contract to sustain the adhesion of discoid platelets under flow, by getting thicker via actin polymerization. Brass asked whether tether retraction is really passive. Jackson answered that cell activation is local, with no global P-selectin expression observed. Turrito asked about the shear gradients, and whether the magnitude of shear attained is the cause instead of the gradient. He proposed varying gradient and magnitude separately. Jackson explained that they have already done this, by varying the angle of the microfluidic stenosis, and also answered how they have proposed that both gradient and peak shear magnitude are important.

16:20-16:40 Lawrence Brass (Univ. of Pennsylvania, USA): Using intravital confocal fluorescence microscopy to probe the microheterogeneity and permeability of the platelet response to laser and mechanical injury.

Brass started by stating that microheterogeneity (variations in time and space of activation state of indiv. Platelets in the thrombus) and porosity (the ability of soluble molecules to pass in and out of the thrombus) are two important terms for his talk. He showed in vivo movies with platelets in red and P-selectin (activated platelets) in green. Red shows up minutes before green, but even after 1 hour there are still P-selectin (negative) platelets. Brass briefly presented on laser vs. mechanical injury (previous data was using the laser injury model), and qualitatively they behave the same. Fluorescent albumin shows that plasma leaks out before and after the clot has formed, and heterogeneity in the permeability of the clot. Fluorescent dextran (10 kD) has also been used. Brass reviewed previous electron microscopy data showing close contacts between platelets. The last results related to an inner core of the thrombus in which the porosity has been reduced. Heemskerk asked a question on secretion of factors by platelets. Brass noted that there is a need for additional in vivo markers of platelet activation. Jackson asked about platelet activation and porosity. There was a question on where does the P-selectin come from (i.e., maybe not from granules), but soluble P-selectin binding to platelets is unlikely because it does not show up on unactivated cells.

16:40-17:00 Tom Diacovo (Columbia Univ., USA) and Michael King (Cornell Univ., USA): The utility of a humanized mouse system for multiscale analysis of thrombus growth, platelet convection, and drug efficacy.

Diacovo began the talk by presenting the motivation for developing the humanized mouse model, and other (ex vivo) means to measure human platelet function. He briefly described the new mouse model that can utilize human platelets that form clots, whereas due to a transgenic mutation mouse platelets do not participate: thus the first in vivo studies using human platelets. The published work was cited for further details. Diacovo showed clinical data that demonstrates that the humanized mouse model is sensitive enough to differentiate diseased cells from normal cell function in thrombus growth. King gave the second half of the talk, focusing on detailed analysis of the fluid mechanics in Diacovo’s in vivo system, and computational models of platelet adhesion. Models that cover the scale of whole vessel, individual cells, and adhesion
receptors (molecular scale) were presented, including the “platelet adhesive dynamics” model which recreates the stochastic behavior of platelet rolling. Some preliminary sensitivity results were shown, indicating how the model predictions of platelet rolling depend on shear rate, or an increase or decrease in the receptor number. Neeves asked a question on whether platelet deformation would be important in the model. King answered that the capabilities are there, as well as the effect of tether deformation as has been done for leukocytes. Another question was asked about modeling the effects of drugs and inhibitors, both King and Diacovo answered that it is possible and planned in both the computer model and mouse model.

17:00-17:20 Atsushi Yamashita (Univ. of Miyazaki, Japan): Disturbed blood flow induces plaque erosion and thrombus formation.

Dr. Yamashita gave background on plaque rupture vs. plaque erosion, and shared histological examples of both. Balloon injury of rabbit femoral artery was used to induce SMC-rich neointima within 3 weeks, with higher TF activity. Disturbed blood flow was introduced by occluding the vessel via external constriction. Neointima SMCs align parallel with blood flow. Erosive injury occurs even within 15 minutes after narrowing. More TUNEL-positive apoptotic cells were found in the neointima layer only after narrowing. Fibrin deposition occurred in the SMC-rich neointima (after narrowing), but not in the normal intima vessels. Thrombotic occlusion occurred in the injured+narrowed vessels within 3 hours. The blood itself showed no differences in in vitro assays.

17:20-17:40 William Olbricht (Cornell Univ., USA): In vivo imaging of cerebral circulation in mouse models of polycythemia vera and essential thrombocythemia.

Dr. Olbricht gave some brief background on the two clinical conditions with elevated hematocrit and platelet count. The clinical treatment is to reduce hematocrit via phlebotomy. The focus of this talk was on microvascular occlusions in brain. The classic viscosity dependence on hematocrit measured in rheometers is not necessarily applicable in the complex capillary bed in the brain. In vivo two-photon microscopy in mouse cortex was used to measure differences in blood flow under these conditions. RBC speed can be measured in capillaries by fluorescently labeling the plasma (no signal within the RBC due to exclusion). “Stalled” capillaries are observed using this technique. Transgenic mouse models are available that recreate the human PV and ET diseases, or EPO can be injected in normal mice to elevate hematocrit without increasing leukocytes or platelets. The different mice show different fractions of capillaries stalled, over hundreds of vessels analyzed. In the PV mice, stalls are often due to microthrombi or leukocyte plugs, not so in control mice. RBC speed in nonstalled capillaries is different in experimental mice. A question was asked about reducing hematocrit in the EV mice, but it has not been done yet. Turrito asked about the implications of the PV mouse for clinical observations.
Control of Anticoagulation

23 July 2011

Chairman: Trevor Patrick Baglin (UK)
Co-chairmen: Walter Ageno (IT), Job Harenberg (DE), Clive Kearon (CA), John Olson (US), Gualtiero Palareti (IT), Sam Schulman (CA)

Introduction and update on activities:

Current Active SSC Registries

1) Recurrent venous thromboembolism in anticoagulated patients with cancer
Sam Schulman, Anna Falanga
Nil to report from meeting. Update to follow.

2) Splanchnic vein thrombosis
Walter Ageno, Francisco Dentali, Sam Schulman
Enrollment commenced June 2008. Web-based registry. Planned sample size 550. Number recruited 574. 32 centres from 11 countries. Baseline data on 94% of patients. Completed 2 year follow up on 27.5%.

3) Cerebral vein thrombosis
Walter Ageno
Nil to report from meeting. Update to follow.

Planned new SSC Registries / Working Parties
None.

Current SSC Standardization Projects

1) Performance requirements of Point-of-Care monitors
AMHP van den Besselaar. Nil to report from meeting. Update to follow.

2) A working party to delineate methodology/endpoints/harmonization of present and future studies on perioperative AC management and bridging therapy
Alex Spyropoulos. The proposals for methodology and harmonization were presented at the meeting by Dr Spyropoulos. A written recommendation will be submitted to the committee for approval and subsequent submission for publication.

3 Evaluation (Standardisation) of tests to evaluate effect of factor Xa inhibitors (rivaroxaban)
Completed. Dr Job Harenberg presented the results of a multicentre evaluation of assays undertaken on behalf of the SSC using plasmas spiked with rivaroxaban between 50 and 750ng/ml One PT assay, 4 chromogenic anti-Xa assays and the Prothrombin induced clotting time were evaluated. No APTT assay was evaluated. CVs were reported for each assay. It is proposed that the results of the evaluation will be available through the website and the report may be published independent of the SSC as an original article.
4) Evaluation (Standardisation) of tests to evaluate effect of factor IIa inhibitors (dabigatran)
Dr Elaine Gray outlined a multicentre evaluation of assays undertaken on behalf of the SSC using plasmas spiked with dabigatran.

**Planned SSC Standardization Projects**

1) Evaluation (Standardisation) of tests to evaluate effect of factor Xa inhibitors (apixaban)
This is to be considered before being formally proposed. Action: Chairman (Dr T Baglin).

**SSC Publications in Past Year**


**Planned SSC publications**

1) Standardisation of platelet thrombography - a recommendation from the SSC Subcommittee for control of anticoagulation - JTH 2011; in press

2) Laboratory measurement of rivaroxaban and dabigatran
Several presentations at the meeting addressed the issue of measuring new oral anticoagulants. Three scenarios in which measurement might be needed were identified:

1. Emergency admission when it is necessary to determine if patient is anticoagulated or not, e.g. surgical admission requiring emergency surgery;
2. Compliance, e.g. detection of drug
3. Failure of treatment in relation to intensity of treatment, e.g. thrombosis occurring during treatment in obese patient where dose intensification may be indicated.

Drug-specific and scenario-specific tests may/will be required. It was agreed in the meeting in response to requests from participants that an official recommendation from the subcommittee was required as soon as possible, i.e. before the end of the year. Dr Trevor Baglin (chairman) will lead the project and submit a formal project submission.
Additional SSC Special Projects
None

Educational Activities
It is proposed that an education programme will be included at the next SSC meeting in Liverpool.

Items requiring 2011 SSC Approval (standards or publications)
None

SSC - Subcommittee for Control of Anticoagulation
The meeting was held on Sunday July 24th at 08.00 - 12.00. An update of activities was presented by the Chairman before the following presentations:

- SSC Registry Report: Splanchnic vein thrombosis WALTER AGENO
- Quality of anticoagulation control during VKA therapy: effects on risk of bleeding and treatment failure GUALTIERO PALARETI
- Interpretation and management of INR results from patients treated with VKA antagonists, a study among clinicians from 14 countries ANN-HELEN KRISTOFFERSEN
- How should INR calibrants be used? JOHN OLSON, STEVE KITCHEN
- Update on novel non-oral anticoagulants - including idraparinux, idrbiotoparinux HARRY BULLER
- SSC Project Report: Determination of anticoagulant effects of rivaroxaban: towards a recommendation for assays JOB HARENBERG
- SSC Project Report: Determination of anticoagulant effects of dabigatran: towards a recommendation for assays ELAINE GRAY
- Effects of the oral, direct thrombin inhibitor dabigatran on commonly used coagulation assays TOMAS LINDAHL
- Effects of the oral, direct factor Xa inhibitor rivaroxaban on commonly used coagulation assays ANDREAS HILLARP
- Systematic review of perioperative heparin bridging therapy: implications of harmonization of outcome definitions and bivariate endpoints ALEX SPYROPOULOS

Presentations were followed by an open discussion to identify issues to be addressed either through official SSC recommendations or through the new Clinical Guidance Group. In addition to measurement of new oral anticoagulant drugs the issues identified were:

- Which patients should be treated with the new anticoagulants first?
- What to do in case of bleeding?
- How to try to assess/improve compliance.
- Follow up during treatment - when and by whom?
- Instructions and documents to be given to patients.
- Pharmacokinetics/dynamics / implications for bridging
- Influence of renal function and practical considerations
- Interactions with drugs/diet
- Different doses for different patients
- Side-effects
- Information (efficacy/safety) to be collected during treatment (for phase IV studies)
- Recommendations for the national regulatory agencies regarding the introduction of the NOAC in clinical practice and observational phase IV studies

The Chairman will send a proposal to the Clinical Guidance Committee for a consensus/position statement on clinical issues.
Disseminated Intravascular Coagulation

24 July 2011

Chairman: Hideo Wada (JP)

In the education session, Dr. Shinichiro Kurosawa made a presentation, entitled, “Disseminated Intravascular Coagulation due to Anthrax; A Pre-Clinical In Vivo Study.” The causative agent of Anthrax, *B. anthracis* was actually used for bioterrorism on the U.S. ground in 2001. National Institute of Health considers *B. anthracis* as the most important agent of bioterrorism and the development of countermeasures against Anthrax is a national priority. Anthrax is an acute infection and without proper treatment, it leads to sepsis, DIC, organ failure (OF) and shock, and ultimately death. It is well documented that *B. anthracis* produces both Lethal toxin and Edema toxin. The paradigm of the field is that the toxin is the primary cause of death. This is evidenced by the fact that most of the efforts are directed toward targeting and neutralizing protective antigen (PA), a common and required component of Anthrax toxin. In case reports from 2001 attack, all the fatal cases had bacteremia, and almost all patients had DIC, coagulation abnormalities including prolonged prothrombin time (PT) and activated partial thromboplastine time (APTT), elevated D-dimer or microangipathic hemolytic anemia (MAHA). Dr. Kurosawa established a non-human primate model of anthrax sepsis similar to the *E-coli*-injected baboon model established by Drs. Hinshaw-Taylor. The intravenous challenged model caused dose-dependent DIC similar to that observed in patients and also the original baboon *E-coli* model. In order to delineate the role of Anthrax toxin, the animals were challenged with toxin-negative *B. anthracis* strain and compared with toxin-positive strain. The difference between the lethal doses of these two groups was not striking, indicating that the role of toxin may not be as great as previously thought. In order to test whether animals were dying from sepsis, the animals were treated with activated protein C (APC), the only FDA approved drug for severe sepsis. All baboons without APC died, while those pre-treated with APC survived. Taken together, a conclusion was made that Anthrax causes septic DIC and APC may be a potential therapeutic.

Dr Marcel Levi talked about the “Pathogenesis of septic DIC”. DIC in sepsis causes OF and bleeding. In sepsis, endotoxin and inflammatory cytokines activate monocytes and endothelial cells to express tissue factor (TF). It has been reported that the messenger RNA levels in monocytes were elevated in meningococemia and TF was detected by an anti-TF antibody in several tissues such as kidneys in these patients. TF, Xa and thrombin activate protease activated receptor (PAR). P selectin released from activated platelets induces TF expression. Decreased ADAMTS13 increases the ultra large multimer of von Willebrand factor (ULM-VWF), and this was observed in sepsis. APC, TF pathway inhibitor (TFPI), antithrombin (AT) and thrombomodulin (TM) have both anti-coagulant and anti-inflammatory activities. As a potential, new factor, the volume loss of the Glycocalyx was observed in sepsis as a result of the activation of coagulation.
In the chairperson’s report, Dr Wada introduced 4 working parties for examining the relationship between trauma and DIC, standardization of fibrin related markers (FRMs), establishment of non-overt DIC and establishment of DIC treatment guidelines.

Dr. Jecko Thachil talked about “Mimics of DIC in the intensive care unit”. He showed the hemostatic abnormalities present in critically-ill patients with DIC and those present in patients with liver disorders, macrophage activation syndrome, MHAH, systemic vasculitis and massive blood loss.

Dr. Satoshi Gando presented “DIC in trauma” It was reported that the administration of tranexamic acid reduced the risk of death in bleeding trauma patients associated with DIC. He showed that the plasma levels of tissue type plasminogen activator (tPA), fibrinopeptide Bβ15-42 (FP Bβ15-42) and plasmin plasmin inhibitor complex (PPIC) were markedly increased during the early phase of trauma thereafter depressed, while those of PA inhibitor-1 (PAI-1) and FPA were persistently increased during all phase of trauma. A trauma patient's survival may depend on the ability to control two opposing conditions; bleeding during the early phase and thrombosis during the late phase of trauma. He stated that most hemostatic abnormalities in trauma were due to DIC. After the presentation, it was discussed whether the hemostatic abnormalities were due to trauma or DIC in trauma?

Dr. Toshiaki Iba presented “Multicenter prospective analysis of the efficacy and safety of AT treatment”. He analyzed the resolution rate, survival rate and bleeding in 1435 cases treated with 1,500 units or 3,000 units of AT. The resolution rate was 55.4% in those treated with 1,500 units of AT and 69.6% in those treated with 3,000 units of AT. The survival rate was 65.2% in those treated with 1,500 units of AT and 75.4% in those treated with 3,000 units of AT. The frequency of bleeding was low in patients treated with either dose. These data suggest that administration of 3,000 units of AT should be recommended.

Dr. Shousaku Nomura talked about the “Therapeutic effects of recombinant thrombomodulin (rhTM) in DIC patient with hematologic malignancy”. He classified DIC in hematological malignancy into 4 types; the leukemia type, tumor-lysis type, infection type and transplantation type. TF and Anexin II mainly cause DIC in the leukemia or tumor-lysis type. DIC due to infection is generally caused by inflammatory cytokines and is frequently associated with systemic immune response syndrome (SIRS) and OF. He introduced many markers, such as sP-selectin, CD40L, PDMP, RANTES, MCP-1, microparticle (MP), HMGB-1 and sVCAM-1, which increased in DIC due to infection. The transplantation-related DIC may include vascular occlusion disorder (VOD), graft versus host disease (GVHD) and thrombotic microangiopathy (TMA). Finally, he reported that rhTM was effective for DIC due to hematological malignancy.

Dr. Nigel Key talked about “Microparticle assays in human endotoxemia.” In this model, MPs may be released from platelets, monocytes, and endothelial cells. A new functional assay for TF activity on MPs demonstrates findings that are analogous to a previously publish assay from his lab (Aras O, et al, Blood 2004). After LPS infusion, MPs-TF levels peaked at 2-4 hours, with a decline thereafter. However, using the recently standardized ISTH approach to measure platelet-derived MPs by flow cytometry (Lacroix, R et al. JTH 2010), the number of MPs was surprisingly most elevated at the 24 hour time point in 2 of the 5 subjects thus far.
Dr. Jorn Nielsen talked about “Controversies in DIC scoring”. The mortality was significantly higher in DIC patients in a RCT for APC and AT. Both APC and AT significantly improved the survival rate in the patients with DIC. In the non-overt DIC scoring system, the mortality was significantly higher in the patients with high non-overt DIC scores. He stated that the DIC related states, such as TTP, capillary leak syndrome (CLS) and hyperfibrinolytic state are important for the diagnosis of DIC. He proposed simplify the scoring system to include platelet activation, CLS and FRMs. The platelet activation marker includes the platelet number and ADAMTS13. The CLS markers are PT, albumin, AT and PC. The marker for FRMs is D-dimer.

Dr Zhaoyue Wang talked about the “Involvement of tissue factor and annexin II in pathoclinical profiles of acute promyelocytic leukemia”. He retrospectively examined the frequency of DIC in more than 1,400 cases of leukemia. The frequency of DIC was 39.6% in leukemia and 80% in patients with acute promyelocytic leukemia (APL). He examined the levels of TF and anexin II in leukemic cells, and NB4 and HL-60 cell lines based on the Xa activity, flow cytometry results, mRNA and western blotting analysis. ATRA and ASO improved DIC by inhibiting the expression of TF and Anexin II. The hypofibrinogenemia in APL is suggested to be caused by Anexin II.
Registry of Exogenous Hemostatic Factors

24 July 2011

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

Manjunatha Kini chaired the session as Mary Ann McLane could not attend the meeting.
Seven members of the registry were in attendance plus about 8-15 guests.
Welcome: Francis S. Markland, Chair

Introduction of new member of the registry: Dr. Ivo Francischetti

Minutes of the last meeting (Boston 2009) was approved.

Publication of the subcommittee

1. Classification and nomenclature of C-type lectin related proteins (snaclecs)

New inventories / activities
Two areas were considered

1. Nomenclature / classification / activities of L- amino acid oxidase will be considered. Ken Clemetson will take the lead in the project.
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.

Any other business

1. Novel exogenous factors: There have been a number of new anticoagulants from snake venoms as well as from saliva of hematophagous animals. Prof. Kini introduced some of them and discussed about imminent explosion of new factors with improved technology.
2. Fifth International meeting on Exogenous Factors Affecting Thrombosis and Hemostasis: It was planned to 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. We are planning for 100-120 participants.

Next meeting
The subcommittee agreed to meet in Amsterdam (2013).
The meeting was adjourned at 11 am.
Factor VIII and IX

23 July 2011

Chairman: Flora Peyvandi (IT)
Co-Chairmen: C. Alok Srivastava (IN), Jan Astermark (SE), Kathelijn Fischer (NL), Charles Hay (UK), Claude Negrier (FR), Johannes Oldenburg (DE), Midori Shima (JP), Edward Tuddenham (UK), Leonard Valentino (US)

The meeting started at 9.00 hours on the 23rd of July, 2011 with the Chairman’s welcome speech to over 300 participants. She thanked all Co-chairs and Dr. Alok Srivastava, the previous Chair, for his significant contributions to the activities of the subcommittee.

Welcome and Introduction to the program – F.Peyvandi and A. Srivastava

A summary of the activities of the last 5 years was reported by Dr. Alok Srivastava. Dr. Peyvandi reported the updates of each of the six Projects started in 2009:

- Consensus definitions in Haemophilia: First draft of recommendations is available on ISTH website
- Consensus definitions in Rare Bleeding Disorders: First draft of recommendations is available on ISTH website
- Standardization of methods for performing the Clot Wave Form Analysis
- Standardization of methods for performing the Thrombin Generation Test
- Standardization of methods for performing the Thromboelastogram
- Pharmacokinetics: Manuscript in preparation, will be available on ISTH web-site
- Potency labeling of clotting factor concentrates: Information on the current practice for post-infusion testing is being gathered through a questionnaire circulated to expert clinical laboratories

She mentioned how arrival of different new products (replacement and by-passing therapies) for the treatment of hemophilia A and B to the market necessitates the establishment of a specific program for the future of FVIII and FIX subcommittees. Construction of a well-organized plan in terms of standardization of global assays, assessment of PK, safety and efficacy and development of novel clinical scale (tool) to evaluate therapeutic efficacy of new drugs remains essential. In addition, she highlighted the importance of novel technologies such as large scale genomic sequencing in the field of hemophilia research and care.

The new Project on “Clinical trial design for hemophilia” chaired by Donna DiMichele was also presented. She also described a recent NIH meeting on the role of global assays in hemophilia A. The meeting concluded that collaboration between two scientific committees is recommended (SSC and NIH) in order to avoid duplication of activities and ensure establishment of a single guideline. Both groups have to come up with a specific recommendation on how to use the global assays in hemophilia.

Session 1: 9:00 – 13:00
Jerry Powell - New Products for treatment of haemophilia – changing options

Hemophilia is a rare genetic disease due to deficiency in the function of one of the coagulation factors, resulting in life-threatening spontaneous bleeding when factor activity is less than 1%. Current optimal treatment provides frequent infusions of the missing factor protein to prevent any spontaneous bleeding episodes. Problems arise because the factor activity still falls to dangerous levels prior to the next infusion, resulting in the risk of spontaneous intracranial hemorrhage and permanent neurological damage, and often intravenous access devices are required to sustain the frequent intravenous infusions, especially in small children, with medical complications of sepsis and thrombosis. In addition, development of neutralizing inhibitor formation remains a problem. Several approaches are in development to enhance the duration of efficacy of infused clotting factors. The first to complete clinical trial was pegylated liposome FVIII. Approaches to increase the half-life of infused clotting factor are currently exploring several innovative changes to the clotting factor, including: 1. fusion of the factor molecule with a portion of the immunoglobulin molecule, 2. fusion of the factor molecule with albumin, 3. addition of site specific polyethylene glycol moieties to the native factor molecule, and 4. modification of sialic acid portions of the factor molecule. Each of these approaches has promising pre-clinical data for increased half-life and corresponding prevention of bleeding in animal models. A completely different approach to preventing bleeding in hemophilia involves inhibition of the tissue factor pathway inhibitor, potentially allowing the coagulation cascade to bypass the need for clotting FVIII or IX, and activate FX directly. Key questions, for any of these innovative approaches, regarding which is more effective to prevent bleeding in different clinical settings will be addressed in current and planned clinical trials. Important questions include which of these new factor products will prove most effective for small children, for prevention of spontaneous bleeding in active adults, or in preventing bleeding during surgery; what are potential benefits: avoid post-surgery bleeding or thrombosis episodes. Although no product specific side effects are anticipated, the clinical trials will need to monitor for any unexpected interactions or altered metabolism with long term administration of these modified factor products. As the clinical trials progress, the other question will be whether any new product can reduce the rate of inhibitor development. For that answer we can only proceed thoughtfully through clinical trials. Further progress will depend on close interactions between the developers of the new clotting factors and the community of hemophilia patients in order to complete the critical clinical trials to address which way forward is best for hemophilia.

DISCUSSION: Author’s personal review: exposure of more than nine days might be enough for the inhibitor development instead of 50 ED. This was discussed with the audience and the author mentioned that this is his personal experience and not based on the evidence-based data.

Elena Santagostino - Evaluation of safety and efficacy of new products (clinical and laboratory problems)
Recombinant DNA technology and bioengineering strategies have produced novel hemophilia therapeutics with improved functional properties such as increased potency, resistance to inactivation, prolonged plasma half-lives and reduced immunogenicity. In addition to these products developed to facilitate replacement therapy and prophylaxis, other non-replacement, haemostatic agents are also advancing from preclinical to clinical trials.

The clinical investigation plans currently in place to assess the safety and the efficacy of new products for patients with hemophilia have been often discussed because a more uniform standard is still needed between studies conducted in the USA and in Europe, particularly taking into account the body of data required in the context of this rare disease. The application for marketing authorization of new products with modified biological properties poses a number of additional challenges to regulatory agencies, manufacturers, physicians and patients.

Since pharmacokinetic (PK) data are considered the most important surrogate endpoints for the efficacy of a new replacement agent, these should be provided using the same functional assay for analysis of the patient’s plasma and the product. The same assay should also be used for PK studies comparing the “new” with the “old” product.

The clinical investigation plans have been designed according to a stepwise approach in order to involve in specific studies an increasing number of previously treated adults, followed by paediatric patients and, finally, by previously untreated patients (PUPs). PK, safety and efficacy data must be available in a relevant proportion of study subjects in order to start the next trial. This design offers obvious advantages in terms of safety assessment but represents a challenge with respect to the small size of hemophilia population and the demanding schedule of visits and blood drawing. Furthermore, the application of this plan to the investigation of long-acting products raises a number of challenges, i.e., the prolonged PK assessments, the need for a wash-out period for PK studies and inhibitor testing and the time needed to complete the follow-up period (at least 50 exposure days). Even the approved concept of exposure day used to define the schedule of inhibitor testing could become a matter of discussion in the clinical setting of treatment with a long-acting product.

The efficacy evaluation of the product poses also additional challenges: other than PK studies, clinical response should be analyzed, as well as the choice of the functional assays to test the product and the patient’s plasma need particular attention.

In the light of these complex issues, what seems predictable is that the marketing authorization will be delayed for both, adults (waiting for the study completion in all age groups) and PUPs (waiting for the indication after the completion of the specific, post-marketing study). Paediatric patients and PUPs are few and have a limited compliance to demanding study procedures; however they represent the target population for these new products promising easier prophylaxis with less frequent infusions. Post-marketing surveillance through international registries and harmonized data sets could facilitate the collection of high-quality data from a large patient sample as it is needed to exclude altered immunogenicity and to confirm long-term safety of new therapeutics for hemophilia treatment.
In conclusion, harmonization of study design is needed to achieve sufficient data of body clinical data.

DISCUSSION: A prolonged follow-up is mandatory, in particular for new molecules. At the same time FDA and EMEA should share schemes and rules with the help of clinicians providing an assessment scheme for clinical aspects and PK.

FDA AND EMEA REGULATORIES: CLINICAL TRIALS REQUIREMENTS

Nisha Jain - Clinical Trial Designs for Factor VIII and IX Products - FDA

Treatment of Hemophilia A and B has evolved over years: from blood transfusions in 1800s, cryoprecipitate in early 1900s, human plasma derived concentrates in mid 1900s, recombinant factors in late 1900s to modified recombinant factors in 2000s. Over the years safety of the products for treatment of hemophilia A and B has improved. The risk of development of inhibitory antibodies and lack of adherence to prophylaxis regimens because of frequent infusions still remains the main challenge in the treatment of hemophilia patients. The modified FVIII and FIX products are bioengineered to improve the biosynthesis and secretion of the FVIII/FIX molecule, improve the functional activity, extend the half-life and reduce neo-antigenicity. The half-life of currently available FVIII and FIX products is less than 20 hours. Modified FVIII/IX products currently under development exploit several strategies to extend the half-lives such as PEGylation, albumin fusion and Fc fusion. Licensure of these modified products poses some challenges: what criteria should be used to evaluate efficacy of these products?, should the criteria for safety evaluation for inhibitor development be different than what is currently accepted for the licensed products, can the currently available standard potency assays be used to establish potency of these products. If the dosing frequency changes are substantial for these products an adequate massive education program for both patients and healthcare professionals needs to be launched. These products may provide advantage over the currently licensed products by decreased frequency of dosing because of prolonged half-life. However, safety and efficacy data are needed in order to determine the advantages of these products over currently licensed products.

Anneliese Hilger - Clinical trial requirements for FVIII/FIX – EU-regulatory perspective

The EU-requirements on clinical development for FVIII and FIX 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 (with differences on patients number requested for each group - >12y, 6-12y, 0-6y - for FVIII and FIX). Guidance is also provided for authorised products where a significant change in the manufacturing process has been made. Clinical trial data, 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 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 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. Guidelines could be finding soon (www.ema.europa.eu).

DISCUSSION on both regulatory presentations: It was discussed whether there could be some problems with study feasibility, in particular with regard to the required 50 PTP children at time of submission in Europe. A. Hilger stated during the discussion that this is a legal requirement. Is there any estimation of the number of required patients for all upcoming clinical trials? Do we need to make a priority list? Regulators answered that they were following the current guidelines / requirements but they also acknowledged that while the principles of safety and efficacy requirements cannot change the further development of specific requirements e.g. with regard to numbers of patients and design of clinical trials can be discussed. Some of these issues will be taken up by the Clinical Trail design project group.

BUSINESS PROGRAM

Section 1. Rare Bleeding Disorders (RBDs)
Chairpersons: F. Peyvandi (Italy) and A. Srivastava (India)

Flora Peyvandi - European network on RBDs (EN-RBD): what has been obtained and next steps

Rare Bleeding Disorders (RBDs) include the inherited deficiencies of such coagulation factors as fibrinogen, factor (F)II, FV, FV+VIII, FVII, FX, FXI and FXIII, and are usually transmitted in the autosomal recessive manner and due to their rarity, sometimes present significant difficulties in diagnosis and treatment. Their frequency in Europe is from 1:500.000 to 1:2 million in the general population but it this increasing due to the high rate of immigration from the Middle East and North Africa where the incidence is significantly higher. The limited available data make it difficult to draw an exact picture of each single RBD. The relationship between laboratory phenotype and bleeding severity in patients with RBDs was explored. Data from 592 patients in the EN-RBD were retrospectively collected over three years. Clinical bleeding episodes were classified into four categories according to severity (ranging from asymptomatic to severe bleedings – grade III). In 513 patients, a linear regression analysis showed that Fibrinogen, FV+III, FX, and FXIII had the best linear correlation between activity level and clinical severity; FV and FVII deficiencies showed a good correlation whereas FXI deficiency showed no such correlation at all. For FII, number of patients was too small to draw any correlation. Our study remains the first to classify RBDs on the basis of clinical and laboratory parameters, and to highlight significant correlations between the laboratory phenotype and clinical bleeding severity. The main limitation of our study is the retrospective nature of data collection. The observed heterogeneity between different RBDs underlines the need for a prospective data
gathering tool that allows for adequate assessment of individual RBDs. Undertaking such a task is one of the future aims of the EN-RBD.

DISCUSSION: Are the categories of bleeding (asymptomatic, grade I, II and III) the criteria recommended for classification of patients? Other than that a bleeding score has been developed, but is currently under validation (the bleeding score developed for VWD1 was not suitable for RBDs).

Mark van Geffen - Nijmegen thrombin and plasmin generation assay in RBDs

Rare bleeding disorders (RBDs) are a heterogeneous group of diseases with varying bleeding tendency, only partially explained by their laboratory phenotype. We have performed simultaneous thrombin and plasmin generation measurements on 41 patients affected with RBDs to investigate whether parameters of the NHA were sensitive to bleeding tendency. In individual groups our analysis showed for patients with prothrombin, FV and FX deficiency and major bleedings an area under the curve (AUC) below 20%. FVII deficient patients had a prolonged thrombin lag-time ratio of 1.6±0.2 (P<0.05) and normal AUC (92 -125%). FXIII deficient plasmas resulted in a reduced thrombin peak height of 59±13% (P<0.05) and normal AUC (90±14%) and afibrinogenemic patients demonstrated plasmin generation of 2-29% of normal. To demonstrate sensitivity of the NHA in this cohort, patients were divided based on their bleeding tendency (major or minor bleeding) and whether their coagulation factor deficiency belonged to the common (FII, FV and FX deficiency) or the non-common (FVII, Fg and FXIII deficiency) pathway. Thrombin lag-time ratio (P<0.001), thrombin peak-time ratio (P<0.01), thrombin peak height (P=0.01), AUC (P<0.0001), plasmin peak height (P<0.05) and plasmin potential (P<0.05) demonstrated significant differences between these groups whereas the FLT ratio did not show any significance. These results provide information over thrombin and plasmin generation phenotype and some NHA parameters show sensitivity to bleeding tendency in the various groups. In conclusion, these results could contribute to more effective clinical management for each individual group.

DISCUSSION: By this new method, only graphs are shown as results and no number or ranges are given. How could this be handled? Enlarging numbers could help in analysis of RBDs by NHA assay.

Clinical trials in RBDs:

Sigurd Knaub - Clinical development plan of a double-virus inactivated fibrinogen concentrate in patients with hereditary fibrinogen deficiency – EU and US requirements

Hereditary fibrinogen deficiencies are rare and present clinically as afibrinogenaeemia or hypofibrinogenaeemia (type I deficiencies, i.e. quantitative defects), or dysfibrinogenaeemia (type II deficiencies, i.e. qualitative defects). Afibrinogenaeemia and hypofibrinogenaeemia are predominantly associated with bleeding symptoms, while dysfibrinogenaeemia may be clinically silent or associated with a mix of bleeding and thrombosis. Plasma-derived fibrinogen concentrate, cryoprecipitate or fresh frozen plasma (FFP) are effective for treating bleeding associated with congenital fibrinogen deficiencies; plasma-derived fibrinogen concentrate is the
treatment of choice. Octapharma® is developing Octafibrin – a highly purified, lyophilised, human plasma fibrinogen concentrate for the treatment of congenital fibrinogen deficiencies. Octafibrin if formulated without added albumin and the purification process used in the manufacturing includes two separate virus inactivation/removal steps. A solvent/detergent (S/D) treatment step inactivates transfusion-relevant enveloped viruses and a nanofiltration step, using a PlanovaTM 20 nm nanofilter, is effectively removing enveloped and non-enveloped viruses. None of these steps carry the risk of denaturing plasma proteins. There are no specific guidelines for the clinical development of fibrinogen concentrates in the proposed indication from either the EU or USA. The clinical program for evaluating the efficacy and safety of Octafibrin has been based on the “Guidelines on the Clinical Investigation of Human Plasma-Derived and Recombinant Factor IX products and discussed with an EU Regulatory Agency and FDA. The clinical development program for Octafibrin in patients with hereditary fibrinogen deficiency includes a pharmacokinetic (PK) study (FORMA-01), followed by two efficacy studies (FORMA-02 and FORMA-03). FORMA-01 is a phase II comparative study (with Haemocomplettan® P/RiaSTAP® as the comparator) investigating PK, efficacy (laboratory endpoints) and safety of Octafibrin in patients with congenital fibrinogen deficiencies and will be performed both in the US and Europe. FORMA-02, an open, uncontrolled phase III efficacy study will document efficacy and safety of Octafibrin in patients with acute or traumatic bleeding. Data from FORMA-01 and interim data from FORMA-02 are required for EU registration. FORMA-03 will compare the efficacy and safety of Octafibrin with Haemocomplettan® P/RiaSTAP® for on-demand treatment of acute bleeding. FORMA-03 is a phase III study that will be undertaken to fulfil FDA post-licence requirements. Requirements for the clinical development of drugs for ultra-rare diseases in the US and EU are different. Whereas a comparative PK study with a surrogate efficacy endpoint and a post-approval commitment study to clinically validate the surrogate efficacy endpoint, are required for US licensure, both PK and clinical efficacy data are needed for EU registration at the time of submission. Using historical efficacy data for comparison would be an option however, published data are often not detailed enough in order to make a proper power calculation.

DISCUSSION: Different drug companies are coming to the market with new fibrinogen products. Due to a limited number of affected patients, how could the feasibility of studies be confirmed? This is a difficult situation as the number of bleeding episodes for each patient is not high enough (2-3 mean of 0.2 bleed/year; Peyvandi 2006) and a large number of patients evaluated for a long period of time could be necessary.

Ramin Tehranchi - Congenital FXIII Deficiency, Clinical and Regulatory Challenges

Rare bleeding disorders encompass inherited abnormalities of haemostasis that may present significant difficulties in diagnosis and management. The overall frequency of these disorders in the general population is low, which makes it very difficult and challenging to conduct well-controlled randomised clinical trials. Regulatory requirements which have progressively increased over time have resulted in an increase in both trial size and duration, adding further complexity to trial conduct in these rare populations. Congenital coagulation factor XIII (FXIII) deficiency, as an example of a rare coagulation disorder, is an autosomal recessive and life-threatening bleeding disorder with an estimated prevalence of 1 per 2 to 5 million individuals worldwide. A clinical development program has been developed to investigate the efficacy and
safety of recombinant FXIII (rFXIII). This presentation describes the clinical and regulatory challenges in a state-of-art rFXIII development for treatment of an ultra-orphan disease.

Debra Bensen-Kennedy - Challenges in clinical development of novel coagulation factors

Ongoing technical advances in biomedical research have afforded the opportunity to engineer novel agents that may provide advantages to current treatment modalities for patients with rare diseases. However, with this opportunity comes a complex set of challenges. Traditional drug development looks to address relatively common disorders occurring in larger populations. When dealing with common diseases, novel agents can be introduced in a stepwise fashion leading to large studies which can adequately account for underlying variability within the study population and detect small treatment effects. This is not possible with rare disease populations, especially those with disorders of coagulation. In addition to the expected challenge of identifying an adequate number of suitable patients for clinical study, development can be further complicated not only by the need for study globalization but also by issues of endpoint clarity, assay variability and regulatory expectations that differ among countries. When working within the rare disease populations, one must remain cognizant of the impact of the development process on these small groups of vulnerable patients, while maintaining expected development standards. This is true because there are no global rules from regulatory agencies: it is necessary a strong collaboration and strive for standardization (endpoint, assays standardization).

Manuela Scarpellini - Ligneous conjunctivitis and plasminogen deficiency: An experience of an orphan drug development for an extremely rare disease

Ligneous Conjunctivitis (LC) is a rare coagulation disorder characterized by Type I Plasminogen deficiency. The prevalence of this disease is in the range of 1.6 per million inhabitants. A specific replacement treatment is not available and topical Fresh Frozen Plasma (FFP) is the only therapeutic alternative for LC. In this context, Kedrion, as a partner of the National Health System in the Plasma Derivatives Self Sufficiency Program, has been asked by the Italian Medicines Agency and the Medical Community to consider the possibility to develop research projects in this field. Upon this specific request, a program for the development and supply of a human Plasminogen (PLG) concentrate for the treatment of LC has started in 2006 and is currently ongoing. The product has been successfully developed as eye drop preparation and preliminary clinical data showed its beneficial effect in clinical use. On August 3rd, 2007 and June 7th, 2010 Kedrion has obtained the Orphan Drug Designation for this indication in Europe and US respectively. In the last year, two non-clinical studies aimed at evaluating single and 28 days repeated dose ocular tolerance have been performed in rabbits. The results did not show any local toxicity effect. These preliminary encouraging findings show that a PLG replacement therapy is a good alternative to FFP in patients affected by LC linked to type I PLG deficiency. A randomized, controlled, double blind Phase II/III study to evaluate Efficacy and Safety of Kedrion human PLG eye drop preparation has been planned and should start next year. A registry could be valuable to identify patients and collect data for clinical studies.

DISCUSSION: The data presented by Kedrion was very interesting and there was discussion regarding whether the request of the European and US Regulatory agencies to ask for a case control study using placebo as a control, was ethical and reasonable. Since no other therapy is
currently available for such a disease, then it would not be reasonable to treat a patient with a
placebo and the preclinical data could be enough. Moreover, a low number of available patients
make such study extremely challenging. Agencies answered that the presentation of product
seems convincing. However, in absence of data or few available data for preclinical studies,
procedures need to be followed, while in presence of convincing preclinical data clinical trial
design could be re-discussed.

Section 2. PROJECTS REPORTS – 1
Chairpersons: M. Shima (Japan) and A. Srivastava on behalf of J. Oldenburg (Germany)

Claude Negrier - Report of Project on Thrombin Generation (C. Negrier, Y. Dargaud, T.
Lecompte, R. Luddington, A. Wolberg, H. Hemker): Standardization of the method

The aim for the Project was to propose a methodology that may be considered to be the
recommended way to perform the thrombin generation test so as to bring standardization in
studies performed using this method.

The Project recognized that several variations of the method to perform the thrombin generation
test exist in the literature with regard to reagents, instruments and interpretation. However, there
is a common feeling that the most used so far and the one on which most work has been done is
the CAT assay (Hemker et al.). The WP however recommends that a thorough description of the
method should be done in any paper on the subject.

Literature published in 2010/2011 has been carefully followed and some lab data and
information exchanged. Particularly, further activities to minimise the variability of the assay
have been carried out among 5 European centers and the results were published in Thromb Res
2010. The Project recognizes that further activity should be carried out in this direction, and
particularly on the use of a reference plasma, the influence of the type of phospholipid vesicles
or platelet membrane, as well as and the type and concentration of tissue factor used. It was also
felt that the potential impact of temperature should be evaluated and in vitro samples will be
soon exchanged among different European (n=4) and USA (n=1) centers. The educational
material (DVD) has been updated (2 languages) and should support the TGA activities in
different centers worldwide. The availability of the TGA machines has been worked out and
most of the participating centers are equipped yet.

In addition to these standardization activities of the CAT assay, the members of the Project felt
that the current variability of the assay was good enough to initiate translational research. In this
regard, 3 main fields have been thought of:

- Use the common method in 10 centres (5 NA, 5 EU) as a validation exercise for
evaluating single clotting factor deficiency (FVIII, FIX, FVII, FXI…) and try to correlate
with the bleeding phenotype (if score available) – short term
- Replication exercise in 10 centres (5 NA, 5 EU) on the use of the pilot study done in
Lyon, using TGT in surgery carried out in patient s with inhibitors receiving bypassing
agents (NovoSeven®, FEIBA®) – short term.
These 2 first exercises should be completed before claiming a complete validation of the assay and a wider availability of the educational material (DVD produced in Lyon) for this purpose.

- Implementation of TGT in prophylaxis (patients with and without inhibitors) – midterm.

Results of monitoring of FEIBA and rFVIIa were presented and it seemed that the latter worked better.

The amount of work needed to standardise this method goes beyond the time dedicated by the FVIII&FIX SSC, hence collaboration with clinical parties is planned.

DISCUSSION: A document providing the details of pre-analytical and analytical aspects requiring standardization will be prepared by the project group before the end of the year and also an educational video on the performance of this test will be made available. This will be published as a report of the SSC subcommittee on FVIII/IX.


Thromboelastography/Thromboelastometry (TEG) is a global assay used to assess coagulation in a physiologic milieu where all cellular and enzymatic components are preserved. The assay extends beyond the initiation of clotting, and provides information on quantity and quality of clot and fibrinolysis. Modifications of the assay have been used to assess platelet function and receptor inhibition. The applications for use are wide and growing, extending from hematology and transfusion medicine to cardiology. Today TEG is used to provide information on appropriate product replacement in trauma and surgery. It has been shown to significantly decrease the use of blood products in the peri-operative setting. In the field of hematology, TEG is used to define the phenotypic variation in congenital bleeding disorders (CBD) and tailor replacement therapy with factor products. It remains one of the few assays that can be used to assess the effect of bypassing agents making it extremely useful during surgery and individualizing replacement therapy in CBD patients. Assessing combination therapy with bypassing agents/factor products and antifibrinolytics can only be done with global assays like TEG. One of the areas of investigation has been to predict the risk for bleeding in patients with bleeding disorders or liver disease, providing a means to individualize therapy. With the advancement in medical technology and the use of supportive devices such as external cardiac pumps, monitoring of anticoagulation becomes a major challenge. TEG provides a unique method to evaluate effect of multiple anticoagulant and antiplatelet agents used in combination. It is also being considered for monitoring anticoagulation in patients on extracorporeal life support. TEG has significant potential in assessment of hemostasis but a major drawback remains the lack of standardization.

Results of studies on different disorders monitored by TEG were reported (Glanzmann Thrombasthenia, Afibrinogenemia, and FXIII deficiency), TEG was also used to monitor coagulation on ECMO demonstrating an enormous potential for evaluating thrombosis and bleeding.
DISCUSSION: A document describing these results is planned to be published within few months in order to proceed and satisfy the Project mandate.

Midori Shima - Report of Project on Clot wave form analysis (M. Shima, S. Nair, J. Thachil, A. Srivastava): Application to currently available clot detection instruments

Clot waveform analysis is based on the monitoring of transmittance during the routine coagulation assay such as aPTT or PT by automated clot detection instrument. In addition to qualitative evaluation of the clotting function by waveform pattern, it is possible to assess by various quantitative parameters such as coagulation velocity and acceleration. Although the technique was originally developed by MDA-II (Organon teknica) and this is no more available, many of current automated clot instruments have capability of waveform analysis. We extracted raw data from CS-2000i (Sysmex) and processed the data to derive each parameter. Maximum coagulation velocity, acceleration and deceleration may be good parameters for assessment of global clotting function. Parameters were well correlated with FVIII level in severe cases. Variability was observed in moderate and mild. The next important point is to validate the method on such instrument. Parameters were also reflecting clinical severity. Currently the method is under study on other instruments to finalise result in a manuscript.

DISCUSSION: How specific is the method? The method was working well with all PT and aPTT reagents, but, for example, microparticles were excluded from the tested samples, it still needs to be done.

A document describing the pre-analytical, analytical and post analytical variations should be described and published within few months in order to satisfy the Project mandate. During the year 2012, this group could proceed with a study on a normal population and a group of haemophilic patients, preferably with low levels of FVIII <1% as well as heterogenous clinical manifestations in order to understand the sensitivity of this assay in a low range of FVIII, and to correlate minimal FVIII activity with the clinical severity of patients.

Session 2: 14:00 – 18:00

Section 3. CLINICAL ISSUES (inhibitors, prophylaxis, novel therapies)
Chairpersons: K. Fischer (The Netherland) and C. Hay (United Kingdom)

Donna DiMichele - Considerations for optimal clinical trial design for new clotting factor concentrates

After all discussions during the morning sessions, it is clear that new products for treatment of hemophilia A pose several concerns and new questions, in particular for pre-licensure studies.

The Design of Clinical Trials in Hemophilia Project Group (PG) proposed by Flora Peyvandi and Alok Srivastava and now chaired by Donna DiMichele began its deliberations in February 2011 and has continued its work through a series of 7 teleconferences to date.
The mission of this PG will be to determine the optimal prospective pre-licensure and observational post-licensure trial designs for new clotting factor concentrates for hemophilia on the basis of: 1) the harmonized safety and efficacy data required by regulators for registration; 2) the anticipated available study population; and 3) innovative clinical trial design.

This will be accomplished by using consensus definitions (provided to the Group by the Definitions Project); and guidance from regulators in the US and Europe, industry, scientific and methodological experts, as well as clinical investigators. The plan is to initially focus on generating evidence-based recommendations for the conduct of new clotting factor trials in hemophilia A and B and to present these recommendations as they develop to the SSC of the FVII/IX Subcommittee. The PG’s work will be ongoing for a period of at least 2 years and will be further harmonized as appropriate with WFH efforts. After 2 years of activity a manuscript will be prepared and sent as SSC a communication.

Its membership is as follows:
Chair: Donna DiMichele/ Members: Flora Peyvandi, Alok Srivastava, Nisha Jain (FDA); Anneliese Hilger (EMA); Sebastien Lacroix-Desmazes; Frits Rosendaal and John Scott (FDA).

Charles Hay - ITI study: results

The ITI study was a randomised comparison of high and low-dose ITI in good risk subjects. It was stopped on 13/11/09 because of DSMB safety concerns because there were significantly more intercurrent bleeds in the low-dose arm and a recalculation of power suggested that a much larger sample size would be required to prove equivalence. 134 subjects had been recruited at that point. No difference in efficacy could be shown between treatment arms. There was significantly greater bleeding in the low dose arm, largely (85%) limited to the period before the Bethesda titre became negative. Infection had no significant effect on outcome. Of the variables investigated in a logistic regression analysis, only peak historical titre and peak titre on ITI predicted the outcome of ITI.

After the study finished, further data accrual and data cleaning and the very slow process of retrieving samples for central testing and ancillary studies took almost a year. Only then were we in a position to analyse the data. The manuscript of principle results has now been submitted for publication to Blood and subsidiary analyses are in progress. These include pharmaco-economic analysis and modelling and further analysis of the morbidity associated with ITI, focussing on the epidemiology of bleeding and the epidemiology of catheter infection during ITI. Several satellite studies involving study samples are also in progress.

Claude Negrier on behalf of Manuel Carcao - Prophylaxis in patients with inhibitor

Most patients with severe hemophilia will bleed frequently and will develop arthropathy if they are not placed on prophylaxis with factor concentrates. Prophylaxis has consequently become standard of care for non-inhibitor patients with severe hemophilia. Patients who develop high-titre inhibitors have in general not been placed on prophylaxis and instead have continued to be treated on demand and have consequently tended to bleed frequently into joints and develop disabling arthropathy. It should be recognized that treatment of such patients even on demand is very expensive due to the very high cost of bypassing agents.
Until recently there were no prospective or randomized studies on prophylaxis in inhibitor patients. Most of the evidence for prophylaxis in such patients consisted of case reports or small case series. Fortunately in the last few years larger case series have been reported and additionally prospective randomized studies (Konkle et al, 2007; Leissinger et al, 2010) have recently been completed on the use of both bypassing agents for prophylaxis in patients with inhibitors. These studies demonstrate that prophylaxis with bypassing agents in patients with inhibitors appears to be effective in reducing bleeding frequency by about 50-60% in patients who were generally older and have a history of frequent joint bleeding. It should be noted that this is still not as effective as prophylaxis with FVIII/FIX in non-inhibitor patients. The very high cost of prophylaxis with bypassing agents makes it imperative that appropriate studies be undertaken to address the cost-benefits of different bypassing agent prophylaxis regimens in inhibitor patients. But which inhibitor patient should be placed on prophylaxis?

The International Prophylaxis Study group (IPSG) consists of hemophilia experts committed to studying prophylaxis in patients with bleeding disorders. The IPSG was created 10 years ago. Recently it has recognized the increasing importance of, and need for, prophylaxis in inhibitor patients and has established a small group in this area. This expert project consists of experienced hemophilia treaters [M. Carcao (Co-Chair; Canada); C. Negrier (Co-Chair; France); C. Leissinger (USA); E. Santagostino (Italy) and L. Valentino (USA)] whose mission it is to seek new knowledge in this field and disseminate this knowledge globally. In this regard this presentation represents the first presentation of this group at International conferences. This is not an industry assembled group. It is unbiased and has multinational representation; the main limitation is the lack of funds.

DISCUSSION: The results of the studies show that the rate of bleeding is still significant, and costs are prohibitive. Is not this disappointing? Yes, however, the rate is reduced, even if not at an optimal level.

ON GOING CLINICAL STUDIES ON INHIBITOR DEVELOPMENT:

Pier Mannuccio Mannucci – The SIPPET study

Clinical data stemming from a systematic review that has summarized a few retrospective studies suggest that the choice of the source of FVIII used for replacement therapy (plasma or recombinant DNA technology) might have some influence on the cumulative incidence of inhibitors. A more recent systematic review and meta-analysis of the available data found a two-fold increased inhibitor incidence with the use of recombinant factors, but the difference in favor of plasma-derived FVIII was no longer statistically significant when variables such as study design, study period and frequency of inhibitor testing were included as confounders. To tackle this state of current uncertainty on this issue, the randomized prospective SIPPET trial, undergoing now in 19 countries from 5 continents, is planning to enroll 300 previously untreated or minimally treated patients at risk of developing FVIII inhibitors, in order to establish whether or not plasma-derived FVIII products are less immunogenic that recombinant products. At this time, SIPPET has already enrolled nearly half of the planned number of cases.

Marijke van den Berg - The RODIN study (intensity of treatment and prophylaxis)
In the early nineties it has been demonstrated that some plasma products had a higher risk on inhibitor development than other products. From that time onwards many studies collected data on inhibitor formation to correlate with plasma or recombinant products. Since PUP studies are difficult to perform the number of individuals that were included in these studies were too low to investigate properly other risk factors. Data from monozygotic twins have clearly demonstrated that besides genetic risk factors, treatment related risk factors play an even important role. Moreover the advantage of treatment related factors is that they may be changed in an individual patient and as a consequence reduce the inhibitor risk. In the Canal study the effect of prophylaxis as an important factor to reduce the inhibitor risk was clearly stated.

The Rodin study is a joint research effort of 30 haemophilia centers and examines patient-related, treatment-related and environmental risk factors of inhibitor development in previously untreated patients with severe hemophilia A. At the ISTH the first results of the Rodin study will be presented, we preferred for the SCC meeting to give an overview of the patient data both in the Canal study and in the Rodin study. In the Canal study the data were collected up to 50 exposures. Since we could not exclude that some patients developed an inhibitor after 50 exposures, the Rodin study collected data up to 75 exposures. For comparison in this abstract we also made a cut-off for the Rodin study up to 50 exposure days.
Full cohorts of consecutive patients were included with a FVIII activity <0.01 IU/mL born between 1990-2000 for the Canal study and from 2000 – 2010 for the Rodin study. The outcomes were inhibitor development (at least two positive inhibitor titers with a decreased recovery) and high responder inhibitor development (peak titer of at least 5 Bethesda units per mL). We could include n=366 patients with severe hemophilia A for the Canal study and 606 patients for the Rodin study. Data on gene defect were available in both studies for up to 90% of the patients. Also the variability in gene defect was comparable between the 2 studies. The first results on 606 patients show cumulative incidences of all inhibitor and of high responder inhibitor development of 32.0% (95% confidence interval (CI) 28.1-35.9) and 22.2% (CI 18.7-25.8), respectively. In the Rodin study 5 patients developed an inhibitor between 50 and 75 exposure days. Major peak treatment moments at first exposure were associated with a 117% (CI 1.55-3.03) increased inhibitor risk. Patients started earlier on prophylaxis in the Rodin study in comparison with the canal study; however the total incidence of inhibitors did not decrease.

<table>
<thead>
<tr>
<th>Prophylaxis</th>
<th>CANAL, N=366</th>
<th>RODIN (&lt;50 ED), N=606</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular prophylaxis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N pt started regular prophylaxis (within first 50 exposure days)</td>
<td>161 (294) (54.8%)</td>
<td>387 of 576 (67.4%)</td>
</tr>
<tr>
<td>Age start regular prophylaxis, months</td>
<td>20.2 (13.4-35.1)</td>
<td>16.2 (12.2-22.8)</td>
</tr>
<tr>
<td>No. exposure days at start prophylaxis</td>
<td>16 (7-28)</td>
<td>14 (7-23)</td>
</tr>
<tr>
<td>No. exposure days at start prophylaxis (rodin minus 2)</td>
<td>16 (7-28)</td>
<td>12 (5-21)</td>
</tr>
<tr>
<td>N pt who started prophylaxis from first exposure onwards.</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Median dose of factor VIII at start of prophylaxis</td>
<td>500 (500-500)</td>
<td>500 (455-500)</td>
</tr>
<tr>
<td>Frequency of factor VIII at start of prophylaxis</td>
<td>2 (2-3)</td>
<td>1 (1-2)</td>
</tr>
<tr>
<td>Early vs Late prophylaxis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients who started early prophylaxis (≤15 ED)</td>
<td>78 (48.4%)</td>
<td>239 (61.8%)</td>
</tr>
<tr>
<td>Number of patients who started late prophylaxis (&gt;15 ED)</td>
<td>83 (51.6%)</td>
<td>149 (38.5%)</td>
</tr>
<tr>
<td>Number of patients who started early prophylaxis (≤5 ED)</td>
<td>28 (17.4%)</td>
<td>110 (28.4%)</td>
</tr>
<tr>
<td>Number of patients who started late prophylaxis (&gt;5 ED)</td>
<td>133 (82.6%)</td>
<td>278 (71.8%)</td>
</tr>
<tr>
<td>Regular prophylaxis (at least 2x/w)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients who started regular prophylaxis (≥2x/w)</td>
<td>142 (48.3%)</td>
<td>264 (45.8%)</td>
</tr>
<tr>
<td>Age start regular prophylaxis (≥2x/w), months</td>
<td>21.1 (13.4-35.2)</td>
<td>16.2 (12.0-24.0)</td>
</tr>
<tr>
<td>No. exposure days at start prophylaxis (≥2x/w)</td>
<td>18.0 (10.8-31.0)</td>
<td>19 (12-30)</td>
</tr>
</tbody>
</table>
These findings demonstrate that treatment-related risk factors such as prophylaxis and periods of intensive treatment are related to the risk of inhibitor development in patients with severe hemophilia A. Although prophylaxis has an effect to decrease the risk of inhibitor development the incidence of high titre inhibitors was similar between the studies.

Mike Makris - European Haemophilia Safety Surveillance System (EUHASS): update

Adverse event monitoring in persons with inherited bleeding disorders is hampered by low patient numbers. The European Haemophilia Safety Surveillance (EUHASS) was set up as a prospective, multicentre, multinational registry monitoring adverse events.

Since October 1st 2008, acute reactions, transfusion transmitted infections, inhibitors, thromboses, malignancies and deaths in all persons with bleeding disorders were prospectively reported every 3 months by participating European haemophilia centres.

In the first two years data were reported by 64 centres from 27 countries caring for 23,811 patients with inherited bleeding disorders. 12,408 persons had haemophilia A (severe in 5323) and 6968 were treated with concentrate. Rarer bleeding disorder patients were also included eg 346 patients had FX deficiency of whom 44 were treated. 59 different concentrates were used during the surveillance period. A total of 408 events were reported, including 54 acute or allergic reactions of which 4 were anaphylactic; 34/54 reactions occurred within 10min of concentrate administration. Overall there were 95 reports of new inhibitors and 16 of recurrences. No transfusion transmitted infections were reported. 28 thromboses were reported within 30 days of concentrate administration including 5 in FVII deficient patients. Of 83 malignancies 18 were hepatocellular carcinoma and 19 were other gastrointestinal neoplasms. 113 deaths were reported of which 26 were due to bleeding (intracerebral in 17).

Despite the rarity of adverse events to concentrates in persons with rare disorders, the multinational approach allows valuable active monitoring without selection bias and analysis of the safety of multiple products to be rapidly performed. Website: www.euhass.org.

DISCUSSION:

- Is the 2.3 per 1000 patient years inhibitor incidence reported in PTP hemophilic patients comparable with the reported literature? Yes, this is similar to published rates. EUHASS data should be sufficient to identify a problem with a product similar to what occurred in the Netherlands and Belgium in the early 1990s when a product was associated with a much higher rate of inhibitors in PTPs.
- Patients with FVII deficiency are not protected from thrombosis: how can we manage this problem? Since most of the thrombosis cases were associated with a surgery and in some cases even thromboprophylaxis has been performed the use of thromboprophylaxis in FVII deficiency was proposed. Guidelines on the management of surgery in FVII deficiency are required to balance the risk of bleeding and thrombosis in the perioperative period.
- Project is at the final stages, how should the program continue? New applications: EU does not fund the same project to continue the study. An EU application for a related
project that can also cover EUHASS has been made. Moreover, South Africa and Canada are likely to begin collecting prospective safety data using the same protocol and possibly same software this year.

Section 4. PROJECTS REPORTS – 2
Chairpersons: F. Peyvandi (Italy) and L. Valentino (USA)


Although there are established definitions for haemophilia A and B (‘mild’, ‘moderate’ and ‘severe’) it is clear that these are not appropriate for other coagulation factor deficiencies. The task of this Project is to consider whether we can arrive at any consensus for definitions in the other disorders. The initial approach was a two-pronged retrospective review.

First, a detailed literature search was undertaken (Isabella Garagiola and Andrea Cairo - Milan group) of the English language 1990-March 2011. This sought all reports which included laboratory phenotype, results and clinical details for at least 5 patients. This resulted in 45 papers and 39 review articles.

Second, data analysis from four registries gave additional information about more than 4000 patients. These registries were the European registry (EN-RBD), the UK haemophilia doctors’ organization registry (UKHCDO), the North American database (NARBDR) and the Indian registry held at Vellore. These four registries did not collect identical datasets.

The analysis overall demonstrated that the RBDs are heterogeneous and complex and a single unifying classification is not possible. Some general conclusions can be drawn: there is a generally poor correlation between factor level and bleeding tendency for both FVII deficiency and FXI deficiency, and some evidence for this also in FV deficiency. There is a reasonably good correlation between bleeding risk and level for FII, FX and FXIII. FX deficiency and FXIII deficiency are the most serious disorders.

A report summarizing our findings has been circulated to additional experts in the field for comments and there is general agreement that individual factor measurements are insufficient to define the clinical bleeding risk, and that we need to define the role of other global haemostasis measures in the rare disorders. A proposal is put forward shown in the table for definitions:

<table>
<thead>
<tr>
<th>Proposal</th>
<th>Laboratory phenotype</th>
<th>Coagulant activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>Severe</td>
<td>Moderate</td>
</tr>
<tr>
<td>FII, FV, FVIII, FVII, FX, FXIII</td>
<td>&lt;0.1 g/l or undetectable clot</td>
<td>0.1-0.5 g/l</td>
</tr>
<tr>
<td>FXI</td>
<td>&lt;1% or undetectable activity</td>
<td>&gt;10%</td>
</tr>
</tbody>
</table>

But this is a very preliminary study; it is important to develop better tools for prospective study.
of these bleeding disorders, and to establish the minimum amount of replacement therapy required under different clinical circumstances.

DISCUSSION: What about genotype? Is it not responsible for the bleeding along with phenotype? Quite interesting, in particular in countries like India or Iran. But in the preliminary analysis this question has not been analysed. In the European and International database, genotype data were collected, but not yet analysed.

Alok Srivastava and Victor Blanchette - Report of Project on definitions in haemophilia (V. Blanchette, A. Srivastava, M. van den Berg, R. Ljung, M. Soucie, M. Manco-Johnson, N. Key, A. Gringeri)

Evaluation of the safety and efficacy of novel hemostatic agents and of different prophylaxis regimens in individuals with inherited bleeding disorders (focus the hemophilias) requires consistency in definitions of commonly used endpoints. The mandate of the Project was to perform a critical review of relevant information and to prepare definitions of primary and secondary prophylaxis, type of bleeds (joint, muscle, etc), response to treatment of an acute bleed, target joint and high/low responding inhibitors in the context of long-term factor replacement therapy in persons with hemophilia. Reaching a consensus for some definitions proved to be more challenging, for example, the cut-off between normal and a positive inhibitor level, where a value of \( \geq 0.5 \) Nijmegen-Bethesda Units (NBU) was thought to represent a positive test result. Inhibitors were considered transient if they disappeared within six months of first appearance despite antigenic re-challenge. Another area, still unresolved, is the consensus definition of primary prophylaxis. The current proposal is “regular continuous* treatment started before the onset of osteochondral joint disease, determined by physical examination or imaging studies, and before or immediately following the first clinically evident bleed into an index (ankle, knee or elbow) joint”. Finally, the chosen definition of joint bleed, a commonly used endpoint in clinical trials of new factor concentrates or prophylaxis regimens was “an episode characterized by rapid loss of range of motion as compared to baseline that is associated with some combination of the following: pain or an unusual sensation in the joint, palpable swelling and warmth of the skin over the joint”. Response to treatment should therefore be defined as the resolution of the most prominent feature that defined the bleed. The proposed definitions will be posted on the ISTH website for broad review and comment. Final revisions will then be made to the definitions prior to submission as a report of the SSC subcommittee for consideration for posting on the SSC website and publication in the Journal of Thrombosis and Hemostasis.

*Continuous is defined as the intent of treating for 52 weeks/year and receiving a minimum of 46 treatments/year.

During the presentation the author poses the problem of the definition of “severe” haemophilia A based on laboratory phenotype and wonders whether a fourth class of “moderately severe” needs to be added to the currently used classification. Suggestions were also sought for the content of the draft published on the web regarding results of the Project.

Anthony Hubbard - Report of project on potency labelling of clotting factor concentrates (Hubbard, M. Weinstein, R. Seitz, J. Dodt, T. Lee, A. Srivastava)
A Project has been established to investigate harmonisation of the potency labelling of clotting factor concentrates, focussing on factor VIII and factor IX, which are licensed for different markets. The primary objective is to recommend procedures which will ensure equivalent vial contents for products licensed in different countries and the Project membership includes representatives of licensing authorities from both Europe and North America. New products will challenge the current concept where all product are potency labeling with IU. Strategies leading to a common approach in potency labelling, for both conventional and modified products, will be explored taking into account similar initiatives by other organisations including the European Medicines Agency and the European Pharmacopoeia. A second objective of the Project is to provide recommendations for the potency estimation of clinical samples in the assessment of recovery after infusion of concentrates. In connection with the latter objective a survey has been initiated to review current practice in clinical laboratories. The possibility to use global assays to test potency labeling has been considered, but several cons vs pros need to be evaluated. An algorithm to define potency labeling of new products has been developed. Post infusion testing has the same problems as potency labelling, but remains more confusing: do laboratories use standard to test a sample? A survey was sent to more than 20 labs: only 6 answered.

DISCUSSION: how can we list problems and give priorities to solving them? How can we make comparisons when there is no standard? The most important thing is that information on new product pre-licensure is not available to the community. Manufacturers could be involved in in vitro assays in collaboration with SSC. The project group will also attempt to finalize its recommendation over the next 1 year.


During prophylactic treatment of haemophilia A there is a relationship between exposure (plasma level as function of time) to FVIII and reduction of the risk of bleeding. The achieved exposure after a given dose depends on the pharmacokinetics (PK) of FVIII in the patient. Variance in FVIII PK between patients thus contributes to variance in treatment response and/or FVIII dose requirement. Defining prophylaxis as keeping the plasma level above 1% of normal at all times is an oversimplification. Despite this, PK, in conjunction with evaluation of clinical outcome, is a tool for individualizing dosing of FVIII. With the future introduction of long half-life FVIII analogues, PK considerations may also become increasingly important for rational dosing. Proof of concept has been presented for the use of sparse blood sampling and Bayesian analysis for measuring PK in clinical practice and using this information for dose tailoring, potentially making prophylaxis more cost-effective. The user-friendly TCIWorks (www.tciworks.info) software for Bayesian analysis is freely available for download and has been evaluated for FVIII PK. The Project aims to present a guideline publication that will provide a description of recommended blood sampling schemes as well as of the methodology and application of the Bayesian PK analysis. This publication will also provide necessary input information for practical use of the TCIWorks program.

DISCUSSION: The author has been asked to estimate the time needed for the publication of the paper? The author explained that it is going to need some time since the Advate data need to be
accepted for publication and TCIWorks version 2.0 needs to be available and following that they
could make their recommendations.

Section 5. STANDARDIZATION ISSUES
Chairpersons: L. Valentino (USA) and J. Oldenburg (Germany)

Steve Kitchen - Monitoring recombinant FVIII concentrates and role of ReFacto AF laboratory standard

Three samples from moderate/severe haemophilia A patients each after treatment with a different
FVIII concentrate - ReFacto AF, Kogenate (FS) or Advate - were sent to 57 UK haemophilia
centres who determined FVIII in one stage assays (n = 46) or chromogenic assays (n = 10
including 5 different kits). One stage and chromogenic assays were calibrated with a plasma
standard or recalibrated with the ReFacto AF lab standard. Taking all results together,
irrespective of local assay reagents used, chromogenic assays gave significantly greater results
(p<0.0001, 32% difference) in the post Kogenate sample but not in the post ReFacto AF 11%
higher by chromogenic assay) or post Advate samples (3% lower by chromogenic). 15 centres
used APTT reagents (Synthasil)/deficient plasma/reference plasma from Instrumentation
Laboratory in the one stage assay and 20 used all Siemens reagents (Actin FS as APTT reagent).
This made a significant difference to results post ReFacto AF (41% higher by IL reagents,
p<0.0001) and Advate (39% higher by IL reagents, p<0.0001), but not Kogenate (7% higher by
IL, ns). In this study use of the ReFacto AF Lab standard was therefore required for one stage
assays to be in agreement with chromogenic when one stage assays were performed using
Siemens reagents but not when IL reagents were used. Differences between results obtained with
different one stage assay reagents for monitoring Advate have implications for dosing patients, if
confirmed. Several different chromogenic assays were used by participating centres and the CV
of chromogenic results was high, so more data on different chromogenic and one stage assays to
monitor Kogenate are required.

Benny Sorensen - Standardization of low TF and TF+tPA assays for TEG/ROTEM

Establish a recommended method to perform TEG/ROTEM in haemophilia. Variations of the
method are reported in literature, hence a review of the literature is highly needed, to outline
similarities and differences.

Method based on continuous measurement of physical changes during blood coagulation. Ten
papers on 122 patients with haemophilia: TEG/ROTEM can be performed with minute amounts
of TF. Dilution of Innovin causes variable results and variation batch constitute a serious
challenge to standardization.

Results: Clotting time has limited dose response while maximum velocity has dose dependent
response. TEG/ROTEM needs to be developed in the area of hemophilia to evaluate adjunction
of therapy.

The Project has established a list of reagents specification for the method and is currently
evaluating different batches with internal validation.
Future: collaborate with the recently formed NIH working-group on global assays with the first aim to develop a business plan and establish a group of collaborators

DISCUSSION:

- Due to high cost of reagents, will manufacturers continue to provide reagents? The author is continuously negotiating with them, but collaboration is not always certain.
- Be aware that by using Corn Trypsin Inhibitor, the sample cannot be re-used, so collection needs to be done with a specific procedure. But is it required to collect sample in CTI? Yes, otherwise response could be underestimated.
- Dr. Sorensen committed on behalf of the project group that he will work towards providing a standardization document by the end of the year so that labs around the world could attempt to perform this test will lesser variables.

Sanj Raut - New approaches in FVIII inhibitor measurement and standardization

Previous inhibitor studies have shown high variability in measurement of FVIII Inhibitors between laboratories with CVs ranging from 40-200%. The classical Bethesda Assay developed by Kasper et al in 1975 and is still the most widely used inhibitor assay. This assay requires the inhibitor patient’s plasma sample to be incubated with an equal volume of normal pooled plasma, the “Test Mixture”, at 37°C for 2 hours, after which the residual FVIII activity is measured and expressed as a % of the FVIII in a “Control Mixture” (consisting of equal volumes of normal pooled plasma and buffer) which had undergone the same incubation conditions as the “Test Mixture”. An inhibitor titre is then calculated in Bethesda Units (BU) per ml using the % residual FVIII:C based on the definition of the BU, which is that amount of inhibitor that would neutralise 50% of the FVIII:C in normal plasma in a 2 hour period at 37°C. More recently, Verbruggen et al, had modified the assay by introducing buffering of normal plasma pool and using FVIII-deficient plasma in place of the buffer in the “Control Mixture” to give a better specificity. However, recent inhibitor surveys revealed that the inter-laboratory variability still remained unacceptably high.

Our laboratory questioned the need for FVIII deficient plasma, which was introduced into the assay as a like-for-like diluent for the Reference “Control”. Ideally we would like to see identical mixtures in both the Test & Reference “Control” mixtures. However, we hypothesised that by introducing the FVIII-deficient plasma we were actually introducing a variant in the assay and that this variability can be exacerbated by the many different FVIII-deficient plasmas now commercially available. More importantly, as the Inhibitor titre is based on % of FVIII in the Reference (“Control”), we should be able to replace the FVIII-deficient plasma with a more like-for-like diluent such as Buffered Normal Plasma. The unknown inhibitor titre in this assay (South Mimms Inhibitor Assay - SMIA) would now be expressed relative to 200% FVIII in the Reference “Control” (rather than 100% FVIII previously). Furthermore, this approach would remove the variant; and reduce a significant step in the inhibitor assay, but more importantly, significantly reduce the cost of an inhibitor assay.
Surrogate inhibitor patient samples were developed by spiking A/FVIII neutralising MAbs & polyclonal antibodies into FVIII-deficient plasma at a range of different concentrations. The above hypothesis was then tested by comparing Nijmegen inhibitor assays with the SMIA test using these samples. Results showed that, for high titre samples (40 - 5 BU/ml), equivocal inhibitor titres could be obtained using the 2 methods. For low titre (1.0 - 0.15 BU/ml) samples, Nijmegen assays were able to detect inhibitor titres down to ~0.6 BU/ml, whilst SMIA tests were able to detect inhibitor titres down to ~0.2 BU/ml.

SMIA tests can produce equivocal results, without the need of FVIII-deficient plasma and is more sensitive to low titres (up to ~0.2 BU/ml) than compared to the Nijmegen assay. SMIA will have a significant assay step removed from the assay, which will significantly reduce its cost. It will be a very simple and a welcome modification to the assay by clinical testing laboratories. It was therefore proposed that a collaborative study be carried out, on behalf of the FVIII/FIX subcommittee, to assess the inter-laboratory variability and sensitivity of this assay, compared to the Nijmegen assay.

Steve Kitchen - Standardisation of assays for rare bleeding disorders: data from UK NEQAS

There continues to be some variability in the detection rate of abnormalities when different PT and APTT reagents are used for initial screening tests. For a sample with FXI of 25 IU/dl 30/750 (4%) of centres participating in a UK NEQAS survey reported a normal APTT, whilst a further 80 (11%) considered their results to be borderline. Detection of a 23 IU/dl level of factor VII was better, with 94% of centres reporting abnormal PT results. The variability of factor assays for FII, V, VII, X and XI in 260-290 participating centres was similar to that observed for FVIII and IX assays. In all case this variability was greater when factor levels were lower, as indicated by greater CVs, which were in the range 9 -17% at normal factor levels, and 13 to 26% when factor levels were in the 20-30 IU/dl range. Typically centres obtain PT/APTT reagents, reference plasmas and deficient plasmas to construct assays from the same commercial sources. Comparing use of different reagent sets shows that there are often 10 to 20 % differences between results obtained in factor assays. Finally in one survey of assay design 1/3 of centre use only a single test dilution and had more than double the variability of centres using 2 or 3 dilutions (as indicated by between centre CV).

DISCUSSION: since the data are coming from immunodepleted plasma of patients with FXIII deficiency. Flora Peyvandi offered collaboration in order to have data on plasma of congenital FXIII deficient patients with very low levels of FXIII. The role of blanking should also be mentioned.

Closing remarks – Flora Peyvandi

In her closing remarks, the chair thanked all the co-chairs, speakers and attendees for their participation and closed the meeting at 17.50 on the 23rd of July, 2011.

The chair asks the attendees to have a look to the recommendation published on the web; all new suggestions will be very much appreciated.
Factor XI and the Contact System

24 July 2011

Chairman: Thomas Renne (SE)
Co-chairs: David Gailani (US), Keith McCrae (US)

Factor XI and the Contact System, 24. July 2011, 8:00 am, Room A

This was the first SSC session of the Factor XI and the Contact System subcommittee that was previously entitled as the Plasma Kallikrein Kinin-System after having changed name. Similarly to the previous meetings in Boston and Cairo the session was very well attended with up to 210 participants and had lively and constructive discussions. The session was divided into an educational portion with more review type presentations and scientific presentations showing latest not yet published results in the field.

Educational Session

Dr. Hideiko Saito, Nagoja, Japan: In a highly stimulating lecture Dr. Saito presented original data and classical patient histories leading to the identification, characterization and purification of Fletcher factor (now known as plasmakallikrein) and Fitzgerad factor (High-molecular weight kininogen). He also showed original functional in vivo data that characterized the proinflammatory activity of the kallikrein kinin-system in vivo and the importance of the system for leukocyte recruitment and increase of vascular leak.

Dr. James H. Morrissey, Urbana, USA: Starting from his original publication (Smith S. et al., PNAS 2006) Dr. Morrissey reviewed the current knowledge on synthesis, expression, and biochemistry including half life etc. of polyphosphates that have recently been recognized as the endogenous activator of factor XII. He showed functions of the inorganic polymer that is enriched in intracellular vesicles called acidocalcisomes in bacteria. In addition to activating factor XII, polyphosphates propagate factor V activation and interfere with fibrinolysis in a TAFI-dependent manner. Dr. Morrissey concluded his presentation reporting recent data from his laboratory showing polyphosphate-driven factor XI activation that involves increased autoactivation and alpha-thrombin-driven activation of factor XI.

Dr. Thomas Renné, Stockholm, Sweden: He reviewed papers showing activation of the contact system by naturally occurring and synthetic negatively charged polysaccharides such as heparin and dextran sulfate, respectively. Dr. Renné presented data from his group showing a role of mast cell-released heparin for anaphylactic reaction and edema in vivo that were published recently (Oschatz et al., Immunity 2011). Mast cell triggered activation of the plasma contact system resulting in bradykinin formation seems of critical importance for triggering the onset of swellings in hereditary angioedema patients. Similarly to mast cell heparin, contaminants in heparins such as oversulfated chondroitin sulfate trigger contact system activation and account for severe anaphylactic side effects and life threatening reactions of some heparin preparations in 2007-08.
Dr. Joost C. M. Meijers, Amsterdam, The Netherlands: Dr. Meijers introduced structure and biochemical characterization of factor XI. He reviewed the importance of the factor for thrombosis in genetically altered mouse models and in patients with inherited factor deficiency. Based on the critical role of factor XI for thrombosis an antisense-based strategy using ASO was developed to interfere with factor XI expression. Dr. Meijers showed protection from thrombosis in various models and vascular beds in ASO-treated mice that was not associated with increased bleeding. Ongoing work indicated that targeting factor XI in baboons in using ASO provides thromboprotection that is not associated with abnormal increased bleeding.

Dr. Erik Tucker, Portland, USA: The topic of Dr. Tucker’s presentation was the role of factor XI and the contact system for sepsis. He showed that factor XI homozygous gene deficient mice and wildtype animals treated with a factor XI-blocking antibody (14E11) had improved survival and reduced coagulopathy in a polymicrobial sepsis models (CLP) and Listeriosis/Gram-positive bacteria-infection models. In contrast factor XI appeared of minor importance in LPS-induced endotoxemia models. He concluded his presentation showing preliminary results with 14E11 in baboon thrombosis and sepsis models.

Dr. Alvin H. Schmaier, Cleveland, USA: Dr. Schmaier presented a comprehensive overview about the functions of factor XII zymogen form that were generated in his laboratory in the recent years. He showed factor XII binding to cell surfaces involves a multi-protein acceptor complex involving the urokinase receptor. Factor XII zymogen stimulates cell proliferation by ERK1/2- and Akt-dependent pathways. Beta1-integrins seem to have a role in factor XII-driven signalling events in endothelial cells. Dr. Schmaier showed a role of factor XII for angiogenesis in ex vivo assays and correspondingly defective neoangiogenesis in factor XII gene deficient mice. Preliminary data from ongoing experiments suggest a role of factor XII for macrophage migration and wound healing that is independent on the enzymatic activity of the protease and not mediated by bradykinin.

Dr. José Govers-Riemslag, Maastricht, The Netherlands: Dr. Govers-Riemslag showed a comprehensive overview on the role of activated factor XII for thrombin formation and especially thrombus stability. Her central information was that clots that were produced in the presence of active factor XII are more stable and more resistant to fibrinolyses. She showed that the formation of the tighter clots is a direct effect of FXIIa on fibrin formation, independent of additional thrombin formation. She used a set of state-of-the-art ex vivo technology to analyze the effect of factors XII on fibrin clot structure including ex vivo clot lysisassays, microscopy and for elasticity of the clot magnetic tweezers.

Dr. Owen McCarty, Portland, USA: Dr. McCarty presented recent data and ongoing research from his laboratory dealing with the interaction of factor XI with neutrophils and the importance of the protein for signalling cascades in these cells. He showed that active factor XI interferes with oxidative stress-driven pathways in PMN. Using flow chamber real time analysis he analyzed roles of factor XI for PMN migration. Identification and characterization of the structures and receptors involved in factor XI binding to PMNs remain a major challenge in his field.
Dr. Uri Seligsohn, Tel Aviv, Israel: Dr. Seligsohn presented an update on the clinical functions of factor XI. The first part of his clear presentation dealt with the observation that bleeding tendency in factor XI deficient individuals from Israel was not related to factor XI antigen plasma levels. He then compared incidence, clinical presentation, genetics and epidemiology of the three types of hereditary factor XI deficiency (types I, II, III). Dr. Seligsohn concluded his presentation showing that severe factor XI deficiency protects from DVT and ischemic stroke but not from MI. Mechanisms and differences in factor XI-driven procoagulant activities in these distinct vascular beds were discussed.

End of the meeting: 12:10.
Factor XIII and Fibrinogen

23 July 2011

Chairman: Hans P. Kohler (CH)
Co-Chairmen: Moniek P. M. de Maat (NL), Aida Inbal (IL), Muriel Maurer (US), Leonid Medved (US), Marguerite Neerman-Arbez (CH), John W. Weisel (US), Sanj Raut (UK)

Educational Program

Dr Moniek de Maat (NL) gave a comprehensive overview on fibrinogen variants which arise from either genetic mutations/polymorphisms or protein changes due to proteolytic degradation, alternative splicing or posttranslational modifications, and their implications for cardiovascular disease were emphasised.

Dr Hans Kohler (CH) reviewed congenital and acquired FXIII deficiencies. He summarised the available diagnostic tests and pointed out their pitfalls, especially in the low-activity range. The clinical relevance of low FXIII levels was also discussed, in particular the relevance of heterozygous FXIII deficiency which is not well understood.

SSC Program

Dr Sanj Raut (UK) presented the results from two international collaborative studies for the value assignments of the 3rd International Standard for fibrinogen, plasma, and the 2nd International Standard for fibrinogen, concentrate. Over 20 labs took part in these studies. Inter-lab variability was low with results showing tight distribution. Fibrinogen potencies for the candidate materials were proposed and the proposals were discussed.

Dr Shirley Uitte de Willige (NL) summarised the current knowledge on the alternative splicing variant of the fibrinogen γ-chain (γ’). Fibrinogen γ’ has been shown to influence thrombotic risk, bind thrombin and FXIII, inhibit platelet aggregation, and influence clot structure. Novel findings on γ’ ratio in arterial and venous plasma samples from patients with coronary artery disease and controls free from CAD, both proven by angiography, were also presented.

Dr Tatiana Ugarova (USA) presented new findings on non-adhesive properties of fibrinogen matrices. In contrast to fibrinogen monolayers, fibrinogen multilayers (6-7 layers) exhibit low adhesion of platelets and leukocytes due to weakened integrin-mediated mechanotransduction. This may have implications for thrombosis/haemostasis and biomaterial science.

Dr Barbara Cardinali (USA) presented new insight on the concerted action of fibrinogen and FXIII activation by thrombin. Release of fibrinopeptides A and B and FXIII activation peptide were monitored over time and the influence of fibrinogen variants was investigated. A surprising finding was that FXIII activation proceeded faster in the presence of γ’/γ’ fibrinogen compared with γA/γA fibrinogen.
Dr Aida Inbal (IL) showed the results from the first phase 3 clinical trial on recombinant FXIIIA in patients with congenital FXIII deficiency. Forty one patients aged 7-60 were enrolled and 33 completed the study. The monthly dose was 35 IU/kg. No spontaneous bleeding was observed and the bleeding rate due trauma was significantly reduced. Four patients developed non-neutralising transient antibodies without any clinical consequences. Thus, rFXIII proves to be efficacious and safe.

Dr Lene Hørlyck (DK) gave more information on the production and characterisation of rFXIIIA. It is expressed in yeast and purified by several chromatographic steps resulting in a highly purified protein without any blood or tissue contaminants. Its native conformation and intact structure and function have been confirmed. Non-clinical and clinical trials have been successfully conducted and approval by the authorities is expected soon. Thus, the long-awaited first rFXIII product will be available to patients.

Dr Hans Kohler (CH) and Dr Sanj Raut (UK) presented a proposal for value assignment for FXIII B-subunit (total and free B) to the WHO 1st International Standard FXIII Plasma (02/206). It is important to establish the level of total and free FXIIIIB as the half-life of FXIIIA depends on the amount of available FXIIIIB. In addition, free FXIIIIB may have so far unknown functions in binding and regulating FXIII and other plasma proteins, and FXIII substitution therapy with rFXIIIA also relies on FXIIIIB. It was proposed to proceed as follows: 1.) Set up of ELISA methods using antibodies specific for free and bound FXIIIIB, 2.) collaborative study involving laboratories including manufacturers, clinical labs and control labs to compare potency values calculated against locally collected plasma pools for calibration of the current WHO 1st IS FXIII Plasma, 3.) to submit the report to FXIII and Fibrinogen SSC Subcommittee, WHO-ISTH/SSC Liaison Group, and WHO/ECBS for endorsement/approval and value assignment. This proposal was well received and FXIII experts strongly agreed on the importance of this project. In the discussion the following points were raised: Dr Diane Nugent recommended to confirm by immunoblot that all free B is aspirated when using the indirect assay principle with aspiration after binding to anti-tetramer antibody. However, this assay principle will only be used in case no antibody specific to free B is available. Dr Laszlo Muszbek emphasised the importance of expressing FXIIIIB concentration as mass concentration, as this is vital to estimate the true amount of free B for complex formation. Dr Akitada Ichinose also pointed out the need for FXIIIIB standardisation in regard to diagnosis and treatment of B-subunit deficiencies, as this condition is probably underdiagnosed. These important points will be taken into consideration for the final project outline.

Dr Sanj Raut (UK) presented the results of an international collaborative study to calibrate the ISTH/SSC secondary plasma standard lot 4. The aim of this study was to value assign functional FXIII activity and antigen (A2B2). Twenty labs participated in this study. The results showed good agreement between labs. Potencies of 0.76 IU/ml (activity) and 0.74 IU/ml (antigen) were proposed.

Dr Laszlo Muszbek (HU) presented novel findings on the interaction of FXIII subunits in plasma and other body fluids. The aims of this work were 1.) to establish binding parameters for the interaction of A and B subunits and 2.) to investigate whether there is free A in plasma and other body fluids. Binding studies were performed by ELISA and surface plasmon resonance. Results
of both methods consistently gave KD values around 10-10 M. Furthermore, it was shown that a small percentage of plasma FXIII A-subunit exists in non-complexed free form, while in cerebrospinal fluid and tears most A-subunit is free. Free A2 in plasma is in inactive form but can be activated by thrombin. This work revealed important new insights into basic FXIII biochemistry and physiology.

Dr Akitada Ichinose (JP) gave an update on the nationwide study on acquired FXIII deficiency. He addressed issues regarding nomenclature of acquired FXIII deficiency and proposed to distinguish between acquired deficiencies due to inhibitors or due to other causes including consumption. In the Japanese study, 26 patients with acquired deficiency due to inhibitors have been enrolled so far. Most patients had developed antibodies against FXIII A-subunit, only 3 patients had antibodies against B-subunit. The latter condition presents difficulties in diagnosis, as it tends to be overlooked or misjudged as thrombocytopenia. Clearly, more work is needed on acquired FXIII deficiencies.
Fibrinolysis

24 July 2011

Chairman: Ann Gils (BE)
Co-chairmen: Jonathan Foley (US), Dirk Hendriks (BE), Colin Longstaff (UK), Osamu Matsuo (JP), Nicola Mutch (UK), Michael Nesheim (CA), Craig Thelwell (UK), Tetsumei Urano (JP)

Educational session:
The SSC meeting started off with an educational session on the clinical significance and therapeutic opportunities to target PAI-1, TAFI and α2-antiplasmin.

PAI-1: Clinical significance and therapeutic opportunities. Ann Gils (BE)
Ann Gils gave a short overview on the basic characteristics of Plasminogen Activator Inhibitor-1 (PAI-1), the different conformations of PAI-1 and its role in fibrinolysis. She cited a number of papers to demonstrate the physiological relevance of PAI-1. The focus of the presentation was on potential therapeutic molecules that target PAI-1. First, she showed a number of in vivo studies in which highly specific antibodies towards PAI-1 were used. Subsequently, she cited a number of in vivo studies in which either Tiplaxtinin, S35225 or TM5275 were used.

Is TAFI a target? John Morser (US)
Thrombin Activatable Fibrinolysis Inhibitor (TAI) deficient mouse in models of abdominal aortic aneurysm, airway hypersensitivity and rheumatoid arthritis have shown that lack of TAFI has significant deleterious effects. In the latter two models the substrate for TAFIa was shown to be complement factor 5a (C5a) and not fibrin. In rheumatoid arthritis patients progression of the disease correlates with the SNP at nucleotide 1040 that encodes amino acid 325. In mice that were deficient in both TAFI and apoE that had been treated with streptozotocin developed tumors while singly deficient mice did not. John Morser demonstrated that although TAFI has been validated as a target, chronic use of a TAFI inhibitor could be problematic because of the effects in these models. John also suggested to rename TAFI to CPB2. The SSC subcommittee will consider this suggestion.

Alpha2-antiplasmin: The role of Alpha2-antiplasmin on the development of fibrosis.
Yosuke Kanno (JP)
Dr. Kanno first gave an introduction on the characteristics of fibrotic disease. Fibrotic disease represents one of the largest groups of disorders for which there is no effective therapy. Some studies have shown that levels of plasmin-α2AP complex in plasma are elevated in fibrotic diseases. However, the roles of α2AP in fibrotic diseases are still not understood. He examined the experimental fibrosis in mice with a deficiency of α2AP by using bleomycin and found that the α2AP deficiency attenuated bleomycin-induced profibrotic proteins, TGF-α production and the development of fibrosis. In addition, he found that α2AP induced TGF-α expression and the development of fibrosis through PEDF-R binding and iPLA2 activation. Regulation of the α2AP expression may be an effective antifibrotic therapy.
SSC session

EQA for point of care D-dimer measurements. Ian Jennings (UK)
Point of Care testing (POCt) is becoming widely used in many countries, including the UK, and over 3000 centres now participate in the UK NEQAS POCt programme for INR measurement. Ian Jennings has also seen increasing interest in, and use of, point of care systems for D-Dimer testing, and a pilot EQA programme has been established for two of the point of care methods. In house studies have shown good correlation of lyophilised EQA material between point of care and laboratory-based D-Dimer methods. To date, 4 pilot studies have shown centres using the same point of care method do not agree on the result or the interpretation obtained on the same sample. This has implications in the use of these methods for VTE exclusion, and highlights the need for D-Dimer standardisation and EQA for point of care testing.

Update on WHO international Standards: Urokinase, PAI-1 and D-dimer. Colin Longstaff (UK)
A project to replace the current WHO IS for High Molecular Weight Urokinase
The existing WHO IS for High Molecular Weight Urokinase (87/594) is becoming depleted and a replacement is needed. The project to make a replacement IS has been endorsed by the WHO Expert Committee on Biological Standardisation (ECBS) and introduced previously to the Fibrinolysis Subcommittee of the SSC. Material has now been sourced from a manufacturer, trial fills completed and methods validated. The schedule for replacement involves a definitive fill of around 5000 ampoules in September 2011 and a collaborative study beginning in November 2011. A report of the study will be presented to the SSC in 2012 and it is expected that the new IS will be approved at the WHO/ECBS in October 2012. Additional participants are urgently needed to take part in the collaborative study.

Quantitation of PAI-1 Antigen in Plasma
A collaborative study was performed in 2007 which compared a number of different methods, both commercial and non-commercial ELISAs, for the determination of PAI-1 antigen in 5 plasma pools sent to a group of 12 laboratories. Results showed that the different methods gave widely different values for PAI-1 antigen although ranking of the pools was generally good and a method was proposed which allowed harmonisation of results from different methods onto a common scale. However, consensus values for ng/ml PAI-1 antigen in plasma are not acceptable and work since the original study has focussed on establishing a real gravimetric value for PAI-1 antigen in 1 or more of the original plasma pools in the study. Biacore methods have been explored and seem to have the required sensitivity but have shown up some problems that may help explain the variability in ELISA methods. Recent work has focussed on a proteomics approach which involves trypsin digestion of proteins in plasma and quantitation of peptides unique to PAI-1 and using synthetic C-13 labelled peptides as calibrators. Initial results are promising.

A project to develop an IS for D-dimer in plasma
A project group has been established, connected with the Fibrinolysis Subcommittee of the SSC with the aim of developing an IS for D-dimer in plasma. The core project group is Colin Longstaff (UK), Carl-Erik Dempfle (De), Ian Jennings (UK), Steve Kitchen (UK), Piet Meijer
Several other SSC subcommittees have also expressed interest in the project. The project to develop an IS has also been approved by the ECBS at WHO. A pool of 280 ml of small pools of patient plasma with high levels of D-dimer has been collected and a trial fill performed where 0.5 ml aliquots were freeze dried to give 519 ampoules. Some of these are currently being investigated in accelerated degradation trials to assess the long term stability of this type of preparation. A further collaborative study is planned in 2011/12 to explore the usefulness of this batch of trial filled material as a standard in assays performed in a number of laboratories along with some patient samples. If this study is successful more D-dimer plasma will be collected to prepare a larger volume and several thousand ampoules of a candidate IS. Further studies are also envisaged to characterise the material in this preparation to help with the future replacement strategy of an IS for D-dimer in plasma.

**PAI-1 deficiency-Eugloblin clot lysis assay. Tetsu Urano (JP)**

Dr. Urano reported the identification of two independent PAI-1 deficient patients. The two patients had distinct gene abnormalities. Their ECLT were very short, and more importantly, he did not see a calcium-dependent shortening of ECLT, in which inactivation of PAI-1 plays an important role, in their plasma. Since undetectable plasma levels of PAI-1 may not be enough to suspect PAI-1 deficiency, such supportive data obtained by functional assay were helpful to move on to genetic analysis.

**Microplasmin degrades fibrinogen like plasmin but fibrin degradation is impaired. Paul Y. Kim and Jeffrey I. Weitz (Ca)**

Plasmin (Lys?Pn) and microplasmin (Micro?Pn) are being used for catheter-directed fibrinolytic therapy. Unlike Lys?Pn, however, Micro?Pn consists only of the protease domain and lacks all five kringle domains. Micro?Pn has been a molecule of interest for fibrinolytic therapy as its rate of inhibition by α2-AP is 2 orders of magnitude slower than Lys?Pn, leading to an increased half-life. In addition to degrading fibrin (Fn), both agents degrade fibrinogen (Fg). This suggests that kringle?mediated interactions with Fg or Fn are not essential for efficient degradation. Paul Kim's data suggests that kringle domains are important for efficient Pn?mediated degradation of Fn, but not Fg. Additionally, kringle domains are more important for Pn?mediated degradation of the β? and γ?chains of Fn or Fg than the α?chains. He concluded that if used for catheter directed fibrinolytic therapy, Micro?Pn may produce significant Fg degradation, which could decrease hemostatic potential.

**The kinetics of TAFIa catalyzed cleavage of C-terminal lysine residues from fibrin degradation products and removal of plasminogen binding sites. Jonathan Foley (CA)**

Partial digestion of fibrin by plasmin exposes C-terminal lysine residues, which comprise new binding sites for both plasminogen and tissue-type plasminogen activator (tPA). This binding increases the catalytic efficiency of plasminogen activation by 3000-fold compared with tPA alone. The activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates fibrinolysis by removing these residues, which causes a 97% reduction in tPA catalytic efficiency. Jonathan Foley studied the kinetics of TAFIa-catalyzed lysine cleavage from fibrin degradation products and the kinetics of loss of plasminogen-binding sites. He showed that TAFIa binds both Glu-Pg and Lys-Pg.

**In vitro evaluation of profibrinolytic properties of TAFI and PAI-1 inhibitors. Tine**
Ann Gils gave an overview of methods for evaluating the profibrinolytic effect of TAFI and PAI-1 inhibitors in vitro and elaborates specifically on the experimental setup of the in vitro clot lysis assay and thromboelastographic measurements using citrated plasma and whole blood, respectively. Based on examples, the challenges of the in vitro clot lysis assay are discussed, such as buffer effects, species-dependency and pool-to-pool variability. This presentation also shows our experience on the evaluation of TAFI or PAI-1 inhibitors using thromboelastographic measurements.

Taiichiro Seki (JP)
Taiichiro Seki investigated the role of TAFI in liver regeneration focusing on hepatocyte survival and death using TAFI-gene silenced primary hepatocytes. TAFI siRNA suppressed TAFI mRNA expression in primary hepatocytes by 90% of that in control hepatocytes. Apoptosis was induced in control hepatocytes during primary culture; however, it was reduced in TAFI-silenced hepatocytes. Cleaved caspase-3, which is an executioner at the downstream of apoptosis, was decreased in TAFI-silenced hepatocytes in comparison with control-hepatocytes. Conversely, phosphorylated-Akt suppressing apoptosis and promoting cell survival and proliferation was increased in TAFI- silenced hepatocytes.

Nuala Booth (UK)
Different models reveal different aspects of the fibrinolytic system. Most studies are performed on plasma or fractions such as euglobulin. Plasma, either free of or containing platelets, can be studied in clot lysis assays. These systems show sensitivity to PAI-1, a2-antiplasmin and TAFIa and they give some insight into the relative contribution of each of the inhibitors. The Booth laboratory has used the Chandler loop to make model thrombi from whole blood, which show similar contributions of the major inhibitors. This system shows clearly that the protease activity is strongest at the thrombus head; where no proteases are added the main contributor is uPA. Using this system, even for plasma rather than whole blood, gives remarkable sensitivity to cross-linking by factor XIIIa, which cross-links active a2-antiplasmin to forming fibrin. The Chandler whole blood system has been extended by making thrombi on strips of porcine aorta, in the Badimon chamber, allowing a marked effect of shear to be observed.

Ellen Vercauteren (BE)
Ellen Vercauteren and colleagues set up a mouse thromboembolism model to evaluate TAFI inhibitors in vivo. In this well-characterized mouse thromboembolism model tissue factor injection led to (1) an increase of mice displaying abnormal physical activity or death, (2) obstruction of lung vessels by platelet-fibrin thrombi as shown in histological analyses, (3) fibrin deposition in the lungs as quantified by ELISA and (4) a massive systemic activation of coagulation that could not be counterbalanced by the fibrinolytic system as demonstrated by coagulation and fibrinolysis markers. However, administration of a TAFI inhibitor in this model resulted in (1) a decrease of mice displaying abnormal physical activity or death, (2) a reduced amount of platelet-fibrin thrombi, (3) a decreased fibrin deposition in the lungs and (4) an increase in plasmin generation indicative for an acceleration of fibrinolysis. In conclusion, this mouse thromboembolism model is an appropriate model to assess the profibrinolytic effect of TAFI inhibitors in vivo.
Alternative pathway for fibrinolysis: clinical significance and therapeutic opportunities, leukocyte elastase. Seiji Madoiwa (JP)
Seiji Madoiwa reported that plasma levels of cross-linked fibrin degradation products by leukocyte elastase (e-XDP) were significantly increased in patients with sepsis induced DIC.

Proteolytic and genetic variation of the alpha-2-antiplasmin C-terminus in myocardial infarction. Shirley Uitte de Willige (NL)
Shirley Uitte de Willige developed 2 new ELISAs to measure the antigen levels of free total α2AP and free C-terminally intact α2AP to investigate whether α2AP antigen levels or α2AP C-terminal cleavage were associated with myocardial infarction (MI) in 320 male MI survivors and 169 age-matched controls. Her data show that levels of free full-length α2AP were decreased in MI, that the percentage of C-terminally cleaved α2AP was unaltered, and that Arg407Lys did not influence α2AP levels or MI risk.
Mechanism of Coagulation in Tumour Progression and Metastasis

Janusz Rak, Canada

Dr. Janusz Rak (CA) spoke on the mechanisms of coagulation in tumor progression. Metastatic cancers emerge in the course of progressive biological changes hardwired in the cellular genome by a succession of oncogenic mutations. These changes not only affect the intrinsic properties of cancer (stem) cells, but also profoundly impact their interactions with the vascular system, through a multitude of proinflammatory, proangiogenic and procoagulant alterations, which are central to tumor dissemination. Several facets of the coagulation system participate in these events, including thrombin, fibrin, PAI-1, COX-2 and many others. However, tissue factor (TF) can be regarded as a paradigm, common denominator and a key effector of cancer-related vascular alterations. Indeed, the expression of several oncogenic proteins (e.g. K-ras, EGFR, HER2, MET, p53, PTEN and others) drives the upregulation of TF, as well as the abnormal expression of its key interacting molecular partners (FVII, PAR-1, PAR-2) in several types of cancer cells (e.g. in colorectal carcinoma or glioblastoma). This change leads to the exaggerated procoagulant and signaling activity of the TF/FVIIa pathway in transformed cells. Moreover, oncogenic pathways promote cellular vesiculation, whereby large numbers of TF-containing microparticles (microvesicles) are emitted from cancer cells into their surroundings, and may enter the systemic circulation, participate in cancer coagulopathy and mediate intercellular transfer of TF activity. Interestingly, tumor cell-derived microvesicles may also contain active oncoproteins and mediate propagation of a quasi-transformed cellular phenotype. These observations suggest that oncogenic transformation, acting in concert with microenvironmental and iatrogenic factors, may provoke a series of distinct changes in the haemostatic system of cancer patients, a process that could be biologically consequential regardless of the accompanying thrombosis. The prognostic and therapeutic implications of this linkage are a subject of active explorations.

Role of tissue factor and biomarkers: ready for prime time?

Jeffrey Zwicker (USA)
Dr. Zwicker spoke on the role of tissue factor as a potential biomarker for cancer-associated thrombosis. Although the association between cancer and thrombosis is well established, the acceptance of primary thromboprophylaxis strategies has been limited due to low overall event rates. A number of biomarkers are currently under investigation to identify those patients at highest risk including circulating tissue factor bearing microparticles, soluble p-selectin, platelet count and other markers of coagulation activation. A number of analytic issues remain and the utility of targeted thromboprophylaxis based on biomarker profiles or risk models requires investigation in prospective clinical trials.

Targeted Thromboprophylaxis Using Clinical Prediction Models

Alok A. Khorana (USA)

Dr. Khorana spoke on the role of clinical prediction models in identifying high-risk cancer patients who could potentially benefit from thromboprophylaxis. Although cancer patients are universally regarded as being at high risk for VTE, the risk varies between individual cancer patients and even in the same cancer patient over time. Multiple risk factors and biomarkers predictive of cancer-associated VTE are being evaluated. Dr. Khorana reviewed the evidence supporting the use of a clinical prediction model (Khorana et al, Blood 2008), including multiple validation studies performed in the past two years. He also reviewed data to support a proposed expansion of this model by the Vienna group (Ay et al Blood 2010) and from the PROTECHT study (Verso et al, ISTH 2011). Finally, a host of thromboprophylaxis studies have been recently reported in cancer outpatients and these data were reviewed as well.

Subcommittee Activity Updates

Dr. O’Connell updated the Subcommittee on issues related to the definition, prognosis and treatment of incidental VTE. The utilization of highly sensitive multi-row detector CT scans for staging malignancy in cancer patients has led to more frequent detection of incidental pulmonary emboli (PE). These PE are frequently symptomatic but unsuspected by the treating physician. While prospective data are lacking, it appears that incidental PE located proximal to the subsegmental branches of the pulmonary arterial tree adversely impact overall survival among cancer patients. Moreover, patients with PE-related symptoms have poorer outcomes when compared to those with incidental PE which are truly asymptomatic. Incidental subsegmental (SS) PE may be indicative of clot burden in other anatomic locations. While truly asymptomatic and isolated SS PE probably don't adversely impact survival, it is not clear whether they can be left untreated. A unified approach to classification and terminology is necessary in order to properly include incidentally detected PE in clinical trials which report VTE as outcomes or adverse events.

Dr. Ay updated the Subcommittee on the role of D-dimer in diagnosis of malignancy-associated VTE, prediction of VTE and prognosis of cancer patients, independent of VTE. D-dimer is a well known and frequently used biomarker, which indicates the global activation of the blood coagulation system. It is a degradation product of cross-linked fibrin, and is formed after thrombin-generated fibrin has been degraded by plasmin. Increased levels of D-dimer have been observed in many conditions, including, infection, inflammation, surgery, trauma, pregnancy and
cancer. As D-dimer levels are elevated after clot formation, the measurement of D-dimer is routinely used in conjunction with clinical parameters in the initial assessment of a suspected acute VTE. A few studies have investigated the usefulness of D-dimer testing for diagnosis of VTE in cancer patients. The sensitivity and negative predictive value (NPV) of a negative D-dimer for excluding the diagnosis of VTE have been found to be high and comparable to non-cancer patients. The specificity, however, is lower than in non-cancer patients. In the Vienna Cancer and Thrombosis Study (CATS), high levels of D-dimer have been associated with occurrence of VTE in cancer patients. Subsequent studies have confirmed the association of high levels of D-dimer with risk of future VTE in cancer patients. Therefore, D-dimer might be a potential biomarker for prediction of VTE and may improve risk assessment of VTE in cancer patients. Several studies have also investigated the association of activation of blood coagulation, as reflected by D-dimer, with prognosis of disease in cancer patients. The global activation of the haemostatic and fibrinolytic system has been reported to correlate with a more advanced tumor stage, unfavorable outcomes and a poor prognosis.

Dr. Carrier updated the Subcommittee on a project focusing on standardized definitions of VTE outcomes for oncology trials. The association of venous thromboembolism (VTE) and cancer therapy presents a challenge for recognition because many randomized controlled trials or clinical studies are not powered to reveal a significant relationship or safety concern. Systematic reviews and meta-analyses are required to assess the risk and have to use events reported using different “non-standard” definitions of VTE. Cancer clinical trials are using pre-established definitions from toxicity classification systems: 1) National Cancer Institute’s Common Toxicity Criteria; 2) WHO Criteria; or 3) National Cancer Institute Common Terminology Criteria for adverse events. These classification systems are widely adopted and used in cancer clinical trials and are used primarily to grade the severity of the event. None of the toxicity classification systems outline the diagnostic criteria for DVT or PE, nor the location or extent. All these VTE-associated factors are important to consider as they are associated with different risks of recurrent events, embolizations and prognosis. The primary objective of this ISTH Subcommittee activity is to propose a standardization of the symptomatic VTE definitions including the diagnostic criteria required to report a DVT or PE. Cancer clinical trials could then adequately report VTE and assess the risk and safety concerns. Secondary objectives are to suggest a standardized method for reporting symptomatic recurrent VTE and bleeding episodes.

Ongoing Studies and New Proposals

Dr. Khorana outlined ongoing studies that are evaluating tissue factor (TF) as a therapeutic target for cancer, independent of anticoagulant effect. Two drugs are currently in early phase studies. ALT-836 is an anti-TF monoclonal antibody that is currently being tested in a phase I study in advanced solid tumors, based on promising preclinical data suggesting inhibition of pancreatic and other cancers. PCI 27483 is a TF/VIIa inhibitor that has completed phase I testing and is currently in randomized phase II enrollment. Data from these studies should provide information regarding the potential of this novel anti-cancer approach.

Dr. Farge updated the activities of her proposal to establish VTE guidelines in cancer-associated thrombosis, with input from an international working group. Much work has already been done with support from the French INCa group with respect to infrastructure and methodology.
support. Dr. Farge outlined the systematic review and proposed grades of recommendation of these forthcoming guidelines.

Dr. Agnelli provided a perspective on new oral anticoagulants and potential use in oncology. Several agents are now available for use for a variety of non-cancer indications. However, caution needs to be exercised in relation to drug-drug interactions, compliance and monitoring. Cancer-specific studies are necessary because cancer patients comprise 10% or less of recent studies.

Updates on ongoing clinical trials were provided by several investigators. Dr. Carrier provided information regarding study design and enrollment for a) PERIOP-01, an extended duration perioperative LMWH study to evaluate recurrence-free survival in colorectal cancer, and b) SOME, a randomized study of screening strategies for occult cancer in patients presenting with unprovoked VTE. Dr. Meyer presented updated study enrollment for TILT, a randomized study of adjuvant LMWH in patients with resectable lung cancer which is three-fourths on the way to full enrollment. Dr. Kamphiusen updated the Subcommittee on a) CATCH, a trial of LMWH vs warfarin therapy for extended treatment of cancer-associated VTE which is currently in enrollment and b) LONGHEVA for post-6 month treatment of cancer-associated VTE. In this latter study, cancer patients who have received 6-12 months of anticoagulation for VTE will be randomly assigned to 6 additional months of LMWH or VKA (target INR 2.0-3.0). Allocation to treatment will be done centrally by block randomisation via a web-based system (concealed allocation) and will be stratified by center. The primary efficacy outcome is symptomatic recurrent VTE. The primary safety outcome is major bleeding.

Based on the above presentations, there was support that the subcommittee consider position papers on the following issues:

1. Establishing diagnostic criteria and guidance for incidental venous thromboembolism. This is important to improve the accuracy and consistency of reporting the incidence of this outcome in clinical studies.
2. Providing guidance on the role of D-dimer testing in diagnosis, prediction and prognosis of VTE in cancer.
3. Encouraging the inclusion of venous thromboembolism as a standard safety outcome in oncology trials of new agents. This could potentially involve collaborating with NCI and WHO to update existing definitions of VTE which are not reflective of widely accepted clinical criteria and hinder meta-analyses of oncology clinical trials to establish linkages between specific therapeutic agents and VTE.

The meeting was adjourned at 18:00.
Lupus Anticoagulant/Phospholipid-Dependent Antibodies

23 July 2011

Chairman: Thomas L Ortel (US)
Co-Chairmen: Tatsuya Atsumi (JP), Ph de Groot (NL), Bas de Laat (NL), Vittorio Pengo (IT), Jacob Rand (US), Guido Reber (CH), Armando Tripodi (IT)

The subcommittee meeting opened after the Educational Session with three primary areas for Discussion during the session.

Lupus Anticoagulant Testing. The first set of presentations discussed issues related to the Subcommittee Communication published in 2009 updating the recommendations for lupus anticoagulant testing (Pengo V, et al., J Thromb Haemost, 2009; 7: 1737-1740). Dr. Ian Jennings reported on compliance in the United Kingdom with the 2009 Guidelines by using a questionnaire to the UK NEQAS participants and a proficiency testing exercise circulated six months after the guidelines were published. They found several areas on non-compliance, including issues related to pre-analytical (citrate concentration; double-spinning plasma); analytical (test selection and determination of cut-off values); and post-test (lack of test interpretation) stages. Proficiency testing found excellent accuracy for LA negative and strongly positive plasma samples, limitations with “weakly” positive samples.

Dr. Piet Miejer followed Dr. Jennings with a presentation on compliance with the Guidelines based on data collected from the ECAT surveys. He found that laboratory diagnostic accuracy did not appear to improve with an increasing number of tests for lupus anticoagulants being performed. He also noted considerable heterogeneity in the results obtained from mixing studies. He also presented data on the problems that clinical laboratories have with ‘borderline’ results, with poor correlation for consistently obtaining the same result when testing the same plasma sample (up to 15% of laboratories resulted a sample as negative on one analysis, yet positive on a second analysis!). The question this raised is whether there might be problems related to the definition of the cutoff values for negative vs. positive results.

Dr. Katrien Devreese presented the last talk in this section, reviewing her data on the importance of mixing studies in the diagnosis of lupus anticoagulants. In particular, the issue was whether integrated tests, which do not include a mixing step, are acceptable. She found that the mixing study was actually quite important to include, even with an integrated sample, to help distinguish samples with factor deficiencies from samples with a lupus anticoagulant.

Anticardiolipin and Anti-β2-glycoprotein I Antibody Standards. These discussions were in follow-up to presentations at last year’s meeting in Cairo, with a primary focus on developing appropriate standards and calibrators to improve diagnostic results obtained by the ELISA-based assays for phospholipid-dependent antibodies.

Dr. Silvia Pierangeli summarized final results from the 13th International Congress on Antiphospholipid Antibodies, which was held in last year in Galveston, TX. The wet workshop at this conference compared polyclonal and monoclonal standards from several different sources,
using antiphospholipid antibody-positive and negative serum samples, and found limitations with the monoclonal preparations. Dr. Bas de Laat followed Dr. Pierangeli, updating the subcommittee on ongoing efforts to develop anti-\(\gamma\)-2glycoprotein I domain 1-specific monoclonal antibodies that could be used as potential tools for standardizing anti-\(\gamma\)-2-glycoprotein I ELISA results. Critical issues included conformation of the immobilized \(\gamma\)-2-glycoprotein I. Dr. de Laat proposed a project that would compare as many assays/standards as possible against multiple patient samples (recruited and collected from multiple investigators) to assess individual assays, standards, and calibrators.

**Diagnostic Strategies for APS.** The last session focused on developing a clinical and laboratory-based strategy to improve the diagnosis of APS. Dr. Kotaro Otomo from Hokkaido University presented the Antiphospholipid Score, which combines the results from anticardiolipin, anti-\(\gamma\)-2-glycoprotein I, anti-prothrombin/PS antibodies, and lupus anticoagulant testing into a ‘score’ to assess the likelihood that an individual patient has APS.

Plans for 2011-12.

Follow-up: Continue to assess impact of the 2009 Guidelines for Lupus Anticoagulant testing.

**Subcommittee Communication:** Plan to prepare a manuscript for submission on performance of anticardiolipin and anti-\(\gamma\)-2-glycoprotein I antibody ELISA-based assays, including discussion of patient selection, pre-analytical variables, analytical variables, and post-testing issues, including interpretive analyses.

Perinatal/Pediatric Hemostasis

23 July 2011

Chairman: Paul Monagle (AU)
Co-chairmen: Anthony Chan (CA), Janna Journeycake (US), Christoph Male (AT), Ulrike Nowak Gottl (DE), Paolo Simioni (IT), Guy Young (US)

The paediatric/perinatal subcommittee had not been productive over the last decade, with no formal publications during that time. The 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 report will outline that the SSC has been successful in achieving these three goals over the last 12 months.

Report:

1. Education Program

Education program was very successful. Topics well received and over 120 people attended the session, most of whom then remained for the business meeting. 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 see some children to update themselves.

2. SSC business

The agenda of the SSC has focussed on thrombosis so as to not overlap with other SSC. We are involved in the collaborative work towards bleeding score.

I recognize that at present we are not pursuing a perinatal agenda adequately and this will need to be addressed in the future, although perhaps in combination with the women’s health SSC.

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)

In the last 12 months:
One position paper has been accepted to JTH

*Definition of clinical efficacy and safety outcomes for clinical trials in deep venous thrombosis and pulmonary embolism in children.* L. Mitchell, N. Goldenberg, C. Male, G. Kenet, P. Monagle, and U. Nowak-göttl, on behalf of the perinatal and paediatric haemostasis subcommittee of the scientific and standardization committee of the international society on thrombosis and haemostasis

In addition, one registry has been finalized and final publication is in progress. (Seven bleep registry)

Three further position papers were presented this week for final comment having already been reviewed and approved by the whole SSC. These will now be submitted:

1. *Recommendations for developing uniform Laboratory Monitoring of Anticoagulants in Children.* Fiona Newall, Anthony Chan, Vera Ignjatovic, Paul Monagle on behalf of the perinatal and paediatric haemostasis subcommittee of the scientific and standardization committee of the international society on thrombosis and haemostasis

2. *Developmental Haemostasis: Recommendations for laboratories reporting paediatric samples.* V. Ignjatovic, G. Kenet, P. Monagle on behalf of the perinatal and paediatric haemostasis subcommittee of the scientific and standardization committee of the international society on thrombosis and haemostasis

3. *Recommendations for definition and standardization of post-thrombotic syndrome (PTS) outcome measurement in clinical investigations of paediatric deep venous thrombosis.* N. Goldenberg, L. Brandao, J. Journeycake, S. Kahn, P. Monagle, S. Revel-vilk, A. Sharathkumar, and A. Chan, on behalf of the perinatal and paediatric haemostasis subcommittee of the scientific and standardization committee of the international society on thrombosis and haemostasis

Three further papers are in draft form having been presented for discussion this week.

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.

There are a number of suggestions for new projects. Many of these were brought forward by people new to the SSC.

**Potential position papers:**
1. Scoring system for non limb PTS in children.
2. Monitoring UFH during extracorporeal circulation

Potential clinical guidance papers

1. Diagnosis of VTE in children
2. Indications for Thrombophilia testing in children
3. Assessment and management of DIC in children
4. Approach to investigation of the bleeding child

Potential Registry

1. Homozygous protein C deficiency (purpura fulminans)
Plasma Coagulation Inhibitors

23 July 2011

Chairman: Steve Kitchen (UK)
Co-chairmen: Elisabetta Castoldi (NL), Elaine Gray (UK), Tilman Hackeng (NL), Richard Marlar (US), Piet Meijer (NL), Laurent Mosnier (US)

Educational programme _ Chairs S Kitchen, E Gray.

Thrombophilia: Who should be tested?

Trevor Baglin (UK)

APC resistance: biological basis and acquired influences

Elisabetta Castoldi , Maastricht, The Netherlands

Problems with Laboratory Assays for Protein C, Protein S and Antithrombin.

Richard A. Marlar, USA

Minutes/Summaries

Thrombophilia: Who should be tested?

Trevor Baglin (UK)

The evidence related to risk of recurrence after provoked and unprovoked VTE was reviewed and the rate that would justify anticoagulation was discussed. It was concluded that investigation of a patient for heritable thrombophilia should be the exception rather than the rule. Recent UK guidelines based on the available published evidence from studies in many countries had recommended that indiscriminate testing for heritable thrombophilia in unselected patients presenting with a first episode of venous thrombosis is not indicated (strong evidence) and that decisions regarding duration of anticoagulation in unselected patients should be made with reference to whether or not a first episode was unprovoked (strong evidence). The same document also recommends that testing for heritable thrombophilia in unselected patients with a strong family history or unprovoked recurrent thrombosis may influence decisions though a validated recommendation on patient selection is not currently possible. Case finding of asymptomatic relatives with low risk of thrombophilia such as FVLeiden or F2G20210A is not indicated.

APC resistance: biological basis and acquired influences

Elisabetta Castoldi , Maastricht, The Netherlands
The protein C pathway is a pivotal anticoagulant system responsible for the proteolytic inactivation of coagulation factors (F) Va and VIIIa, the essential cofactors of the prothrombinase and intrinsic tenase complexes, respectively. Cleavage of FVa and FVIIIa by activated protein C (APC) is stimulated by the APC-cofactors protein S and FV.

APC resistance is defined as a poor response of plasma to the addition of exogenous APC in vitro (Dahlbäck et al, PNAS 1993). Although it was initially discovered in a single thrombotic patient, it was soon recognised as the most prevalent risk factor for venous thrombosis. Most cases of APC resistance are attributable to the FV Arg506Gln mutation (FV Leiden) (Bertina et al, Nature 1994), which abolishes one the APC-cleavage sites in FV(a). However, several other genetic and acquired factors contributing to APC resistance have been described in recent years, most of which act by altering the levels of coagulation factors and inhibitors modulating APC activity. The numerous APC resistance assays currently available are based on different principles and are therefore differentially sensitive to the manifold determinants of APC resistance.

This educational lecture reviewed the multi-factorial etiology of APC resistance and discussed its implications for thrombosis risk and for APC resistance testing with different functional assays.

Problems with Laboratory Assays for Protein C, Protein S and Antithrombin. Richard A. Marlar, USA

PC, PS and AT assays are the most common assays performed for the evaluation of patients with potential thrombophilia. These assays appear to be essentially straight forward in their methodology. However there are numerous direct and indirect parameters that can affect these assays and their reported results. External variation of the plasma samples (pre-analytical variables), assay methodology differences (analytical variables), and differences in reference intervals (post-analytical variables) can complicate the diagnostic capabilities of the laboratory. This is on top of evaluation that do not take into account the potential complex genetic abnormalities that the assays are trying to accurately detect. This presentation will review the sample and assay variables that can be troublesome for proper PC, PS and AT assay performance and a brief discussion of genetic defects that can hamper assay performance. Finally, recommendations for the best assay methods will be presented along with the best protocol for confirming the positive diagnosis of one of these thrombophilic deficiencies.

Subcommittee Business section

Session 1 Chairs P Meijer, R Marlar

Instability of Protein S in blood samples.

Kieron Hickey UK

Whole blood samples from normal donors were stored at room temperature for up to 72 hours prior to processing (centrifugation at 2000G for 10 minutes). Plasma aliquots were frozen at -80 oC and assays of free protein S (FPS) performed. The FPS antigen as determined by latex
immunoassay utilises beads coated with C4B binding protein to capture FPS and a monoclonal antibody to human protein S to initiate aggregation. In 10 normal donors there was a 14% reduction in FPS at 24 hours (relative to time 0), rising to 37% at 72 hours. In contrast FPS antigen determined by ELISA (method of Woodhams) was unchanged in that time.

A second study utilised PS activity assays from Siemens, Instrumentation Laboratory and Precision Biologic. Whole blood samples were stored for up to 24 hours at room temperature prior to processing. For all three methods there was a reduction of 3 to 5% in PS activity after 4 hours, rising to a 13-15% mean loss after 24 hours.

In this second study FPS antigen by latex immunoassay was reduced by 6% at 12 hours and 11% at 24, confirming the findings of the first study. ELISA Free PS antigen again demonstrated no change.

Elaine Gray, NIBSC, UK

Data on the calibration of SSC plasma standard lot 4 were presented. All analytes were calibrated against relevant WHO International Standards. SSC plasma standard lot 3 was included in the exercise for an assessment of continuity and stability of lot 3. Overall there was good agreement between unitage for lot 3 between the original calibration and the current study although the free PS Ag was associated with a difference of 8% thought to relate to the replacement of the WHO international standard (from first to second).

In relation to lot 4 for PC activity there was no significant difference between clotting and chromogenic PC assay data. Inter-laboratory variability was assessed by geometric coefficient of variation (GCV) which was good for PC antigen (6.9%) and activity (3.6%). The PS activity data included 5 sets of Instrumentation Laboratory Pro S results and 6 sets of Staclot data. The difference between results with these two methods was significant but the overall inter-laboratory GCV was only 7% and participants had approved the proposed assigned single value including all results. Free PS data included 10 sets of latex immunoassay results and 7 sets of ELISA result. There was no significant difference with a GCV of 6.8%. Total PS assay data was also precise (GCV 3.8%). For AT there was no significant difference between results with IIa and Xa based assays and a single value was assigned. AT antigen assays were acceptably precise. All participants had agreed with all the proposed potencies. There were no objections to these assigned potencies from the Plasma coagulation inhibitors subcommittee.

Protein S assays: need for standardisation

Ian Jennings, UK NEQAS (Blood Coagulation) UK

UK NEQAS (Blood Coagulation) data show a fall in centres performing PS activity assays between 2001-2010, with a concomitant rise in free PS assays. The latter is entirely due to laboratories performing latex-based assays. Between-laboratory agreement for free PS antigen assays is good, with CVs around 10% in proficiency testing exercises. However, for PS activity assays, marked differences between results obtained with different kits are still seen. These differences are apparent for samples distributed in both NEQAS and ECAT surveys, and could
not be explained in an in-house investigation. A multicentre study investigating PS activity in both frozen and lyophilised plasmas is planned. This will be submitted for approval as a subcommittee project.

**Biological Variation for AT, PC and PS: their relation to analytical quality**

**Piet Meijer (ECAT, The Netherlands)**

Sources of analytical variation were discussed including pre analytical factors related to the subject or sample collection and handling. Analytical variability could be random involving inherently unpredictable variations in test results or systematic errors which are typically constant or proportional to the test result. Data from a study of biological variation were presented. This involved 15 samples collected over 1 year from each of 40 normal subjects. The within subject CV was 4 to 9% for AT, PC and PS determinations. Analytical variation of these assays was in the range 1 to 6%. Data on long term analytical imprecision using results of the ECAT quality assurance program indicated CVs of around 7% for chromogenic assays of AT and PC, with CVs of 10 to 16% for clot based and antigen assays of PC and PS.

**Evaluation of a novel total Protein S assay system for screening of protein S type II deficiency**

**Prof Hiroko Tsuda Japan**

A novel assay system for activity and antigen levels of total protein S (PS) for automated analyzers was described. To test usability of this assay to screen for type II PS deficiency in Japanese venous thromboembolism (VTE) plasma samples of healthy volunteers and confirmed type II deficiency patients was assessed. Reference intervals (average ± 2SD) of healthy male (n = 98) were 19.3 - 32.8 μg/mL for total PS antigen and 18.8 - 32.3 μg/mL [PS equivalent] for total PS activity, respectively, and those of female (n = 89) being 16.0 - 29.0 μg/mL and 14.8 - 28.8 μg/mL [PS equivalent], respectively. The mean specific activity for healthy individuals (n = 187) was 0.99 and limit of reference interval was 0.79. Specific activities were found to be < 0.69 for specimens from patients with confirmed PS type II deficiency and those administered warfarin. The automated total PS assay system was considered an effective screening tool for PS type II deficiency.

**Session 2 Chairs Elisabetta Castoldi, Tilman Hackeng**

**Mutation databases for PC, PS and AT.**

**Carolina Pintao (The Netherlands)**

Hereditary deficiency of the coagulation inhibitors protein C, protein S and antithrombin is rare and associated with increased risk of thrombosis. The genetic basis of the deficiencies is highly heterogeneous, almost every family segregating a different mutation.
The exponential growth of data generated by high throughput sequencing and genome-wide association studies, as well as the growth of knowledge and understanding of the relationship between genotype and phenotype increases the need for constantly updated and comprehensive mutation databases.

Two mutation databases currently aim to provide information on genetics with different overlapping priorities: the Online Mendelian Inheritance in Man (OMIM), and the Human Gene Mutation Database (HGMD) (Cardiff, UK). The first is aimed on genotype-phenotype knowledge, but is not comprehensive on adding variations, focusing on the first and most relevant ones. The second is aimed on adding every public available mutation associated with a gene, but has little phenotypic information (Samuels and Rouleau, Nat Rev Genet, 2011).

In contrast, the so-called locus-specific databases (LSDs), which are typically curated by experts on a particular area, aim to provide comprehensive information associated with a particular gene or disease. Databases following this model have been released before for protein C (1995), antithrombin (1997), and protein S (2000) deficiency, on behalf of the International Society of Thrombosis and Haemostasis.

This presentation is aimed to discuss the need to update (and maintain) the databases of mutations for protein C, antithrombin and protein S deficiencies.
Platelet Immunology

23 July 2011

Chairman: Andreas Greinacher (DE)
Co-Chairmen: Beng Chong (AU), Yves Gruel (FR), Donald Arnold (CA), Hartmut Kroll (DE), Yoshiaki Tomiyama (JP)

Animal models for immune thrombocytopenia
J. Semple (Toronto, Canada)
Mechanisms of ivIgG in immune thrombocytopenia – lessons learned from in-vitro studies and animal studies
Lazarus (Toronto, Canada)

A proposal was made that the platelet immunology SSC should summarize laboratory parameters, which could be included into treatment trials on ITP to assess whether the pathways found in mice are also affected in humans.

Part 2. Autoimmune and Alloimmune Thrombocytopenia

Role of complement in the pathomechanism of ITP

Ulrich Sachs (Giessen, Germany) presented a summary on the role of complement in the pathomechanism of ITP. After a short overview focusing on the detection of complement fixed to autologous platelets from ITP patients, he presented and discussed data from two current studies investigating the capability of plasma from ITP patients to fix complement to allogeneous test platelets. The findings included that ~ 50% of ITP patients are capable of activating the complement system on the surface of allogeneous platelets. There is evidence that the activation follows the classical pathway. GPIIbIIIa antibodies were able to activate complement, GPIbIX antibodies did not. This specific capability may contribute to the heterogeneity in clinical presentation of ITP and, probably, treatment response. He concluded that studying complement activation in clinical trials of ITP might prove helpful in order to further elucidate the biological and thus, medical relevance of complement activation. Furthermore, the finding that complement can be fixed to platelets by ITP sera that according to currently available standard methods do not contain platelet reactive antibodies, may indicate that current techniques for autoantibody detection are insufficient. He concluded that further efforts in standardizing autoantibody detection methods and increasing the test sensitivity are warranted.

DISCUSSION:
The effect is likely caused by low affinity/low avidity antibodies. Currently there are no clinical data, whether complement activation capability of antibodies correlates with the thrombocytopenia.
Platelet immunology SSC Statement on the antibody testing in trials on immune thrombocytopenia (ITP)
The subcommittee worked on a recommendation on how laboratory testing for platelet autoantibodies could be included into clinical trials in immune-thrombocytopenia. This had been discussed during the last two SSC meetings, a manuscript as SSC recommendation has been drafted, which contains the following main points:

Consensus recommendations of the ISTH platelet subcommittee for the implementation of platelet antibody testing in ITP clinical trials.

<table>
<thead>
<tr>
<th>Key element</th>
<th>Consensus recommendations</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticoagulant</td>
<td>ACD-A</td>
<td>Minimizes platelet loss, prevents platelet activation.</td>
</tr>
<tr>
<td>Volume of collection</td>
<td>30mL (adults); 10mL (children)</td>
<td>Sufficient sample volume is required for adequate platelet yield. The proposed collection volumes pose minimal health risk and no ethical concern.</td>
</tr>
<tr>
<td>Test method</td>
<td>Direct antigen capture assay (ACA; MAIPA)</td>
<td>Direct tests are more sensitive than indirect tests; glycoprotein-specific assays are more specific than PA1gG.</td>
</tr>
<tr>
<td>Monoclonal antibody</td>
<td>Commercial or in-house anti-GPIb and anti-GPIIIa, or anti-GPIIbIIIa and anti-GPIb and anti-GPIX.</td>
<td>The majority of antibodies are specific for GPIIbIIIa or GPIbIX. Limitations of sample size, platelet count and monoclonal antibody availability would limit testing other glycoproteins at this time</td>
</tr>
<tr>
<td>Centralized testing</td>
<td>Yes</td>
<td>Centralization of processing and testing is necessary to reduce the variability introduced by different laboratories.</td>
</tr>
</tbody>
</table>

This approach was discussed by the subcommittee members and accepted with the following additional remarks:

DISCUSSION: Dr Kaplan indicated that the ISBT working party on platelet immunology has done a lot of work on platelet antibody testing, which needs to be included into the proposal. It was made clear during the discussion that the Platelet Immunology SSC working group on platelet antibody testing in ITP clinical trials based the proposal on the work of the ISBT working party.

Predictive parameters for the fetal status in anti-HPA-1a managed pregnancies

G. Bertrand G., C. Kaplan (Paris, France) presented a retrospective study including 75 HPA-1bb women with feto-maternal incompatibility (239 pregnancies, 66 index cases and 89 managed pregnancies). When measured in early gestation and before therapy, the maternal alloantibody concentration was predictive for severe fetal thrombocytopenia. Follow-up of the concentration during managed pregnancies may reflect a modulation of the maternal anti HPA-1a IgG production and transportation across the placental barrier, and could also result from IVIG therapy modulation. The area under curves were predictive of severe neonatal thrombocytopenia.
During the last annual SSC meeting of the ISTH (May 2010, Cairo), variations in predictive thresholds of antibody concentration were observed between different reports. Consequently an inter-laboratory assay on quantitative MAIPA has been organized. Four sera were quantified using the WHO reference serum (Ref 03/152), following a suggested protocol or an “in-house” MAIPA protocol. Similar concentrations were obtained by the 4 participants, even if slight variations were observed between the two protocols.

Thus the differences of thresholds in antibody titers in the MAIPA found in different studies cannot be explained by the methodology but must be related to the cohorts. This requires further workup.

DISCUSSION: the design of the study was questioned, especially its power to find an association of the maternal alloantibody concentration at delivery and thrombocytopenia in the fetus. The antenatal treatment modulation was also discussed, especially concerning in utero transfusions according to the maternal alloantibody concentration.

Part 3. Heparin-induced thrombocytopenia

IL10 gene polymorphisms and the risk of HIT.

Y. Gruel (France) investigated whether single nucleotide polymorphisms (SNPs) in IL10 promoter and variations in IL10R and IL10G microsatellites are associated with the synthesis of Abs to PF4 and a variable risk of HIT. They studied 82 patients with HIT and two control groups, one (Abneg) with 85 patients who had undergone cardiopulmonary bypass (CBP) with high doses of heparin, but without Abs to PF4. The other one (Abpos) comprised 84 patients with significant levels of PF4-specific antibodies after CPB but no HIT. Genotypes and allele frequencies of 3 SNPs, rs1800896 (-1082A>G), rs1800871 (-819C>T) and rs180072 (592A>C), were similar in HIT patients and controls. The polymorphic CA repeat microsatellites IL10R [5325CA(11_15)] and IL10G [8134CA(14_29)] were also analyzed. The short G20 allele of IL10G was more frequent in Abneg patients (8.2%) than in Abpos (2.9%) and HIT patients (3%)(OR 0.29; 95% CI [0.12-0.70], p < 0.006). Combinations of the 3 SNPs with IL10G and IL10R variations defined nine different combined haplotypes (cH1 to cH9). The pair of haplotypes cH1/cH8 comprising the short G20+R13 alleles was less often represented in HIT (4.9% vs. 13.6% in controls)(OR 0.33; 95% CI [0.11-0.97], p=0.036), and levels of Abs to PF4 in Abpos patients were lower in cH1/cH8 subjects (median OD 0.67 vs.1.21 in non-cH1/cH8 individuals, p=0.019). These results suggest that IL10 promoter microsatellite polymorphisms may influence the immune response to heparin and the risk of HIT.

DISCUSSION: Question on statistical correction for multiple testing

Evaluation of the platelet microparticle assay for the diagnosis of HIT

F Mullier (Namur, Belgium) (presentation given by Dr Chatelain); assessed the release of procoagulant platelet microparticles (PMPs), considered as the major step of HIT comparing PMP generation assay (PMPGA) (Mullier et al. Thromb Haemost. 2010 Jun; 103(6):1277-81) with ELISA, Light Transmission Aggregometry (LTA), 14C-Serotonin Release Assay (SRA) and clinical outcomes.

Sera or citrated-platelet-poor plasma of HIT-suspected patients (n=72) were incubated with
citrated-whole blood from healthy donors with/without unfractionated heparin (UH: 1 or 500 IU/ml). PMPs were quantified and characterized using a FACS Aria® flow cytometer. In positive HIT patients, PMPs expressing phosphatidylserine (PS+) are generated following immune complexes formation with low UH concentration whereas PMP rate decreases significantly in presence of high UH concentration. The ratio of PMPs PS+ between low and high heparin concentrations can be used as a diagnostic parameter with an optimal cut-off ratio at 2. The correlation between PMPGA and SRA is markedly more significant (p<0.0005, n=57 including 10 positive SRA) than LTA (p=0.0267, n=39) and ELISA (p=0.0022, n=58). Sensitivity and specificity of PMPGA were 70.0 and 97.7%, respectively, calculated with SRA as reference. Combining clinical outcome to biological testing, PMPGA sensitivity and specificity reached 100 and 88.9%, respectively. PMPGA can be considered as a confirmation assay of HIT.

Which “gold standard” for the diagnosis of HIT: a clinical score, an expert opinion, or SRA? Brigitte Tardy, presentation given by T Lecompte (France)

Only a subgroup of patients clinically suspected for HIT have a positive EIA test and a positive functional assay. EIAs have a poor specificity. Blinded expert assessment on HIT cases shows a lower sensitivity and specificity of the SRA. A study is currently performed in France, Belgium and Switzerland, SRA centrally performed. Cases assessed blinded for lab results by experts. Correlation coefficient between experts was rather high, but differed between experts and local physician. In the SRA were more cases positive than in the judgement of the experts. The study shows that both is necessary, a clinical picture of HIT as well as a corresponding antibody.

DISCUSSION: the study confirms that for the diagnosis of HIT in clinical studies both is needed, a clinical presentation consistent with as well as the corresponding antibodies.

Heterogeneity of the heparin dependent antigen in some atypical patients with clinical characterized HIT, and incidence in immunoassay reactivity
J Amiral (France) discussed discrepancies between diagnostic assays for HIT. The vast majority of patients with HIT are reactive with most of the diagnostic assays. Although when heparin antibodies are present, their functional activity (platelet activation) is lower to much lower than the immune reactivity (many being asymptomatic).

- Pathology is primarily associated with IgG isotypes, although some characteristic cases are associated with IgA isotypes (mainly in cancer patients). The impact of IgM isotypes remains unclear.
- Some patients, reactive with one assay and not with the others have strong antibodies to PF4 alone (and weak to PF4 heparin). Clinical presentation is atypical but very severe. The PF4 antibodies are thought to be pre-existing, and their pathogenicity developed by presence of heparin during treatment.
- Some patients (mainly cardiac surgery) can develop antibodies to protamine sulfate or to protamine sulfate complexed with heparin. These patients are also atypical, but develop severe adverse effects, especially when reactivity is targeted to protamine sulfate complexed with heparin. These patients had (as far as it can be documented) more than 1 exposure to protamine sulfate for neutralizing heparin.
• Some patients have IL 8 antibodies (and more rarely NAP 2), with also atypical clinical presentations. IL 8 antibodies are thought to be pre-existing, and heparin therapy reveals the pathogenic effect.
• In few cases, with severe dys-immune diseases, sticky samples can induce false positive reactions. Using a control plate is useful for analyzing these patients. These sticky samples can also co-exist with severe infections, inflammations, presence of amyloid substance, or circulating immune complexes.

It was suggested that the capture antigen assay using immobilized but flexible heparin can be useful for detecting the atypical cases.

Whole blood aggregometry using the Multiplate analyzer for the diagnosis of HIT.
MC Morel-Kopp (Sydney, Australia) presented the results of the first Australian multi-centre study on HIT diagnosis using a new whole blood impedance aggregometry approach which is easy to perform with rapid turn-around time and has been shown to be more sensitive than LTA. Hirudinized blood was used. WBIA and SRA were performed on 181 samples positive for H-PF4 antibodies. Sixty five samples showed platelet activation by both assays. Variability in the high heparin step was an issue with the SRA: for 9 WBIA positive samples, serotonin release with high dose heparin dropped by at least 50% but was still >20%; these were retested after a half dilution and 8 became positive. Ten samples were discrepant: 1 strongly positive (89% release) and 6 weakly positive by SRA (average release 56%) were WBIA negative. When samples were retested using a random donor only 2 remained SRA positive, suggesting that a cut-off at 20% release is perhaps too low. Three samples were SRA negative but strongly WBIA positive; two were retested by SRA with 0.5IU/ml heparin and 1 became positive. Using the strict definition for SRA positivity, WBIA had a sensitivity of 90.3%, specificity of 89.0% and PPV of 84.4%. With a modified SRA definition (cut-off of 50% for low dose heparin and drop >50% for high dose heparin) the sensitivity and specificity would be 93.7%, 97.1% respectively.

Multiplate and HIT: opportunities and limits.
I. Elalamy (Paris, France) reported about his experience with heparin-induced platelet aggregation in whole blood assessed by Multiplate® (Heparin-Induced Multiple Electrode Aggregometry, HIMEA). HIMEA and PRP based platelet aggregometry were prospectively compared to serotonin release assay (SRA) in 200 well characterized consecutive patients suspected for HIT. HIMEA is at least as sensitive and specific as PAT (72.7 vs 68.2 %) for detection of HIT platelet-activating antibodies. It had similar NPV (96.7 % vs 96.1 %, respectively) than SRA. The combination of an immunological assay with HIMEA, presented as an easy-to-perform, rapid and reliable functional assay, could be a feasible option in non specialized laboratories for HIT diagnosis optimization.

A suggestion was made to the Platelet Immunology SSC to organize an international workshop comparing the multiplate assay with washed platelet assays.

Interassay variability of the high heparin step
A. Greinacher (Greifswald, Germany) showed that an in-house PF4/heparin EIA showed inhibition with high heparin in a smaller number of sera compared to the GTI EIA. The reasons are currently unknown but are most likely related to slight differences in assay design. Thus the validity of the high heparin step should be assessed for each of the different EIAs independently.
Standardization of quality control of functional HIT assays
T. Warkentin, J.C. Moore (Hamilton, Canada) The greater diagnostic specificity of a functional test for HIT antibodies—the serotonin-release assay (SRA)—vis-à-vis the polyspecific PF4/polyanion EIA was reviewed; in brief, published data indicate that EIA+/SRA- patients do not have HIT. Regarding steps for SRA quality control, the following seven assay variables were discussed: (a) peak reaction conditions (type of heparin and heparin concentration[s]); (b) use of buffer control; (c) use of Fc receptor-inhibiting monoclonal antibody; (d) reporting of per cent serotonin-release results; (e) use of pedigree versus random platelet donors; (f) use of positive HIT controls, including weak positive controls; and (g) use of PF4-dependent EIA as a “quality-control” maneuver. The merits of these variables in optimizing test sensitivity and specificity were discussed.

DISCUSSION: what is the clinical impact of a positive SRA test in an asymptomatic patient?
Dr Paolo Gresele reported on the preliminary results of an online survey on how congenital platelet function disorders are diagnosed worldwide. The survey represents the first step towards the development of methodological guidelines, the main ongoing project of the Platelet Physiology Subcommittee. About 200 centres filled up the online questionnaire, providing information on how they select patients who need to undergo a diagnostic workup for platelet function disorders, what first-step, second-step and third-step diagnostic tests they use. The results were analyzed anonymously to participant identities. As expected, the survey revealed a high variability in diagnostic practices worldwide: as a consequence, methodological standardization is necessary. The information gathered in this survey will be helpful in the development of guidelines.

Dr Dermot Kenny gave a brief review of a 96 well platelet function assay his group developed in 2008. He then outlined a rationale for testing platelet function in disease states other than bleeding disorders and cardiovascular disease. He presented data from assays of platelet function in patients with inflammatory arthritis, patients with HIV, patients with HIV taking the drug abacavir, patients with recurrent miscarriage and finally in patients with pulmonary hypertension. The profiles of platelet function were different in these disease cohorts relative to normal controls and also demonstrated disease specific phenotype relative to disease activity. He concluded that platelet function testing is of interest to the internist.

Dr Diego Mezzano and Dr Cathy Hayward illustrated their most recent personal experience with methods to study platelet secretion. Platelet secretion defects are common platelet function disorders that may, or may not, be associated with aggregation abnormalities. These conditions are emerging to have clinically important bleeding risks and their management sometimes necessitates platelet transfusion.

Dr Mezzano reviewed methods that are used to measure the platelet secretion of serotonin. The method based on loading platelets with radiolabeled (3H or 14C) serotonin has the disadvantage of exposing the operator to radiations and is not feasible in all patients (for instance, in patients who lack platelet delta granules). A method based on the measurement of endogenous serotonin by HPLC by electrochemical detection proved to be reliable, quick and cost-effective, provided that the laboratory is already equipped with the necessary, rather expensive instrumentation.

Dr Hayward presented an update on the diagnosis of platelet function disorders based on dense granule ATP release assays, using light emission triggered by reaction of the released ATP with D-luciferin, catalyzed by firefly luciferase. The released ATP is quantified against an ATP standard. ATP release shows considerably more variability than aggregation assays (Havelow et
al, Lab Hematol 2007; 13:59-62; Pai et al, AJCP 2011; in press) and this is reflected by fairly broad reference ranges. Strong agonists (thrombin, collagen) induce more dense granule ATP release than other agonists and show the least variability. Data from a prospective study of ATP release for bleeding disorders (Pai et al, AJCP 2011; in press) indicates that the findings are more predictive of a bleeding disorder, or an inherited platelet function disorder, when abnormalities are present with more than one agonist. Furthermore, the test detects platelet function disorders even when aggregation findings are normal. Receiver operator curve analyses indicate that there is little added value to measuring release with multiple concentrations of an agonist. Among the best agonists for detecting common platelet function disorders due to dense granule release assays are: 6 μM epinephrine, 5.0 μg/ml collagen and 1 μM thromboxane analogue U46619.

When dense granule release is reduced with strong agonists, the differential diagnosis includes dense granule deficiency in addition to secretion defects due to other causes. Overall, ATP release assays can be very helpful to diagnose bleeding disorders. However, caution is warranted in that the reagent commonly used to measure ATP release, Chronolume?, potentiates some aggregation responses that are submaximal. Evidence has now emerged that the reagent can alter platelet aggregation findings for congenital platelet disorders.

Dr Tim Warner described the 96-well platelet aggregometry test that was developed in his laboratory. The test relies upon the detection of changes in light transmission through suspension of platelets, either in platelet rich plasma (PRP) or in washed platelets, caused by platelet agonists; i.e. agonists that cause platelet aggregation lead to a decrease in light scatter through the suspensions and so a decrease in measured absorbance. For 96-well plate aggregation testing, agonists are first placed into the plate, in a similar concentration range to those used for traditional light transmission aggregometry (LTA). Agonists are placed first as there are too many agonist wells to be pipetted after the addition of platelets, followed by the addition of the platelet suspension. The plates can then be placed into a kinetic plate reader which will shake the plates and then read the changes in absorbance as a marker of platelet aggregation over time. The time course of the aggregation is generally slower than that seen in traditional LTA, most probably because the mixing is less vigorous and because the mixing is stopped each time the plate is read, unlike LTA in which mixing and reading take place at the same time. The advantage of 96-well plate aggregometry is that controls, e.g. unstimulated PRP and PPP, and test samples, PRP plus agonists, are run together removing problems of variability in PRP responsiveness over time. In addition, by making use of the many wells available on the plate full agonist curves to multiple platelet agonists can be rapidly developed. If a luminescent plate reader is available the assay can also be used to determine ADP/ATP release by use of luciferase reagents as in LTA, and samples can be retained at the end of aggregation experiments for determination of e.g. the production of TXA2. As samples are already in 96-well format there is very easy transfer to other standard plate based assays. Finally, platelet adhesion in the plates, subject to pre-coating of the plates, can be easily read by the addition of p-nitrophenol phosphate and the detection of the formation of p-nitrophenol by platelet acid phosphatase. In our most recent experiments, we have produced 96-well plates with lyophilized and immobilized platelet agonists. These plates can be stored at room temperature and following the simple addition of PRP can be used for the rapid screening of platelet aggregation responses; in these experiments plates can be vortex mixed for 5 minutes and then the assay read as end point assay of platelet aggregation in any plate reader. This may well be the best use of 96-well plate aggregometry, a
rapid across the board screening of platelet responses in a standardized manner, leaving traditional LTA for more in-depth kinetic analyses of platelet function.
Predictive Variables in Cardiovascular Disease

23 July 2011

Chairman: James Douketis (CA)
Co-chairmen: Gordon Lowe (UK), Karel Moons (NL), Frederick Spencer (CA), Alberto Tosetto (IT), Richard White (US)

Educational and SSC Activity Update Session

- This was a well-attended session, taking place on July 23rd from 9AM-1PM; it was co-chaired by Drs. James Douketis and Richard White.
- The session began with the Educational Session, devoted to a comparison of the incidence, and determinants of VTE and cardiovascular disease in different racial groups (whites, blacks, Asians); the presenters were Drs. Rich White (USA), Shinya Goto (Jp) and Mary Cushman (USA).
- The second part addressed ongoing SSC collaborative activities relating to determinants of cardiovascular disease (Dr. Olivia Wu, UK), D-dimer to predict stroke risk (Dr. John Eikelboom, Can) and ongoing studies in this area (Drs. Marc Rodger, Can, Dr. Paolo Prandoni, It, and Dr. Geert-Jan Geersing, Neth).
- The session ended with a spirited debate by Drs. White and Cushman on the pros/cons of thrombophilia testing in patients with otherwise unprovoked VTE.

Subcommittee Activities

- The committee continues to promote a collaborative project assessing D-dimer and other determinants of recurrent VTE, with the aim of better distinguishing patients at low and high risk for disease recurrence. To date, three papers relating to this work have been published (J Thromb Haemost, Ann Intern Med, BMJ), and a fourth is submitted for publication (JAMA).
- Annual meetings of this project have been held since 2009, with a meeting in Kyoto held on July 23rd and attended by 9 collaborators. Ongoing studies assessing D-dimer and other predictors of recurrence, including DULCIS, REVERSE-2, MORGAGNI, and VISTA were discussed, with feedback provided to the lead investigators.
- The committee has promoted and contributed to the development of a new patient-level pooled data analysis of studies assessing the utility of residual vein occlusion (RVO) to determine recurrence risk after DVT; this work aims to clarify an area where there is considerable uncertainty.
- Additional studies by this collaborative project are in progress using pooled patient-level data relating to (a) development of a clinical prediction guide for recurrent VTE, (b) determinants of recurrence risk after provoked VTE, and (c) effects of thrombophilia markers (factor V Leiden) on recurrence risk.
This year, the SSC VB meeting was focused on microparticles. The session was divided into two sections: the Educational session, devoted to microparticle function, generation and role in disease, and the business session, focused on Microparticle determination and standardization. This business session was divided into three parts addressing respectively: Part A, "Standardized strategies". Part B "Functional assays", and Part C "Novel methodologies." The success of this SSC VB was attested by the presence of at least 200 participants during the whole session.

The Educational session, chaired by Michael Berndt and Nigel Key, covered the relevance of microparticles (MP) as clinical biomarkers of vascular disease, the multifaceted role of endothelial derived MP and the role of MP in cancer.

Guus Sturk (NLD) presented an overview of the definition of different vesicle subtypes including exosomes, microparticles and apoptotic bodies. Particular attention was paid to the methodological recommendations related to their isolation from different biological fluids but also to the most appropriate markers and methods used for phenotypic characterization and enumeration. He highlighted their involvement in various biological functions and also reviewed the pathological circumstances associated with altered MP levels. The role of MP as potential prognostic markers to identify patients with vascular risk but also as a therapeutic monitoring strategy to evaluate the effect of medications impacting the cardiovascular system, was underlined.

Francoise Dignat George (France) presented the multiple faces of endothelial derived MP (EMP), that can be viewed as a “miniature version” of endothelial cells, expressing a large repertoire of antigens and receptors involved in hemostasis, inflammation, angiogenesis, and immune regulation. She provided in vitro and in vivo evidence that EMP behave as vectors of bioactive molecules such as TF, EPCR, adhesive molecules, MMPs, proteases, NADPH Oxidase, thereby playing a key role in the tuning of vascular homeostasis. She also reported more recent data challenging the presumed deleterious role of EMP and providing evidence that EMP also promote cell survival, exert anti-inflammatory effects, counteract coagulation processes or induce endothelial generation. She reviewed the current mechanisms involved in EMP formation, and concluded by opening a debate on the protective or deleterious role of EMP that bring new insights into the understanding of endothelial-associated diseases. She suggested that these observations could open novel pharmacological approaches to manipulating EMP generation.

Janusz Rak (Canada) began by commenting that the terminology ‘microparticles (MP)’ and ‘microvesicles’ (MV) is often used as interchangeable descriptions of all cellular vesicles,
especially in the cancer field. He provided a characterization of the major proteins, lipids and nucleic acids (DNA, mRNA, microRNA) that may be transferred horizontally between cells. Particular attention was paid to oncogenic pathways that stimulate the production of MV/MP harboring TF and that are possibly involved in thrombotic complications in patients with gliomas. Moreover, he provided data showing that the cargo of MV include several other receptors, antigens and bioactive molecules such as growth factors that are capable of stimulating tumor progression, invasion, angiogenesis and metastasis. MP from tumor cells harbor molecular information linked to cancer-related processes, and may serve as a reservoir of prognostic and predictive biomarkers to monitor tumor progression and responses to targeted therapies.

The Business Session was focused into 3 parts covering recent advances in standardization, (part A) new horizons raised by functional assays (part B), and technical challenges raised by emerging technologies (part C).

Part A, chaired by M.Berndt and F Dignat –George: Standardization strategies:

Nigel Key (US) presented the result on the SSC VB Working group devoted to the standardization of the pre-analytical step. The objective of the study was to compare the inter-laboratory variability using a common pre-analytical protocol (A) versus a non-standardized protocol (B). 14 laboratories participated to the workshop. Preliminary results showed that (1) a standardized protocol results in a reduction of the inter-laboratory variability in MP analysis (more markedly for flow cytometry analysis); (2) Tube volume and plasma volume that remains above the buffy layer and centrifugation conditions are major pre-analytic parameters that impact MP analysis. However, the inter-laboratory variability remains significant in some cases even when protocol A was apparently correctly applied suggesting the impact of other unidentified parameters. In this first report on the workshop, only platelet MPs (most sensitive to pre-analytic variables) or related functional properties were analyzed. The impact of pre-analytics protocols on the other MP sub-population was planned to be analyzed.

Romaric Lacroix (F) presented the progress reached on microparticle enumeration using the latest generation of flow cytometers. He showed that the new generation of flow cytometers (Gallios) displays improved FS resolution and decreased background noise compared to current device (FC500). This allows resolving previously undetectable small size MP. A new standardization protocol was proposed using calibrated beads to reproducibly measure both large and small MP. He showed that the fact to include the MP subset of small size gives access to a new information: significant correlations between old and new instruments were observed for platelet- and erythrocyte-derived MP but not for leukocyte- and endothelial-derived MP and Leu-MP. Moreover, he reported that significant difference in leukocytes-MP between patients with stable and unstable atherosclerotic plaques were only evidenced using the new standardized protocol.

PART B : Functional assays Chaired by F Dignat –George and M Berndt

Alisa Wolberg (US) described the cellular and plasma activities that regulate thrombin generation and fibrin formation, structure, and stability. A central hypothesis of her group is that MP exhibit procoagulant functions similar to their parent cells. Platelet MP (P-MP) were
prepared from platelets stimulated with calcium ionophore, TRAP, or TRAP+convulxin, and monocyte and human monocytic THP-1 MP (M-MP) were prepared from cells stimulated with lipopolysaccharide. Isolated MP were assessed by transmission electron microscopy, nanoparticle tracking analysis, flow cytometry, tissue factor (TF) activity, prothrombinase activity, thrombin generation, and clot formation, density, and stability. M-MP and P-MP exhibited similar shapes and diameters. M-MP expressed functional TF, supported prothrombinase activity, and triggered shorter thrombin generation lag times than buffer controls. Compared to controls, M-MP supported faster fibrin formation, higher fibrin network density, and higher clot stability in the presence of tissue plasminogen activator. In contrast, P-MP did not exhibit TF activity and supported lower prothrombinase activity than M-MP. P-MP supported contact-dependent thrombin generation, but did not independently increase fibrin network density or stability. Interestingly, P-MP increased rates of thrombin generation and fibrin formation when mixed with THP-1-derived MPs. It was suggested that these data indicate that M-MP and P-MP differentially modulate thrombin generation and fibrin formation, structure and stability, suggesting unique contributions to thrombosis.

Philippe Poncelet (F) proposed a novel assay for measuring the plasmin generation of MP. The objective was to replace the classical method based on high speed centrifugation to purify MP from plasma. This method was time consuming and no reproducible. The new prototype assay extracts MP from plasma by immune-magnetic separation to measure the MP-linked plasmin generation in plasma samples. Higher recovery and better reproducibility were found for the new method compared to centrifugation-based method. Using this test, he showed that plasma from healthy donors display a very low activity while samples from intensive care unit patients have highly variable and sometimes dramatically elevated levels of plasmin generation. He concluded that the new assay presents better characteristics to study the relevance of this MP-dependent activity in human pathologies.

PART C: Novel methodologies, chaired by Nigel Key and Michael Berndt

Don Gabriel (US) provided an update on ISADE (Invitrox Surface Antigen Detection and Enumeration). ISADE is a Mie light scattering device that detects and counts MP down to a size of 0.15 microns. The resolution between MP of similar size is at least 0.01 microns. Accuracy and reproducibility appear to be excellent. At present, this technology can enumerate and size MP, but has not been adapted to allow the cellular source of MP to be distinguished. Pre-analytical variables found to affect results include the magnitude of the centrifugal field and the time of exposure to the field. High fields promote MP aggregation. Aggregated MP can then distort the size distribution and give misleading information. Dr Gabriel presented data to illustrate that the efficiency of PMP production can vary with the choice and concentration of agonist, as well as mechanical shear. Other potentially important variables include temperature and buffer conditions. In addition, preliminary results from the MP size distribution in patients with acute liver failure were presented. Although statistical analysis has not been completed for this pilot trial of 56 patients, the number of MP seems higher in patients with acute liver failure compared to normal controls. Notably also, the presence of MP in the 0.2 to 0.26 micron size range was more prevalent in hypothermic patients with circulatory shock. Three of the 56 patients had MP expressing hepatocellular biomarkers by flow cytometry.
Edwin van der Pol (NL) presented the ability of two new, advanced methods (nanoparticle tracking analysis (Nanosight NS500) and resistive pulse sensing with a pore diameter of 1 µm (iZon qNano)), to measure the size and concentration of a standard population of human urine vesicles by comparison with two established techniques (transmission electron microscopy and flow cytometry). He showed that Nanoparticle tracking analysis and resistive pulse sensing detected a concentration of 1.4?10^10 ml^-1 and 3?10^10 ml^-1, respectively, which is 1000-fold higher than the observed concentration with flow cytometry. By combining the collected information with a model based on the Mie theory for the calibration of flow cytometers, he proposed a corrective factor of the sizing value of polystyrene beads for the standardization of MP detection with flow cytometry. He also brought some evidence that the detection of large vesicles by flow cytometry would be due to the simultaneous presence of multiple vesicles in the laser beam.

Chris Gardiner (UK) presented an update on nanoparticle tracking analysis, a technology based on the measurement of Brownian motions using the last version of a laser instrument (Nanosight). This technology measures particles with a size between 50 nm and 1 µm. Analysis of preparations of particles from human plasma indicated that there are 1-5 x 10^10/mL cell-derived vesicles in healthy people, a concentration of particles that is approximately 1000-fold greater than estimates by conventional flow cytometry. Most “microparticles” are lipoproteins that are undetectable by flow cytometry approaches. However, the fact that particles other than real MPs or exosomes such as lipidic vesicles may interfere, advocates for specific labeling. Quantum dot labelling can be problematical. Bright high-affinity, specific fluorescent labels are necessary for phenotyping. Multiparameter measurement of micro/nanovesicles is achievable. NTA may be useful for determining the resolution of flow cytometry.

Suzanne Osanto described the use of a combination of microfluidics and AFM to detect MPs directly in plasma. Tenfold-diluted EDTA PPP was flown through a microfluidic channel with a controlled pressure driven laminar flow to allow direct contact with anti-human CD41 antibody-coated mica. MPs bearing CD41 antigen were captured on this surface and subsequently detected by AFM operated in fluid tapping mode. The majority of the captured MPs have diameters (dsph) similar to prior results obtained with MPs immediately isolated from fresh PPP. High-speed centrifugation needed to isolate MPs did not influence the size distribution of MPs. Use of the microfluidic system also increased the efficiency of capturing MPs. This microfluidic system was applied to count PMPs in plasma from healthy donors, and was compared with a drop method. Considerably more CD41-positive MPs per 100 µm^2 surface were detected when plasma was run through the microfluidic system than when measured by the drop method. A linear dose-response curve between the number of CD41-positive MPs per 100 µm^2 surface and the MP concentration was observed.

Given that the release of procoagulant platelet microparticles (PMP) is considered a hallmark of HIT, Christian Chatelain (BE) presented a novel assay based on PMP generation (PMPGA) for the early diagnosis of type II heparin-induced thrombocytopenia (HIT). This diagnosis of HIT remains challenging in clinical practice.

The performance of this PMPGA was compared with ELISA, Light Transmission Aggregometry (LTA), 14C-Serotonin Release Assay (SRA) and clinical outcomes. Sera or citrated-platelet-poor
plasma of HIT-suspected patients (n=72) were incubated with citrated whole blood from healthy donors with/without unfractionated heparin (UH: 1 or 500 IU/ml). PMPs were quantified and characterized using a FACS Aria® flow cytometer. ELISA (PF4 Enhanced®), LTA, PMPGA, SRA and clinical outcomes data were compared by Chi-Square tests and ROC Curves.

In HIT patients, PMPs expressing phosphatidylserine (PS+) are generated following immune complex formation with low UH concentration whereas PMP rate decreases significantly in the presence of high UH concentration. It was proposed that the ratio of PMPs PS+ between low and high heparin concentrations be evaluated, with an optimal cut-off ratio of 2.0. The correlation between PMPGA and SRA is markedly more significant (p<0.0005, n=57 including 10 positive SRA) than LTA (p=0.0267, n=39) and ELISA (p=0.0022, n=58). Sensitivity and specificity of PMPGA were 70.0% and 97.7%, respectively, calculated with SRA as reference. Combining clinical outcome to biological testing, PMPGA sensitivity and specificity reached 100 and 88.9%, respectively.

Those cases that were discordant between SRA and PMPGA were analyzed by clinical outcome. Given its good correlation with 14C-SRA performance and clinical outcome, PMPGA has to be tested in larger scale studies as a potential new biological reference confirmation assay for HIT.

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 now 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.

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. 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. The most critical remaining question is: **How representative is this newly accessible part of the MP ‘iceberg’ for the clinically relevant biomarkers we are seeking?** Challenging these recent technical evolutions in pathological situations is a mandatory step to validate their real impact in clinical practice. This question suggests a direction for future collaborative working parties in the VB SSC that were proposed during Kyoto. The project proposal is to dedicate a new Collaborative Workshop on the capacity of new and existing methods to resolve the “clinically relevant MP” population. Using the recently defined standardized preanalytical protocol, the Core laboratory will prepare frozen aliquots of PFP from normal and pathological samples (cancer, sepsis and CVD…), that will be aliquoted and sent to participating labs. A laboratory expert in one technology among the current (flow cytometry, procoagulant assays) and emerging options (NTA, DLS, AFM, ISADE…….) will measure MP levels under blinded conditions. Thereafter, the core laboratory will collect data from each expert lab, and evaluate the respective value of each methodology in the evaluation of both normal and pathological samples.
von Willebrand Factor

24 July 2011

Chairman: Jeroen Eikenboom (NL)
Co-chairmen: Thomas Abshire (US), Imre Bodo (HU), Jorge DiPaola (US), Emmanuel J. Favaloro (AU), Yoshihiro Fujimura (JP), Paula D. James (CA), Bernhard Lämmle (CH), Reinhard Schneppenheim (DE)

Audience: approximately 250 educational session, 150 business session

Summary of VWF Subcommittee Approvals and Projects

- Project Standardization of VWF propeptide estimation: calibration of reference plasma, assignment of VWFpp value to WHO 6th IS FVIII/VWF Plasma (07/316) and SSC Lot #3. Project will continue to calibrate SSC Lot #4. SCC Official communication will be written.
- Registry on Acquired Von Willebrand Syndrome (www.intreavws.com): during 2011 the website is being re-organized and updated. Ongoing.
- Project “Desmopressin in the management of Von Willebrand disease: biological response versus clinical efficacy”, enrollment closed, data analyzed, manuscript in preparation.
- Project “Standardization of quantitative bleeding scores”. This ISTH-endorsed BAT was published as an Official Communication of the SSC in 2010: Rodeghiero et al., ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. J Thromb Haemost, 2010;8:2063-2065. Work will be continued in the standing committee “Bleeding Assessment Tools”. A proposal was presented for an initial validation study. It has to be decided whether this validation study will be a project within the VWF Subcommittee or will be an activity of the new standing committee “Bleeding Assessment Tool”.
- New ideas:
  o development of an international reference preparation for ADAMTS13 activity
  o comparative study between different VWF ‘activity assays’

Educational program
The educational program was well attended and got a lot of positive feedback.

**Bleeding scores and Bleeding assessment tool**

Session Chair: Paula James (CA)

- Update on activities of bleeding score working group

Paula James (CA) (on behalf of Alberto Tosetto) provided an update about the activities of the bleeding score working group. This group was established as a collaborative effort between the VWD, Pediatric and Women’s Health Issues SSCs and has met regularly since 2008. The initial mandate of the group was to draw on the experience of its members to develop a single bleeding assessment tool (BAT) that could be used in a variety of settings. This ISTH-endorsed BAT was published in the J Thromb Haemost in 2010 and Dr. Barry Coller from Rockefeller University is working with his team to make it web-accessible for research studies. A proposal was presented for an initial validation study. It has to be decided whether this validation study will be a project within the VWF Subcommittee or will be an activity of the new standing committee “Bleeding Assessment Tool”.

- Online bleeding assessment tool

Barry Coller (USA) reported on the Bleeding History Phenotyping Initiative at Rockefeller University and the system they have developed to support the International Society on Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH BAT). He described: 1) the rationale for an ontology structure, 2) the study of 500 healthy individuals conducted with the Rockefeller questionnaire, 3) the ongoing study of 100 individuals with mild bleeding disorders, 4) the elements of the agreement between Rockefeller and ISTH, 5) how ISTH members can access the ISTH-BAT, 6) the results of the first 50 healthy individual using the ISTH-BAT at Rockefeller, and 7) potential cooperative studies that could be conducted with the ISTH-BAT at multiple sites.

**Standardization of assays**

Session Chair: Bernhard Lämmle (CH)

- Working party on standardization of VWFpp assays

Tony Hubbard (UK) reported on behalf of the working party on standardization of VWF propeptide assays. Determination of the ratio for VWF propeptide/VWF:antigen (VWFpp/VWF:Ag) is undertaken to identify conditions associated with decreased half-life of VWF in the circulation (e.g. type 1 von Willebrand disease with increased clearance, acquired von Willebrand syndrome). Although there is an agreed international unitage (IU) for VWF:Ag, there is no agreed IU for VWFpp. Laboratories therefore rely on local or “unofficial” reference preparations for VWFpp estimation with potential for considerable inter-laboratory variability as well as problems of long-term continuity. The SSC/ISTH Sub-committee on VWF has established a Working Party on Standardization of VWFpp Assays with the following objectives:
- to assess the inter-laboratory variability of VWFpp (and VWF:Ag) estimates
- to calibrate a reference plasma with an agreed unitage for VWFpp

These objectives were addressed through a multi-centre study where 13 laboratories tested two common freeze-dried plasma samples (WHO 6th IS FVIII/VWF Plasma and SSC/ISTH Secondary Coagulation Standard Lot #3) for VWFpp and VWF:Ag relative to their local reference materials.

The inter-laboratory variability for VWFpp estimates was surprisingly low for both common samples (GCVs 8.1%, 8.8%) considering that different local reference materials were used in each laboratory. This variability was reduced even further (GCV 3.0%) when estimates were calculated relative to the same common sample (SSC Lot #3 vs WHO IS) indicating that the different methodologies for VWFpp estimation are in generally good agreement and that a common reference material/unitage could further improve agreement between laboratories. The low inter-laboratory variability for VWFpp estimates also provides the opportunity to assign consensus mean values to both the WHO 6th IS and the SSC Lot #3 of 1.03 IU/ampoule and 1.01 IU/ml respectively. The inter-laboratory variability for VWF:Ag estimates, relative to local reference materials (GCVs 12.2%, 12.0%), was larger than that found for VWFpp estimates despite the availability of the WHO IS for VWF:Ag. This large variability is probably caused by inaccurate calibration of the local reference materials rather than different methodologies since the variability was greatly reduced (GCV 3.5%) when all laboratories compared the same samples (SSC Lot #3 vs WHO IS). This study indicates that increased agreement between laboratories in the estimation of the VWFpp/VWF:Ag ratio may be partially achieved through the establishment of a WHO IS for VWFpp but will also need better harmonisation in the calibration of local references for VWF:Ag.

Project will continue for another year to calibrate SSC Lot #4. SCC Official communication will be written.

- Variation in ADAMTS13 activity assays and the need for standardization

Ian Mackie (UK) reported on the comparison of the commonly used clinical ADAMTS13 activity assays: collagen binding assay (CBA) using full length VWF substrate, in-house Fret-VWF73 assay, commercial Fret-VWF86 and chromogenic VWF73 assays in 159 citrated plasmas from healthy normal subjects and patients being investigated for thrombotic microangiopathies (TMA). They found that:

1. Frozen aliquots of pooled normal plasma, QC and normal subject plasmas gave better agreement between assays than TMA samples.
2. Lyophilised commercial calibrants gave CBA values (probably due to stabilisers such as HEPES).
3. The CBA assay had lower mean values than the peptide substrate assays. Agreement between assays was best in samples with <11% or >55% activity. Only 46% of samples with moderate ADAMTS13 deficiency (11-55%) showed agreement between assays.
4. There were some differences between in-house and commercial assays that could have been due to the accuracy of calibration.
Substrate type, denaturing reagents, protease inhibitors, incubation times, dilution factor, diluent, calibrant, gene mutations, antibody subsets, icterus, haemolysis, lipaemia, abnormal amounts of VWF, factor H, and FVIII could impact differently on each assay. It is unclear which assay has the best clinical utility, but it is important that the same assay is used throughout the clinical management of a patient. The development of an International reference preparation and formal EQA schemes are needed.

- A 10 min-assay for ADAMTS13 activity with an automated biochemistry analyzer

Dr S. Kato (JP) presented the results of a newly developed automated ADAMTS13 activity assay that makes use of colloidal gold particles coated with antibodies (either N10 antibody - monoclonal antibody that specifically recognizes Y1605, which is the C-terminal edge residue of the enzymatically cleaved VWF-A2 domain – or anti-GST antibody) and the substrate GST-VWF73-His. The aggregation of the colloidal gold particles is followed as a measure of the level of cleavage of the substrate by ADAMTS13. Assay has very good inter- and intra-assay variability.

- ADAMTS13 activity assays: Comparison of different methods

Johanna Kremer Hovinga (CH) showed that despite improved ADAMTS13 activity assays we still have variation between different ADAMTS13 activity assay results. Discussed assays are: an improved FRETS-VWF73 assay and a multimer degradation assay. A few examples were shown to illustrate what we can learn from discrepant results. She concluded that we don’t need better assays, but that we have to learn what we can learn from discordant ADAMTS13 activities.

Based on the results of these presentations on ADAMTS13 activity it was suggested to explore the possibilities for the development of an international reference preparation for ADAMTS13 activity. Johanna Kremer Hovinga (CH) will make a proposal for a study and submit that to the subcommittee.

- Collagen binding assay, current status

Jerry Koutts (AU) gave a short update on the Collagen Binding assay and its use as a supplementary assay for VWF activity. The ristocetin cofactor assay represents the main functional assay in general laboratory use, but suffers from relatively high performance time and complexity, poor reproducibility and poor sensitivity for low levels of VWF. The collagen binding assay substantially reduces the diagnostic error rate in VWD when the usual test panel of VWF:Ag, VWF:RCo and FVIII:C is used. This benefit was originally highlighted by data from the RCPA Haematology QAP and recently confirmed by a NASCOLA study. Standardisation issues are currently noted to hamper the broader utility of the assay and commercial kits differ in efficacy.

- Performance data of a particle enhanced VWF activity assay with no need of ristocetin

Juergen Patzke (DE) presented data on the performance of a new ristocetin-independent VWF activity assay (Not available for sale in the US). Fully automated assay applications on several
hemostasis instruments were developed. Reagents are liquid and ready-to-use, the measuring range covers 4-600% for most instruments, the limit of detection is below 2.2 % VWF and Within Device CVs are in general between 2 and 7%. The correlation to the VWF:RCo assay is excellent. All types of von Willebrand disease show a very good comparability of the activity/antigen ratios of the new assay and the VWR:RCo assay.

Based on this presentation it was suggested to make an inventory of all available VWF ‘activity assays’ and to have a comparative study between the different methods. Also nomenclature for the new assays should be decided upon. A potential protocol for such a study will be designed by Juergen Patzke (DE), Reinhard Schneppenheim (DE), Imre Bodo (HU) and Flora Peyvandi (IT) and then submitted to the subcommittee for approval.

**VWF and VWD registries**

Session Chair: Imre Bodo (HU)

- Platelet Type–VWD registry/database (www.pt-vwd.org/)

Unfortunately, Dr Maha Othma (CA) was unable to attend, but she provided us with the following written update on the Platelet Type–VWD registry/database. An international prospective/retrospective PT-VWD study (Hamilton et al, Thromb haemost 2011) reported that PT-VWD constitutes 15% of diagnosed type 2B VWD worldwide. 24% of cases thought to have type 2B VWD don’t show mutations in either genes (VWF or GP1BA). Clinical and laboratory-phenotype assignment remain to be refined worldwide. The PT-VWD registry is available and maintained at www.pt-vwd.org: The registry shows 55 reported cases worldwide, 36 of which are females. To date, 4 mutations reported: three within the VWF binding domain of the GP1BA gene: Gly 233 Val, Met 239 Val, Gly 233 Ser and one (27bp deletion) outside this domain. A fifth mutation; Asp 235 Tyr was recently identified by the Iranian Comprehensive Haemophilia Care Centre (personal communications, added to the registry). Flow cytometry test for diagnosis is published. PT-VWD plasma samples are needed for standardization exercises. PT-VWD animal model is available and is helpful to understand the disease pathology as well as phenotype.

- VWF database (www.vwf.group.shef.ac.uk/)

Dan Hampshire (UK) gave an update on the activities regarding the VWF database. Two major alterations have been made to the online locus-specific database for von Willebrand factor (VWFdb) over the last year: 1) A VWFdb steering committee, consisting of 8 members from 6 different countries, has been established (Dan Hampshire (UK), Anne Goodeve (UK), Reinhard Schneppenheim (DE), Paula James (CA), Dan Bellissimo (US), Luciano Baronciani (IT), Pierre Boisseau (FR) and Steve Keeney (UK)). This committee will oversee classification of VWF variants submitted to VWFdb and will recommend enhancements to VWFdb. 2) A new VWF mutation/polymorphism registry has been created in the Leiden Open Variation Database (LOVD) format following steering committee consultation. The new registry will go live in September 2011 and will contain previously unavailable fields for phenotype documentation, along with a “live summary” of sequence variants on the database and will simplify submission
of variants to VWFdb. In addition to these two major alterations, several other modifications have been proposed and were discussed.


- International registry on acquired von Willebrand syndrome (www.intreawws.com)

Augusto Federici (IT) gave an update on diagnosis and treatment of AVWS. He discussed a recent paper on this (Blood 2011;117:677-6785). During 2011 the website is being re-organized and updated. Ongoing.

**Multicenter studies on VWD**

Session Chair: Reinhard Schneppenheim (DE)/Jorge DiPaola (USA)

- European Project on type 3 VWD

Augusto Federici (IT) presented the aims of the project on type 3 VWD (acronym of the study 3WINTERS-IPS). To enroll 300 VWD type 3 patients in Europe and Iran. The study will run for 5 year and will include a 2 year follow-up of each patient to evaluate the frequency and risk of bleeding. From all patients plasma and DNA will collected.

- EUVWD Cooperative Group

Ian Peake (UK) summarized the goals and activities of the EUVWD Cooperative group. The group was formed by the MCMDM-1VWD Partners following the formal end of the project in 2006 and is responsible for the data and samples resulting from the MCMDM-1VWD project. To date the group has published 16 papers based on the MCMDM-1VWD data and several papers are in preparation.

A project on a novel GPIb binding assay is underway. Working with Augusto Federici and collaborators in establishing the EU/Iran type 3 VWD study (VWD-3WINTERS-IPS). Collaborating with the NIH funded Zimmerman VWD PPG. Considering producing a Principles of Care of VWD document in collaboration with EAHAD. Current membership: Ian Peake (Co-chair), Giancarlo Casterman (Co-chair), Anne Goodeve (Secretary), Francesco Rodeghiero (past chair), Agnes Veyradier, Alain Gladisseur, Imre Bodo, Jeroen Eikenboom, Erik Berntorop, Augusto Federici, Flora Peyvandi, Jorgen Ingerslev, Jenny Goudemand, Javier Batlle, Frank Leebeck, Mike Laffan, Pier Mannicci, Reinhard Schneppenheim, Alberto Tosetto, Ulrich Budde.

- Zimmerman Project (ZPMCB-VWD)

Bob Montgomery (USA) reported on the status of recruitment and frequency of mutations observed in the ZPMCB-VWD project. Until now, 570 index cases have been recruited, including all subtypes. Comparable to previous European and Canadian type 1 VWD studies mutations are very likely to be found when VWF levels are below 20 IU/dL. A striking finding
was the very high frequency of a type 2N mutation (H817Q) in African-Americans: 14% heterozygotes. The factor VIII binding defect is mild, even in homozygotes. The frequency of the well-known R854Q mutation in Caucasians was 2.5% heterozygotes, confirming earlier European data.

- Potential importance of the CHARGE loci in VWD patients

David Lillicrap (CA) reported on the results of recent genetic analysis of several large type 1 VWD cohorts which suggest that genetic loci other than VWF likely contribute to low plasma VWF levels. These contributions may be as either the primary pathogenic locus or as genetic modifiers of the type 1 phenotype. The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) consortium has recently published the results of their meta-analysis of 5 large genome-wide association studies evaluating genetic associations with plasma levels of FVII, FVIII and VWF. Results on the discovery population of 23,608 were replicated in a second independent population of 7,604. In addition to confirming the role of ABO and the VWF, 5 novel loci were identified in both the discovery and replication studies: STXBP5, SCARA5, STAB2, TC2N and CLEC4M. These results suggest that additional genes associated with both VWF biosynthesis and clearance influence plasma levels of the protein. Studies to further evaluate these associations are now underway.

- VWD International Prophylaxis (VIP) Study

Tom Abshire (USA) reported that there are currently 54 Centers participating in this study, 30 from Europe, 23 from North America and 1 from Asia with 117 patients enrolled in the three arms of the study (64 retrospective, 42 GI natural history and 11 prospective). Initial data for the retrospective study was presented as an oral presentation at ASH, 2010 and a manuscript is in final preparation. For the ASH presentation, there were 42 patients studied (60% type 3 VWD) with equally distributed prophylaxis indications (epistaxis = 10; joint bleeding = 9; GI bleeding = 8; other = 15). The median number of infusions per week = 2 and the median infusion dose = 43 U VWF:RCo/kg. For all indications, annualized bleeding rates decreased from a median of 12 bleeds (pre-prophylaxis) to 3.8 bleeds (during prophylaxis; p < 0.0001). There were two patients who developed an inhibitor; one before initiating prophylaxis and the other while on prophylaxis. The positive effect of prophylaxis on bleeding was similar for both children and adults. The VIP study continues to enroll patients.

- WIN (Willebrand in Netherlands)

Frank Leebeek (NL) reported on the Willebrand in the Netherlands (WiN) study, a nation-wide study of patients with moderate and severe von Willebrand disease (VWD). A total of 806 individuals, both children and adults, have been included. Of these individuals data on bleeding history was obtained by questionnaire. In addition blood was obtained from 649 VWD patients and DNA is available of 725 patients. VWF parameters have been measured in a central laboratory. We recently studied quality of life in both paediatric patients and adults included in the WiN study and observed a significant decrease of QoL in VWD patient compared to reference populations, especially in type 3 patients (De Wee, EM, et al JTH 2010, JTH 2011). In the next years we will investigate the variability in VWF levels and bleeding phenotype in VWD
patients. Our hypothesis is that this may in part be determined by genetic variations that influence VWF:Ag levels in healthy individuals, as has recently been discovered by the CHARGE consortium (Smith N, et al. Circulation 2010). Furthermore studies on specific patients groups within the WiN study population (women, children, elderly) will be performed.
Welcome and Introduction of Co-chairpersons (S. Eichinger)

Sabine Eichinger welcomed all participants also on behalf of the co-chairpersons. She provided an overview of the program and reasons for changes in the program as Benjamin Brenner and his coworker Yona Nadir as well as Claire Philipp could not attend the meeting.

Educational Session (S. Eichinger)

Educational activities are an integral part of the work of the SSC on WHITH. This year’s Educational Session covered three topics:

- Management of thrombocytopenia during pregnancy - A. James, USA
- Diagnosis of VTE during pregnancy - M. Huisman, The Netherlands
- Management of obstetric hemorrhage – C. McLintock, New Zealand

These topics were chosen because of relevance and also to avoid overlap with State-of-the-Art lectures within the congress.

The session was very well attended and a large proportion of attendees continued to stay at our meeting.

Other Educational Activities

Sabine Eichinger excused Benjamin Brenner and briefly mentioned his activities regarding the organization of the 4th International Symposium on Women’s Health Issues in Thrombosis and Haemostasis, which was held in February 2011 in Berlin.

The SSC will also try to expand educational activities within the ISTH in particular to reach out to low resource countries and to address their needs.

Menorrhagia Working Group
The menorrhagia working party has very successfully been led by Claire Philipp together with Peter Kouides, Rochelle Winikoff and Rezan Kadir. Claire Philipp had to cancel her trip in the last minute due to personal reasons. One of the major issues within the group is the validation of the “Bleeding Assessment Tool” which has been developed in a joint project with the Subcommittee on vWF and Perinatal/Pediatric Thrombosis and Hemostasis. One approach would be to use the US prospective data collection in women which was presented by Roshni Kulkarni also on behalf of Peter Kouides.

Databases would also available from Canada as presented by Rochelle Winikoff.

Sabine Eichinger and Claire Philipp are now active in the “Bleeding Assessment Tool Standing Committee” of the ISTH and will pursue validation possibilities of the score.

Rezan Kadir gave an update on activities to improve prenatal diagnosis of haemophilia and presented innovative methods.

Symposium in Honour of Margareta Blombäck

Margareta Blombäck retired as a co-chairperson. She was a major force in the initiation of the SSC on WHITH. That is why we decided to honour her contributions to the society and to our SSC in particular by a special symposium. Jacqueline Conard hosted the session and provided an overview of the enormous achievements of Margareta Blombäck and also a history of the “birth” of our SSC.

Margareta then shared original data from her own lab on the fibrinolytic profile in patients with myocardial infarction with the audience.

Takao Kobayashi eluded on the problem of fibrinogen deficiency during pregnancy.

The symposium was overall well perceived and attended by many long time friends of Margareta.

SSC projects

Diagnosis of VTE during pregnancy is one of the main projects of our SSC. Several presenters were originally planned to contribute to this topic, but could not attend the meeting for personal reasons. Thus, Menno Huisman covered all these presentations in one talk. He introduced the activities with regard to clinical studies that have been going on or have been initiated since last years meeting.

We will continue with this project and enforce collaboration within these studies as these are rare conditions and adequate studies can only be accomplished by multicenter activities.

Finally, Saskia Middeldorp introduced a new study (HIGHLOW) to investigate two different doses of LMWH for preventing recurrent VTE in pregnant women. Again, rare events that will need the contribution of many investigators to fulfil the required number of patients.
The presentation on heparanse procoagulant activity in oral contraceptive users and pregnancy had to be cancelled because of the absence of Yona Nadir.

**Update on Registries**

Andra James provided the current status of registries. There are currently no active registries of our SSC. As explained by Andra James, conduct of registry in general is cumbersome because of lack of funding, legal issues (uncertainties about IRB approval, patient consent), IT aspects and adherence of participating investigators.

**Closing Remarks (S. Eichinger)**

**End:** 1:10 pm
Working Group on Coagulation Standards

24 July 2011

Chairman: Anthony Hubbard

Review of Lot #3 (A Hubbard)

Between July 2010 and end June 2011 a total of 8,840 vials of Lot #3 were issued. Orders were despatched to 21 different manufacturers and 1 external quality assurance scheme. Lot #3 was also used in two collaborative studies (calibration of Lot #4 and the calibration of von Willebrand factor propeptide). Since the issue of Lot #3 commenced in 2006 approximately 48,000 vials have been despatched – this leaves remaining stock of approximately 6,700 vials. At the current rate of issue it is expected that Lot #3 stocks will be exhausted in Q2 2012. The stability testing of Lot #3 comprised both accelerated degradation testing and real-time studies, both indicated excellent stability with predicted mean loss of <0.1 % per year for all of the four test analytes (FV, FVII, FVIII, FIX). The results of this study have been published as an Official Communication of the ISTH (AR Hubbard, S Kitchen, M Beeharry, SA Bevan, A Bowyer. Long-term Stability of the Scientific and Standardization Committee Secondary Coagulation Standard (SSC Lot no. 3), J Thromb Haemost 2011; 9: 1246-8).

Experience of EQA schemes with Lot #3

UK NEQAS (S Kitchen)

Eight vials of SSC Lot #3 were used for “trouble-shooting” purposes between May 2010 and June 2011 relating to assay issues for Protein C activity, Antithrombin and factors II, V and VIII. Use of Lot #3 by one laboratory revealed that their discrepancy for Protein C activity estimates was linked to the routine reference plasma. After re-calibration, their difference from the mean was reduced from 9% to only 3%. A similar improvement was also found for estimates of factor V in a different laboratory where the difference from the median was reduced from 16-19% to 5-9%. Dr Kitchen then reviewed the results from NEQAS surveys based on reference plasmas with the understanding that many laboratories use reference plasmas and reagents from the same manufacturer hence it is not possible to distinguish between differences caused by the reference plasma or the reagents. Some manufacturers had not yet calibrated reference plasmas for FXI or FV relative to the WHO International Standards. The largest differences of around 20% were found between reference plasmas for FV and FVIII, however, some of this difference could be caused by the use of different reagents.

College of American Pathologists (J Teruya)

SSC Lot #3 has been issued in two surveys in the past year – one for VWF estimation and one thrombophilia survey. Dr Teruya presented the data in terms of methods-specific bias from the assigned value. A bias of greater than 10% was found for some methods for the following analytes: FVIII, Protein C activity (clotting 20%; chromogenic 11%), Protein S activity and Protein S total antigen. One clotting method for Protein S activity has produced a 20% bias in
surveys performed over the last 3 years. Inter-laboratory variability did not exceed a CV of 20% for any analytes tested in 2011.

**Progress towards Lot #4**

**Results of the calibration exercise (E Gray)**

Dr Gray presented the results for the calibration of 20 analytes relative to the relevant WHO International Standards (Table 1). One new analyte (FXIII antigen) was included in the calibration of SSC Lot #4. The number of laboratories in the calibration exercises ranged from 8 to 29 and inter-laboratory variability (GCV) ranged from 2.3% (Antithrombin function) to 14.2% (VWF:Ristocetin cofactor). All participants in the collaborative studies and members of the Executive Board of the Working Group have agreed with the proposed assigned potencies. Final approval of the assigned values will be sought in the SSC Business Meeting on 27 July 2011. SSC Lot #3 was also included in the calibration exercise to enable a comparison of estimates obtained in the present study and the original calibration of Lot #3 in 2005. No significant difference (p>0.05) was found for 14 out of 19 analytes. For 4 analytes the significant difference was associated with a difference between means of 6% or less. For Protein S free antigen there was a difference of 8% which was probably associated with replacement of the primary WHO standard since the original calibration. Overall there was good agreement between the original calibration and the present study consistent with the excellent stability record for SSC Lot #3.

**Lot #4 stability testing (A Hubbard)**

The stability of Lot #4 will be assessed through an acceleration degradation study. Vials have been stored at elevated temperatures since December 2009 and the first tests were carried out in February 2011. Four analytes (FV, FVII, FVIII, Antithrombin activity) were tested by NIBSC and the Royal Hallamshire Hospital, Sheffield, UK. The relative residual activities after storage at +45 C indicated greatest loss for FV and FVIII, however, the calculated predictions of loss were contradictory indicating greater stability for these two analytes compared to FVII and Antithrombin. The robustness of the predictions was compromised by the small amount of data and the limited loss of activity for FVII and Antithrombin. It was agreed that more testing should be performed in Q4 2011 in order to provide more reliable predictions on which a shelf-life for Lot #4 could be based.

**Table 1  Proposed assigned values for SSC Lot #4**

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<th>Value (IU/vial)</th>
<th>Inter-lab variability (GCV%)</th>
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<td>Factor</td>
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<td>Antigen(A2B2 complex)</td>
<td>von Willebrand Factor</td>
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