50th Scientific and Standardization Committee Meeting

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# Table of Contents

Animal, Cellular & Molecular Models ........................................................................................................... 2

Biorheology ........................................................................................................................................................ 4

Control of Anticoagulation Subcommittee ........................................................................................................ 6

Disseminated Intravascular Coagulation (DIC) ................................................................................................. 14

Registry of Exogenous Hemostatic Factors .................................................................................................... 21

Factor VIII and Factor IX .................................................................................................................................. 22

Factor XIII and Fibrinogen Joint Meeting ......................................................................................................... 36

Fibrinolysis .......................................................................................................................................................... 40

Haemostasis and Malignancy ........................................................................................................................... 43

Lupus Anticoagulants/Phospholipid-Dependent-Antibodies ........................................................................... 47

Perinatal/Pediatric Haemostasis ....................................................................................................................... 56

Plasma Coagulation Inhibitors ......................................................................................................................... 60

Plasma Kallikrein-Kinin System (Formerly Contact Activation) ..................................................................... 63

Platelet Immunology ........................................................................................................................................ 71

Platelet Physiology ............................................................................................................................................. 75

Predictive Haemostatic Variables In Cardiovascular Disease ......................................................................... 77

Von Willebrand Factor ....................................................................................................................................... 79

Women's Health Issues ..................................................................................................................................... 87

Working Group on Vascular Biology ............................................................................................................... 90

Working Group on Coagulation Secondary Standards .................................................................................. 93
Animal, Cellular & Molecular Models

Chairman: P. Jagadeeswaran, USA
Co-chairs: G. Johnson, USA; C. Kluft, The Netherlands; T. Nichols, USA

The meeting was attended by 50 persons and the Drs. Johnson, Nichols, Kluft and Jagadeeswaran were present. The sessions were chaired by Drs. Jagadeeswaran and Johnson. There were a total of 9 presentations. The following are brief summaries of these presentations.

**Barbara Furie** (USA): Dr. Furie presented her work on the molecular basis for the ferric chloride and laser induced thrombosis in mice. The ferric chloride injury exposes collagen surface while in the laser induced thrombosis, the tissue factor was prominent at the site of the injury.

**M. Dewerchin** (The Netherlands): Dr. Dewerchin presented data on gene targeting studies on vascular abnormalities in neurological phenotypes. Low levels of VEGF in mice were associated with paralysis and axonal loss in sciatic nerve. The onset of paralysis was accelerated by SOD. She also presented data on haplotypes in VEGF gene promoter in ALS patients and found an association to a specific haplotype.

**Tim Nichols** (USA): Dr. Nichols presented sensitivity of assays to measure canine factor IX and factor VIII. He also presented historical perspectives of these assays and their applicability to the study of hemophiliac dogs.

**Pudur Jagadeeswaran** (USA): Dr. Jagadeeswaran presented an overview of zebrafish hemostasis and thrombosis and emphasized the significance of the zebrafish as a genetic model to study thrombosis. He presented data on isolation of zebrafish mutants such as Victoria, Leopold and Nicholas by using laser induced thrombosis assay. He also described the discovery of thrombocyte microparticles and their possible role as initiators of thrombus formation.

**Bruce Furie** (USA): Dr. Furie presented the data on microparticles, their measurement and role in thrombosis. An impressive series of videos presenting the accumulation of endothelial, leukocyte and platelet microparticles in the growing thrombus.

**M. Jirouskova** (USA): Dr. Jirouskova discussed methods for the measurement of platelet function in mice. She emphasized the importance of the method of obtaining blood. Samples obtained from retro-orbital vessels or the vena cava are most satisfactory. Platelet counts in mice must be performed with knowledge of their high counts, small size and strain variability. Platelet activation and aggregation studies have been performed using techniques suitable for human platelets, but a platelet-monocyte aggregation requires a better definition.

**Susan Smyth** (USA): Dr. Smyth presented comparison of human and mouse fibrinolytic components with respect to structures and function. She also reviewed the effects of gene knockout experiments in mice and insights gained in role of uPA, tPA, uPAR and tPAR.
**E.M. Muchitsch** (Austria): Dr. Muchitsch reviewed various coagulation assays in mice such as factor X assay using peptide substrates, factor IX, factor VIII one stage clotting assays. Standardization was performed using human deficient plasmas and verified by using factor deficient mouse plasma. She also presented vWF multimer assays. She discussed the limitations of tail bleeding time.

**Gerhard Johnson** (USA): Dr. Johnson presented the interesting results of the working party on Mouse Hemostatic and Thrombotic Parameters. Contributions to the work of the working party have been obtained in three major categories; platelets, plasmatic coagulation and fibrinolysis. Work on this project is nearing completion, a manuscript is being prepared.

New Business: The report of the working party on Mouse Hemostatic and Thrombotic Parameters was accepted by the Subcommittee for submission, on final completion, to the SSC for approval as an official SSC communication.
Biorheology

Chairman: J.J. Zwaginga, The Netherlands
Co-chairs: T. Diacovo, USA; S. Diamond, USA; E. Grabowski, USA; M. Hoylaerts, Belgium;
G. Nash, UK

The Biorheology SC meeting was divided into 2 distinct parts, the first focusing on methods to
study cell/surface interactions under flow and the second on flow modulated molecular
interactions and devices to study drug efficiency during patient treatment.

*Ex vivo* perfusion studies on the function of endothelial PSGL-1 on monocyte margination,
adhesion, rolling velocity and cluster formation were presented by dr. Zwaginga. Dr. Vink, a
biophysicist, presented challenging data from intra-vital studies, showing an influence of the
endothelial glycocalyx on leukocyte adhesion to the endothelium. The impact of this ‘gel-
like’ structure on the interaction between adhesion molecule/ligand interactions is evident from
its vascular volume and layer thickness, covering the endothelial cell layer. Many studies on
cultured EC, that lack most of the glycocalyx unless cultured under flow, have to be reconsidered
in this light. Endothelial (chronic) activation seems to modulate the glycocalyx composition
considerably, also in vivo, further adding to the necessity to take the glycocalix into
consideration. Gerard Nash made an effort to delineate many experimental factors to be
considered during ex vivo perfusion assays. Especially, the role of red blood cells concentration,
their low-shear aggregation and thus effective particle size on margination of leukocytes and
blood platelets was highlighted. Moreover, the shear rate and shear stress as often hard to predict
in non-Newtonian fluids, such as blood. Finally, experimental conditions as temperature,
anticoagulant use and storage time were advocated as modulators of platelet and leukocyte
adhesion. Blood pressure and pulsatility were noticed as modulating factors hardly incorporated
in ex vivo test devices.

In the second part, Marc Hoylaerts started out to show that important cardiovascular drugs as
aspirin, GPIIb IIIa inhibitors and ADP antagonists have different potencies in influencing
platelet adhesion, thrombus formation and preventing embolization. Devices/ methods to test the
possible in vivo efficiency of these or new drugs should therefore be validated for these different
in vivo outcomes. Low shear or static aggregation tests like in the classic aggregometry, study
different mechanisms than those responsible for platelet activity during (high) shear induced
platelet activation (SIPA). Finally, combinations of different platelet inhibitors were shown to
not always have additive effects but sometimes even have mutually neutralizing effects in ex
vivo test systems. Dr. Grabowski dealt with ex vivo measurements that combined (decrypted)
tissue factor induced thrombin and fibrin formation together with thrombus formation and its
localizing influence on further tissue factor containing micro-particles from the circulation. Dr.
Frojmovic centered his attention on the limited integrated knowledge on how different drugs
behave in the different ex vivo devices that test (parts of) the haemostatic response mechanisms:
the aggregometer, the cone-and-plate viscometer, the couette and flow-controlled perfusion chambers.
Within the discussion part, the subcommittee agreed upon producing a review of devices and assays validating existing data in the light of their critical limitations. Especially the known clinical efficiency of validated drugs and their tested behaviour within the different devices might be a valuable basis for this review, providing a biorheological support for their mode of action. It is the subcommittee’s opinion that single pass perfusion systems testing whole blood of patients, are the best possible approximation for the in vivo situation, therefore deserving most attention.

New and ex-members of the SC are and will be (re-)invited to give new focus to the SC. The Biorheology SC will furthermore strive for synergy with the Vascular Biology and Animal Model SC to minimize in vivo model variability and maximize interpretability of vitro testing. To this end, certain aspects discussed in the “Platelet Physiology” subcommittee that pertain to the definition of assays validating drug resistance, should be matched to include defined shear stress evaluations. The physical presence of members of these groups at each others meetings should be made possible by avoiding that these groups meet at the same time during the subcommittee meetings.
Control of Anticoagulation Subcommittee

Chairman: M. Greaves, UK
Co-chairs: H. Bounaumes, Switzerland; J. Harenberg, Germany; C. Kearon, Canada; M. Laposata, USA; J. Olson, USA; F.R. Rosendaal, The Netherlands; S. Schulman, Sweden; A. Tripodi, Italy; A.M.H.P. van den Besselaar, The Netherlands

Working Party on Duration of Anticoagulant Therapy in Venous Thromboembolism.
Chair: S Schulman & C Kearon

Dr Kearon introduced the topic. He reported that there are few new data on optimal duration of anticoagulant therapy since the last SSC meeting and emphasized the importance of a topic not previously addressed by the working party: the lack of consistency in the classification of bleeding in clinical trials of anticoagulation.

1. Classification of bleeding in clinical trials.

European agency for evaluation of medicinal products perspective. S Schulman.

Dr Schulman reviewed the European recommendations and highlighted some inconsistencies. For example the types of bleeds to be included in trials of thromboprophylaxis [fatal bleeds, those resulting in a fall of haemoglobin concentration of 2g/dl or more, transfusion of 2 or more units of red cells, intracranial bleeds, retroperitoneal bleeds, need to discontinue therapy] were described as “can be included” in trials of treatment. Also, intraspinal and intraocular bleeds were included in trials of treatment but not prophylaxis trials.

It was further noted that a review of trials revealed a lack of consistency in the adoption of these criteria. The term “clinically important bleeding” had been adopted, but not in any consistent fashion.

US Food and Drug Administration perspective. C Francis.

Dr Francis reported that there are regulations dictating the interactions between trial sponsors [pharmaceutical companies] and the FDA. However it is clear that classification of bleeding in clinical trials has not been standardized, with no clear distinctions between categories. Discussions with the FDA had confirmed that no guidelines were in use, in contrast to the situation in Europe. However, consistency with previous trials with the same pharmaceutical was encouraged. Reference was made to a review led by G Raskob which had been sponsored by Sanofi. The objective had been to review the definitions used to date and to define standard criteria for use in orthopaedic surgery and, eventually, general surgery. In orthopaedic surgery 62 eligible studies had been identified. These revealed 18 distinct definitions for abnormal bleeding. Only 6 of the 62 reports included the surgeons’ clinical judgement. 21 of 62 had no explicit criteria to distinguish normal surgical bleeding.
from excessive haemorrhage. These factors rendered comparisons between studies extremely difficult.

Classification of bleeding during prophylaxis after orthopaedic surgery. C Kearon

Dr Kearon recommended that surgical site bleeding should be distinguished from bleeding at other sites and emphasized the desirability of inclusion of blinded assessment of bleeding by the surgeon.

Some proposed definitions for clinically important bleeding were introduced:

- Overt bleeding which is deemed by the surgeon to be excessive and which requires transfusion of at least 2 units of red cells and/or results in at least a 2g/dl fall in haemoglobin
- Or/and, results in wound dehiscence, infection, re-operation or prolongation of hospital stay
- Or/and, contributes to myocardial infarction, stroke or death.

However it was noted that even if consistency of definition is achieved it must be recognized that numerous factors influence bleeding risk which may vary between trials, including the patient characteristics [eg age range], operator technique, surgery type and bias of assessors.

Classification of bleeding during anticoagulant therapy. M Prins.

Dr Prins reviewed the definitions used in the TASMAN study. These had resulted in a huge variation in reports of minor bleeding between study sites [0-22%, regardless of treatment]. This is explained by gross observer differences. Although interpretation of major bleeding in the study appeared reproducible and related to clinical impact, determination of minor bleeding was not reproducible.

The speaker went on to discuss the principles which should be used in improving assessment of bleeding in trials. He suggested 2 levels:

- Major bleeds
- Clinically relevant non-major bleeds, that is, bleeds which are unusual in the non-anticoagulated individual and which demand clinical attention.

Examples were given of sets of criteria which could be used to further define these non-major bleeds, eg:

Epistaxis of greater than 5 minutes or requiring nasal packing.
Haematuria which is spontaneous and/or lasts for more than 24 hours.

2. Risks of bleeding in clinical practice

Prediction rules for bleeding on anticoagulant therapy. R Beyth.

The risk factors for anticoagulant-associated bleeding were reviewed, including duration of therapy, increased age, other drug use, hypertension, malignancy and renal failure. Two prediction models were described: OBRI [Outpatient Bleeding Risk Index, Beyth et al, 1998] and that described by Keijer [1999]. Each has somewhat different criteria and has been applied in a separate sizeable development and validation cohorts. They performed moderately well in prediction of bleeding. OBRI has also been compared with “physician estimates” of risk and superiority of the model was demonstrated, physician estimates being no better than chance.

Dr Beyth concluded by recommending the use of a predictive model in clinical practice, alongside assessment of other relevant criteria such as compliance and anticoagulation stability.

Proportion of major bleeds which are intracranial or fatal according to indication. L Linkins.

Dr Linkins presented the preliminary findings of a meta-analysis of intracranial bleeds during oral anticoagulant therapy. Defined methods were used to review publications of treatment of at least 6 months duration. 9% of major bleeds and 30% of fatal bleeds were intracranial, proportions which are lower than previously considered. An attempt was made also to relate intracranial bleeding to indication for therapy [atrial fibrillation, CVA, CAD, VTE and valve prosthesis]. Although there were some differences in the mean proportions of intracranial bleeds, confidence intervals were wide, with no firm evidence of a relationship between bleeding at the intracranial site and indication for therapy.

Summing up:

Dr Schulman concluded:

- The same definitions for bleeding should be used throughout a program of research with a particular anticoagulant, as a starting point in standardization
- Objective criteria are required which consider the impact of a bleeding episode
- A list of sites of bleeding should be drawn up with definitions of significant bleeding
- “Bleeding leading to discontinuation of therapy” should not be within the major bleeding category, but should be reported separately from bleeding complications

During subsequent discussion there was debate over the difficulties in defining the importance and size of bleeds and comment on potential bias introduced in centres practising autologous predonation, through a lowered threshold for transfusion.

Dr Schulman agreed to undertake further work on these issues, on behalf of the SSC, with a view to improving standardization of criteria for bleeding in clinical trials of anticoagulants.
**Working Party on Standardisation of Methods to Determine Direct Thrombin Inhibitors.**

Chair: M Greaves, MM Samama

Progress on the collaborative study for the validation of the determination of thrombin inhibitors in patients: summary of findings and presentation of a draft publication. J Harenberg/E Gray

A report was given on the use of a range assays on samples from subjects on hirudin or argatroban: APTT [both local and central reagents], ECT [with wet and dry reagents], anti-IIa ELISA and hirudin ELISA.

The wet ECT produced wide cv’s, due to instrument variability. The ELISA also produced wide cv’s. Although the sensitivity of the APTT was lower than that of the dry ECT there was good reproducibility between laboratories. The dry ECT performed poorly with high inhibitor concentrations

*A draft publication has been prepared and will be submitted to the Chair of the SSC subcommittee for approval prior to publication as a full paper.*

Assessment of the antithrombotic effects of direct thrombin inhibitors and LMWH in non-anticoagulated human whole blood. J Harenberg

A method for measurement of TAT and PF4, by ELISA, in blood incubated for 30 minutes at 37 degrees prior to addition of an anticoagulant and antiplatelet cocktail was described. Samples studied were from subjects treated with LMWH, fondaparinux, ximelagatran or warfarin at defined times after dosing. Differences were noted dependent upon type of anticoagulant. In general the direct and indirect thrombin inhibitors were more effective at reducing TAT generation ex vivo under the conditions used.

These results are to be submitted for publication.

Establishment of a Working Group on Thrombin Generation Tests. E Gray/A Lawrie

It was noted that, although TGTs are of interest to the activities of 4 of the SSCs, standardization remains an issue. Proposals were described for the establishment of a Working Group, with membership spanning the relevant SSCs, to take this issue forward. The remit will be to examine the various methods and protocols, including pre-analytical variables, trigger used, phospholipid concentration, substrate employed and quantification modality, and to develop recommendations for standardization.

*The group will report on this project at the 2005 SSC. It will be preferable for this activity to be centred within a single SSC, to be decided.*

Validation and standardization of thrombin generation assays. MM Samama
The wide range of assay variables was highlighted [see above]. Data were reported, from the speaker’s laboratory, on assay performance. Acceptable variations in lag time, ETP, t max and c max were reported. The need for standardization of platelet count was noted, as were differences in results using fresh and frozen-thawed plasma. There were variations between results in healthy volunteers which were not abrogated by use of tissue factor activation. Dr Samama went on to present data on samples spiked with anticoagulants in vitro.

**Standardisation of measurement of unfractionated and low-molecular-weight heparins.**
Chair: M Greaves

**Working Party on Optimal Methods for Assay of Fondaparinux. E Gray.**

A study designed to assess the comparability of anti-Xa assays for the measurement of fondaparinux was described. Data from 6 laboratories, including 3 clinical laboratories, were reported. 5 coded plasma samples were examined.

The results demonstrated low intra-laboratory variation for commercial kits, with greater variation at lower concentrations of fondaparinux. Variation was greater for in-house assays. Inter-laboratory variation was 13-39%. This did not appear to be related to standard used or the pooled plasma employed in calibration.

*These results are to be recorded in a short report which will be submitted for SSC approval.*

Dr Gray recommended the performance of a second study, on patient samples.

**A new heparin binding assay for the quantitation of heparin and LMWH. M Mostert.**

Dr Mostert presented data on a new commercial assay which employs a novel heparin-binding protein. The assay is designed to measure heparin regardless of anticoagulant activity.

**Development of Prothrombinase Induced Clotting Time [PICT] and its validation in the monitoring of anti-Xa and anti-IIa drugs. D Hoppensteadt.**

The assay method was described, in which an activator with a defined amount of Xa, RVV and phospholipids is added to plasma. The assay was applied to samples with heparin, LMWH, and anti-thrombins. Compared with some other assays there was high sensitivity to anticoagulants, including in samples from in vivo studies in primates and humans.

Further studies in clinical situations are planned.

**Monitoring of LMWH using a Point of Care Device. A El Rouby.**

A study of a new approach to heparin assay using a POCT device in the context of patients on LMWH who require a cardiac catheterization procedure was described. 41 subjects were included. All patients had a prolonged clotting time compared with non-anticoagulated
controls. There was good correlation with anti-Xa assay. The new approach may have applications where a rapid indication of clotting time in subjects treated with LMWH is desirable.

Development and standardization of native whole blood, fibrinopeptide A, thrombin-antithrombin complex and prothrombin F1.2 generation tests for the potency evaluation of anti-Xa and anti-IIa drugs. J Fareed

Dr Fareed presented results on FpA generation and other end-points in relation to a range of anticoagulant therapies. He went on to report on preliminary data on a proteomics approach to the study of generation of coagulation proteases.

An update on the development of generic versions of LMWH. J Fareed

Following on from the 2003 SSC Dr Fareed reiterated his concerns regarding the introduction into clinical practice of LMWH with possibly different chemical characteristics from the licensed forms. He expressed the view that current regulations are inadequate, in relation to this issue. The chairman enquired whether the measured differences were within the range of variation between currently licensed products and the response was in the affirmative. Despite this there was some support from the audience for further work on this topic. A submission will be made to the SSC and a decision taken whether this is a legitimate area for future activity by the committee.

Chair: T van den Besselaar/A Tripodi

Introduction of a combined approach to Point of Care Testing PT monitors by IFCC/ISTH/SSC. J Jespersen/FE Preston

Dr Jespersen reviewed the advantages of POC testing and highlighted the problems in establishing mechanisms for internal and external QC.

He noted the establishment of an SSC working party on POCT in 1999, although in response to a question from the floor it was acknowledged that no report had yet been produced. The speaker recommended a joint ISTH/IFCC approach to development of QC with the aims of the SSC working group being amalgamated into this strategy.

The issue of appropriate representation on these groups was raised during discussion. The point was made that POCT for warfarin control was in widespread use, mostly under guidance of community physicians in many countries, but there is no representation from community physicians nor patients, on the proposed working group.

INRs determined following local calibration of coagulometers using certified plasmas: Routine use by participants in the UK NEQAS scheme. S Kitchen.
Dr Kitchen described a study [laboratory coagulometer, not POC based] to address 2 questions:

Do commercially available certified plasmas comply with current SSC recommendations?

Do laboratories using these have better results?

In the QA scheme local calibration was reported by over 100 laboratories. 3 types of calibrants were in use with a wide range of reagents and instruments. Results were generally good and similar for local and manufacturers’ ISIs. Although manufacturers are not yet fully compliant with SSC recommendations it was noted that the full guideline is not yet in print.

Quality Assessment of 2 point-of-care whole blood PT-monitoring systems. M Keown

The speaker reported on anonymised data from CoaguChek and TAS instruments. Reference was made to a recent publication from the group [British Med. J 2003] on discrepancies between displayed and actual INR. In the current report results were encouraging with small sets of 3 or 5 QA plasmas. It was reported also that sets of 5 ECAA QA plasmas have been prepared for use.

Collaborative study on quality assessment of CoaguChek point-of-care PT monitors using ECAA plasmas. C Schiach/T van den Besselaar.

A proposal for an extensive QA study was described in which 5 ECAA plasmas will be tested twice yearly. Patients using POCT will be recruited, although testing will be performed under laboratory conditions. There will be a schedule for earlier repeats if results are discrepant.

The characteristics of the QA plasmas prepared by the ECAA were described.

During discussion some concern was raised regarding the requirement for such an approach to QA for POCT. It was argued that clinical studies continue to show highly satisfactory outcomes in subjects who have adopted POCT for oral anticoagulant management, with the QA arrangements currently in place.

Candidates to replace RBT90: Progress. T van den Besselaar/A Tripodi

It was reported that the planned schedule is being followed. 2 manufacturers have submitted material and 9 of 31 laboratories invited have agreed to assist in calibration, so far. Preliminary stability data on one sample were presented.

*It is anticipated that the replacement material will become available according to schedule, pending satisfactory analysis of characteristics.*

Cost-effectiveness of computer assisted dosing study. L Poller.
Dr Poller reiterated the details of the on-going study. Meaningful data cannot be presented until final analysis has been carried out, to prevent confounding.
Disseminated Intravascular Coagulation (DIC)

Chairman: K. Hoots, USA
Co-chairs: I. Bokarew, Russia; M. Levi, The Netherlands; J. D. Nielsen, Denmark; C-H. Toh, UK; H. Wada, Japan

The DIC Subcommittee of the ISTH met in Venice, Italy, June 18th, 2004 to discuss progress in the implementation of the present iteration of the DIC algorithm. The following sections summarize the steps discussed by the subcommittee, lists the specific ACTION ITEMS that were considered (with assignment of specific responsibilities to subcommittee co-chairs when required).

Action Items (Short-term Objectives)

A. The INR and its Role in Assessment of Coagulopathic States:

Relevance of the INR (in place of or in addition to the Prothrombin as a parameter in the published algorithm of the Subcommittee. [Thromb Haemost 2001;86:1327-30]). This discussion was prompted by observations of several subcommittee members and guests that the clinical laboratories in their respective institutions are no longer reporting Prothrombin time in seconds, listing only the INR results whether or not the patient is receiving anticoagulation with Warfarin. This discussion prompted the following action items by the subcommittee:

1) Dr. Hoots has consulted with other experts in the SSC concerning whether the expressed problems with INR in adequately quantifying a coagulopathy outside the context of anticoagulation apply here. Specifically, Dr. Michael Greaves, chair of the Anticoagulation Subcommittee of the SSC, shares the concerns of the Subcommittee about applying a test that was designed for Warfarin therapy monitoring and subject to variations in both test reagents and instrumentation make its present use in the algorithms problematic. The fact that many labs are switching to thromboplastins with isi’s of approximately 1.0 may make the INR a closer approximation of the Prothrombin ratio which may obviate some of the acknowledged difficulties of employing the INR outside its traditional use. In our future proposed pilot studies in critically ill patients, we will analyses of scoring with both PT and INR and determine confidence intervals around each parameter to assess the overall impact. Clearly, the preference is to use PT or PT-ratio; however, since many local laboratories refuse to release anything but INR, this will continue to be problematic in the immediate future.

2) Further, through this report Dr. Hoots communicated these difficulties to the SSC leadership and during the presentation to the Council directly since the impact of this change by hospital and commercial labs to reporting if only INR goes far beyond choosing an appropriate parameter for the DIC
algorithm. Clinical monitoring of patients, assessment of suspected Factor VII deficiency (congenital or acquired), etc. would all be negatively impacted.

3) To ascertain the impact of using INR on the DIC algorithm, Drs. Toh, Wada, Levi and Dempfle will do retrospective reviews of the local cohorts, comparing the sensitivity and specificity of the DIC algorithm using the PT versus INR and report this data at the Subcommittee meeting in Venice.

4) In particular, Dr. Wada and colleagues have performed some preliminary sensitivity and specificity analyses on cohorts from Japan who have contemporaneously been assessed by both the SSC algorithms and the algorithm developed by Japanese investigators under the aegis of the Japanese Ministry of Health and Welfare (JMHW). To date, the reports are too preliminary to draw conclusions.

5) Finally, we plan to request this INR issue be presentation and discussion topic for future DIC symposia at both the International Critical Care meetings and ASH or ISTH (or both). Because of the roles that subcommittee members and collaborators play (e.g. Dr. Kinasewitz in Critical Care) in these respective organizations, it is felt likely that this can be accomplished within 12-18 months.

B. Outreach to the Medical/Scientific community (particularly critical care physicians) about the proven and potential expanded utility of the DIC algorithm for management of critically ill patients:

A recent review article on sepsis in the NEJM (The Pathophysiology and Treatment of Sepsis by Hotchkiss and Karl. N Engl J Med 348;2:138-150) is indicative of the challenge for hematologists in general, and this Subcommittee in particular, to inculcate the recent understanding about the integrated inflammatory - coagulopathic approach into the current state-of-the-art management concepts for severe sepsis. The article articulated in exquisite detail the perturbations of the reticuloendothelial organ that occurs in ill septic patients and cites the proven efficacy of activated protein C (Xigrisâ) in the treatment. The latter clinical effect is ascribed to down-regulation of thrombin but no mention is made of disseminated intravascular coagulation as the inexorable outcome when the process is not aborted by APC or other therapy. Our challenge is to promote more complete integration of the hemostatic and inflammatory pathways into the pathogenetic and therapeutic intervention of sepsis and other predisposing severe injury states:

1) Accordingly, we plan to write and publish a “DIC Algorithm use in Critically Ill Patients” article in the Critical Care literature with the integral participation of our colleagues in Critical Care and to enlist an accompanying editorial about the use of a hematologic diagnostic instrument by Critical Care Specialists. We plan to link the terminology of the Algorithm in DIC terms to vernacular used in the critical care arena such as Multi-organ Failure Syndrome (MOFS), septic shock,
and ischemia-reperfusion. Drs. Kinasewitz and Toh will take the lead in this endeavor.

2) We plan to monitor the citations of the original T&H publication of the Algorithm as a surrogate for both its acceptance as a definition of DIC as well as a possible indicator of its use in clinical practice.

3) Using our collaborators within the Critical Care community, we plan to use plenary sessions at national and international meetings of these clinicians and clinician scientists to “re-introduce” the concept of DIC which has recently considered the inflammatory excess in ill patients as systemic inflammatory response syndrome (SIRS) and multi-organ failure syndrome (MOFS) and thereby minimized the contribution of coagulopathy to the overall pathogenesis of the syndrome.

4) Similarly, we will consider developing specialty-focused manuscripts about the contributions of microangiopathy and reticuloendothelial perturbation to DIC-associated disease states in the obstetrical/gynecologic (e.g. abruption placenta) and oncologic (e.g. acute promyelocytic leukemia [Aph]) setting.

5) Consideration by the Subcommittee of a submission to Journal of Thrombosis and Haemostasis as an Official Communication of the SSC concerning the need to integrate hematologic and critical care measurements in ongoing assessments of natural history of the severely ill patients.

C. Differentiation of DIC pathogenesis that arise from diverse disease states (e.g. APL versus sepsis):

Since the Japanese have the most extensive experience utilizing scoring systems to diagnose DIC from different diseases and in using them to prognosticate clinical outcome, Dr. Wada and colleagues re-examined their aggregate data from multiple cohorts to assess the specificity/sensitivity of both algorithms in the sepsis patients and in the patients with leukemia patients. These data were presented by Dr. Wada at this Subcommittee session and the conclusions were as follows: 1. Clearly different disease processes that initiate DIC exhibit variable abnormalities in both global tests of coagulation and in biological markers thereof. 2. The data he presented are consistent with the pathogenetic mechanisms in pre-clinical models of sepsis vs. trauma whereby in the former dramatic down-regulation of fibrinolysis is easily measurable and the latter is a much later occurring event. Similar concordance exist between Dr. Wada’s clinical data and experimental animal models with regard to markers of endothelial injury and natural anticoagulant depletion.

DISCUSSION ITEMS which we intend to move to ACTION ITEMS within the next 18-24 months(Medium-term Strategies)
A. Non-Overt DIC:

1) It is our consensus that “trend” scoring of microangiopathy (i.e. does it progress, regress, or remain static in an individual patient?) is the component of the non-overt DIC score that distinguishes it from the overt DIC score. Hence, refinement of these trend analyses using both coagulopathic and inflammatory markers offer significant promise for enhancing the utility of the non-overt algorithm.

2) There is a need to clarify overt versus non-overt DIC: specifically, we must be able to accurately assess the status of the patient at any point in time in his clinical course with regards to inflammatory/coagulopathic activation and, even more importantly, to be able to differentiate a decompensated DIC state from a decompensating one. In this regard, we have agreed that in order not to “oversample” the patient’s status with measurements and thereby confound an accurate assessment (thus reducing prognostication capacity), we stipulate that none of the algorithms which we have developed should be employed more often than every 24 hrs.

**Important Caveat:** **Decompensated** versus **decompensating** DIC

In terms of clarifying the totally decompensated state, it is our consensus that scoring ≥ 5 on the overt DIC scale implies “full-blown” DIC. In terms of applying the overt algorithm, it should be stipulated that there is no diagnostic “penalty” for applying the algorithm the first time in an ill patient. If he scores ≥ 5, he by definition has DIC. On the other hand, if the patient does not meet the scoring criteria for DIC, we then need to switch to the non-overt algorithm in which there is a “penalty” for multiple testing: i.e. any improvement in any biological measurement on re-testing subtracts from the likelihood that a threshold score for non-overt DIC will be achieved. It is also the consensus of the Subcommittee that the non-overt algorithm must not be applied more often than every 24 hours to reduce the likelihood that measurement variability rather than biologic changes in the patient will excessively impact scoring.

Decompensating DIC (as defined by a rising score in the algorithm) could become a surrogate for worsening microangiopathy and clinical deterioration.

Dr. Cheng Hock Toh presented a completed pilot study from Liverpool of the non-overt pilot study which validated to a significant degree both the rationale and the algorithm thresholds for the global markers in the non-overt algorithm. It is expected that this data will be published soon.

B. Fibrin-related markers:

1) We are committed toward an evolution to the use of D-dimers as the specific measurement of fibrinolysis that is to be employed in the DIC algorithms for
overt and non-overt DIC. This intent is predicated on the expectation that the on-going work by this Subcommittee and that of the SSC Fibrinolysis Subcommittee are arriving at standardization of tests and normal ranges that make this progression possible. Further, the broadening availability of quantifiable marker of D-dimer in widespread hospital laboratories around the world makes such a transition possible over the next few years. The refinement of fibrinolytic measurement using D-dimers will rely on “multiples” of the upper range cut-offs of normal to score this parameter for both the overt and non-overt algorithm: e.g. the algorithms will score the lesser number score in the scoring schema if the D-dimer level is at least twice the upper limits of the 95% confidence range on normal; and the higher number score will be given only if the D-dimer measurement is > 5 times the 95% upper limits range. This will clearly require validation using existing datasets from DIC populations which we have assembled. Dr. Carl-Erik Dempfle (ex-officio to the DIC Subcommittee as he is a co-chair of the SSC Fibrinolysis Subcommittee) will oversee these analyses. At this meeting, he presented data that indicate that it is possible to establish clear threshold values of D-dimer to score 0,2,3 in the non-overt algorithm with very predictive results: values less than 2x upper limits of normal (ULN) score 0; values 2x and 5x ULN score 2; values greater than 5x ULN score 3. Using these parameters, his study achieved a high degree of sensitivity and specificity of the overt and non-overt algorithms.

2) Despite the recommendation to encourage implementation of D-dimer as the optimal fibrin-marker for use in the algorithm, we wish to preserve the potential use of other fibrin-related markers (e.g. soluble fibrin monomers). This is important for institutions that have implemented this testing with the algorithm already. Needless to say, these non-D-dimer markers will need to be quantified and scored in an analogous manner to D-dimers (i.e. using 95% upper limit of normal confidence intervals) and will similarly require further validation for establishing sensitivity and specificity.

3) It is worth noting that our data analyses performed to date do not imply that the scoring in the algorithm for fibrin generation will need to be “corrected” or modified based on the type or source of the disease state that is predisposing to DIC; however, this clearly needs to be continuously re-examined.

C. Roles (if any) of other biologic markers in scoring for DIC:

1) It is our consensus that we need at this point to continue to maintain flexibility in permitting new biological markers to be studied for their potential implementation into the scoring of the algorithm for non-overt DIC. We will need to select such markers according to the following criteria: a high specificity to a predisposing etiology or pathogenetic process; and measures a process that is on-going at the time of initial clinical assessment of the patient. We have not as yet ascertained whether such an added marker should
remain intrinsic to the non-overt score or rather in some way modify or refine the initial scoring. Certainly it will be essential to determine in the assessment phase prior to incorporation of any new such biologic marker whether it can be used to guide future therapy. Nonetheless, it will be necessary to establish “cost-efficacy” of any new measurement or test since added costs of patient monitoring clearly will impede use of the non-overt algorithm in the I.C.U. setting. Accordingly, even though we will continue to investigate potential biological markers for incorporation into the non-overt algorithm, until we have the above-sited necessary data we will encourage only GLOBAL tests in the algorithm for clinical management. In parallel we will continue to search for unique markers that specifically link the coagulopathy of DIC with the concomitant inflammatory up-regulation.

2) We will pursue aggressively validation of the algorithm in pediatric populations. Drs. Prasad Mathew and Marilyn Manco-Johnson collaborated to develop and present at this meeting a concept sheet for a natural history study of the DIC algorithms in neonates and other pediatric populations. They plan to pilot this in their own institutions initially, but welcome members of the Subcommittee and others who wish to participate in this endeavor.

3) We will explore possible collaboration with OB/GYN physicians about the potential use of the algorithm in very ill female (pregnant) populations.

4) A major intermediate commitment of the Subcommittee is to assess the potential use of the algorithm in designing and monitoring of clinical trials in critically ill populations. Specifically, with regards to clinical trials of new pharmacologic agents in such patients, we plan to advocate use of the algorithm(s) for stratification and/or establishment of eligibility for randomized clinical trials. This effort will require a study by study and pharmaceutical company by pharmaceutical company education process. It is our hope that initial attempts to use the algorithm in this fashion will result in publications that will support such use in future drug trials in these populations.

One particular clinical trial application of particular interest to the subcommittee is the use of heparin/heparan sulfate in combination with existing anticoagulant or anti-inflammatory drugs such as activated protein C in septic and other critically ill populations. This will require a discerning and unbiased approach to design a trial in which the algorithm assists in stratification of patients between those who may naturally have a need for more aggressive need for anticoagulation (e.g. the ICU patient with high risk or proven DVT) versus the critically ill patient with demonstrable microangiopathy in whom aggressive anticoagulation may induce morbid or mortal hemorrhage. Clearly cogent
rationale for such algorithm use will require data from our pilot projects and also retrospective application of the algorithms to on-going clinical trials.

LONG-TERM PLANS AND CONSIDERATIONS:

The application of new techniques (e.g. genomics and proteomics) to the understanding of the extreme inflammatory state that predisposes to DIC appears to be a logical next step. Once the Subcommittee has achieved wide clinical implementation of the DIC Algorithm(s). The following reflect some of our preliminary scientific discussions with regards to specific avenues for future investigation:

1) We propose multi-site, IRB-approved studies to genotype critical care cohorts for known immunologic, inflammatory, endothelial cell wall, procoagulopathic, natural anticoagulant and fibrinolysis genes and make correlations with clinical mortality in order to examine whether certain genotypes predict a particularly morbid outcome when one controls for other environmental risk factors.

2) We will encourage investigations among ourselves and others that examine the possible application of proteomic techniques in order to study whether there are identifiable discriminate disease responses to injury and/or predisposition to develop microangiopathy.

3) We will advocate for exploring pertinent ligand-receptor interactions in the pathogenesis of DIC.

Respectfully submitted: K. Hoots
Registry of Exogenous Hemostatic Factors

Chair: M. Kini, Singapore
Co-chairs: C. Bon, France; F. Markland, USA; N. Marsh, Australia

The subcommittee met from 2.15 to 4.00 PM. There were two scientific presentations (Factor X activator from Vipera lebetina venom is synthesized from different genes by J. Siigur (Estonia) and Exogenous and endogenous prothrombin activator systems in Australian snakes by R. M. Kini (Singapore).

With regard to future directions, the subcommittee has selected to perform the following functions.

1. We plan to organize the Third International Conference on Exogenous Factors Affecting Thrombosis and Hemostasis in Sydney, Australia (August 13-14, 2005) immediately following the ISTH Congress. This will be similar to the one we organized in Paris in 2001. The attendees also felt that the proceedings we published from the previous Conference was useful and agreed to publish short abstracts.

2. Classification of disintegrins and related proteins. In the recent years a number of disintegrins with different specificities have been isolated and characterized. M. A. McLane, USA and F. S. Markland, USA will be focusing on their classification.

3. Our subcommittee will also start looking into the Classification and nomenclature of C-type lectin related proteins. This will be done by T. Morita, Japan and K. Clemetson, Switzerland.
Factor VIII and Factor IX

Chairman: K. Mertens, The Netherlands
Co-Chairmen: D. DiMichele, USA; J. C. Gill, USA; C. Lee, UK; J. Oldenburg, Germany; JM Saint-Remy, Belgium; A. Srivastava, India; HM van den Berg, The Netherlands

Completed/Submitted Reports and Recommendations

- International Factor VII Deficiency Registry: G. Mariani, complete report in preparation
- 7th International Standard for FVIII Concentrate: S. Raut et al, submitted to JTH
- Registry on Factor IX inhibitors and anaphylaxis: I. Warrier, report in preparation

Clinical Studies in Progress: Co-Chairs: C. Lee and A. Srivastava

Update: Gene Therapy Registry: K. High

Dr. High reviewed the status of gene therapy trials for hemophilia. Five trials (3 for FVIII and 2 for FIX) treating a total of 41 patients have been done. Summary of these trials are available at the US National Institutes of Health, Office of Biotechnology Activities, database called Genetic Modification Clinical Research Information System (Gemcris). The database can be accessed by the public and contains basic information on all gene transfer trials that have been reviewed in the US. It can be searched by disease, vector, or principal investigator. This currently constitutes one of the best resources on hemophilia gene transfer studies, but the data available to members of the public are limited, consisting of scientific and lay abstracts of each trial. Most of the data in hemophilia gene transfer has been published or presented at national meetings.

Overall though no serious toxicity was noted in any of these trials, the major problems have related to poor transduction efficiency and lack or loss of function very soon after infusion. All studies have currently stopped for further evaluation in the laboratory. One approach could be study the effect in larger animals using short course of immunosuppression to avoid rejection and facilitate sustained gene function.

Update: International Prophylaxis Study Group: V. Blanchette

The International Prophylaxis Study Group (IPSG) is a collaborative initiative whose aim is to facilitate generation of new knowledge relating to factor prophylaxis in the hemophilia population. To assist accurate and consistent documentation of the musculoskeletal outcome, the group is also focusing developing new physical examination and MRI based radiological scoring optimized for the detection of early joint disease. Two expert groups were established with this goal in mind: a physical therapy group (Co-Chair: Marilyn Manco Johnson) and a MRI group (Chair: Holger Pettersson). Scoring systems have been developed (the Hemophilia Joint Health
Score and MRI-based joint score) and reliability tests have been conducted. Once this initial process is completed, these scoring systems will be submitted to the Pediatric and Musculoskeletal committees of the World Federation of Hemophilia and the FVIII-IX Subcommittee of the ISTH-SSC for review and endorsement.

Progress Report: Definition of the Clinical Phenotype: D.M. DiMichele

Part of the FVIII-IX Subcommittee’s focus over the past 3 years has been on identifying more physiologically accurate methods of measuring hemostasis in persons with hemophilia and other bleeding disorders. The intended application of these assays would be in 1) patient diagnosis (accurate assignation of clinical bleeding severity in order to determine optimal therapeutic strategies) and 2) patient treatment (cost-effectiveness monitoring and control of replacement therapy and its complications). The consensus of the Subcommittee Working Party resulted in a recommendation to the Subcommittee in 2003 that included 1) the development and validation of these assays; and 2) major application of this technology in the prediction of hemostatic baselines in severe hemophilia A and B patients.

However, such studies need to be validated by a consensus clinical definition of bleeding phenotype, with particular application to the young child with hemophilia. No such definition yet exists. A definition was proposed in 2003 by Dr. DiMichele for the child with biochemically severe (< 1 U/ml) hemophilia A (HA) that included “mild” and “severe” severe HA phenotypes. At this meeting, Dr. DiMichele proposed that this definition be disseminated among various clinical and scientific groups for comment and validation in hemophilia cohorts around the world. Input will be sought from 1) FVIII-IX Subcommittee co-chairs and members; 2) Pediatric Subcommittee of the WFH; 3) European Pediatric Network; 4) Japanese hemophilia physicians; 5) Canadian hemophilia center directors’ organization; and 6) North American Hemophilia and Thrombosis Research Society. Other suggestions were elicited. The subsequent plan is to assemble a working party to review and develop a consensus definition to be presented to this Subcommittee in 2005.

Phenotype versus genotype survey: H.M. van den Berg

Dr. Van den Berg presented data of a study done to investigate variability and determinants of phenotypic variability of severe hemophilia. A large cohort of severe haemophilia patients were investigated to determine clinical variability with regard to data on bleeding episodes, treatment characteristics and long-term outcome. Of 285 patients a total of 4737 follow-up years were available (mean follow-up 16.6 years). Clinical variability was shown in age at first joint bleed and a combination of joint bleed frequency and annual clotting factor use.

The determinants of variation in severe hemophilia were further evaluated in a smaller cohort of 38 patients – 20 with mild and 18 with severe phenotype. The clinical difference among these two groups was significant for age at first bleed (4.6 vs 0.7 years), joint bleeds per year (3.1 vs 4.2) and number of joints with arthropathy (0.6 vs 4.5). The half life of FVIII was also studied in
these patients. Though there was no statistical difference in factor VIII half-life between patients with a more severe and patients with a milder phenotype, a one hour increase in factor VIII half-life was associated with 100 IU/kg/yr less annual clotting factor use. Further evaluation of additional markers of defining phenotypic heterogeneity of severe hemophilia is progressing.

**Final Report: International survey of factor replacement protocols for surgery in hemophilia: A. Srivastava**

This survey, which was initiated to fill the gap in the knowledge of current practices of factor replacement for surgery in hemophilia, has been completed. The two part questionnaire survey (first part for documenting practice policies and the second part for details of specific procedures carried out in the last 1-2 years) received responses from 45 major treatment centers in 23 countries (16 from North America, 14 from Europe, 3 from Australia, 9 from Asia, 2 from South America and 1 from Africa). The data shows that only 15% of centers used continuous infusion exclusively, 42% used bolus infusion only and the other 42% used both with lack of uniformity in the frequency of dosing. The target pre-operative levels were 80-100% at most centers but there was a 5-fold variation in post-operative doses used. The median dose for major surgery was ~ 650 IU/kg with a range of 300-1500 IU/kg. The dosage used in developing countries was 10-30% lower for similar procedures. Antifibrinolytic drugs were used by ~ 70% of centers and fibrin sealant was used by ~ 45% of centers. While most centers reported no major complications, operative bleeding was reported in 3-5% of cases and significant post-operative bleeding occurred in 5-6% of cases at some centers. Wound infection occurred in 3-4% and thrombotic complications in 4-5% patients. These rates appear to be similar in different parts of the world.

The data suggests that there is need for optimization of factor replacement dosage and other practices for surgery in hemophilia. Based on this data, it should be possible to evolve consensus protocols for factor replacement for surgery in hemophilia and test them prospectively.

**Factor VIII Inhibitors: Co-Chairs: D.M. DiMichele and H.M. van den Berg**

**Factor VIII inhibitor assay standardisation: S. Raut**

Dr. Raut presented an overview of inhibitor assay standardisation. The current gold standard assay is the Bethesda Assay or the Nijmegen Modification thereof. However these assays can be cumbersome, labour intensive and can give inter-lab CVs between 30 - 80% or more. Development of an anti-FVIII inhibitor antibody standard was recently proposed by the FVIII-IX Subcommittee and as such three patients samples and the three standards were assayed in a preliminary collaborative study. Results showed that inter-laboratory variabilities continued to be high as observed in previous studies. One standard reduced this variability although CVs were still high compared to normal FVIII assays. The rabbit polyclonal antibody (99/648) appeared to perform better as a standard compared to the other two monoclonal antibody standards.

After discussions at the FVIII-IX Subcommittee of ISTH-SSC, it was decided to seek feedback from participants on whether a larger study be launched using the polyclonal antibody inhibitor
and if so at what optimal titre should such a material be prepared. Dr. Raut proposed that a questionnaire be sent out by NIBSC to all participants, recommending that a larger study be carried out using the rabbit polyclonal antibody (99/648) as the primary candidate standard and to design a more controlled study, in the hope that an inhibitor standard may prove useful as a reference reagent. It is envisaged that perhaps, after the larger study, the candidate material may be suitable to propose as the WHO 1st IS for anti-FVIII inhibitor.

**Update: International ITI study: C. Hay**

Dr Hay reported that the ITI study is now in its second year. 63 centres from 20 countries have agreed to participate, 33 patients have been recruited from 9 countries. Recruitment is building up slowly and some administrations have only now completed the administrative steps to start. Six patients have been withdrawn (3 pre-ITI, 2 failed ITI and 1 lost to follow-up). 26 patients subjected to ITI for median 7 months (range 1-20 months). 16 have negative inhibitor (7 with normal recovery and two tolerant). 2 have failed ITI. 48 non-serious adverse events have been reported, including 9 catheter infections and a variety of intercurrent infections and traumatic bleeds. 29 serious adverse events were reported including 9 line infections in 4 patients resulting in 5 line removals. The remaining SAEs were mostly haemorrhagic. Of 29 SAEs reported 10 were study-related. For further information see website: [http://www.itistudy.com](http://www.itistudy.com).

**European Pediatric Network: PedNet Registry and RODIN study: H.M. van den Berg**

The European Paediatric Network for Haemophilia Management (PedNet) is a collaboration of 23 European paediatricians involved in haemophilia care. The PedNet Haemophilia Registry is an initiative of PedNet members through which a common set of data is collected to improve understanding of the pathophysiology of haemophilia, current clinical management of children with haemophilia, and the safety and efficacy of treatment strategies. Patients with severe (<1%), moderate (1-5%) and mild (5-25%) haemophilia A or B born after January 1st, 2000 with well-documented patient history and treatment data from diagnosis onwards are eligible for enrollment. Data are collected using a patient logbook and assessment forms, which are filled out at every regular visit. The assessment forms are sent to the central database by the Internet ([http://www.pednet.nl](http://www.pednet.nl)). In September 2003 the number of eligible patients at the moment was estimated at 260 patients.

The first study to be performed in the registry is the RODIN study, Research Of Determinants of Inhibitor development among previously untreated patients with haemophilia in Europe. This study aims at examining the roles of potential genetic and environmental risk factors for inhibitor development among PedNet-registered patients with mild, moderate and severe haemophilia A and B. Data collected in the PedNet Haemophilia Registry are treatment characteristics during the first 75 days of treatment with clotting factor (such as age of first infusion of factor VIII, frequency and dose of infusions, type and purity of clotting factor product, possible extravasation of clotting factor), severity and type of bleeds, infections and the use of antibiotics during the first 75 exposure days, allergic diseases, vaccinations, breast feeding, family history of inhibitors, ethnicity and mutations in the genes for the deficient clotting factors. In 5 years we
hope to have registered a sufficient number of patients to answer the research questions of the Rodin study. Data can be submitted by the internet site: http://www.pednet.nl.

**Global PTP surveillance study: D.M. DiMichele**

A possible increase in inhibitor incidence in PTP’s using ReFacto in the post-licensing setting prompted an evaluation of the background incidence of inhibitors in PTP’s at the July 2003 FVIII-IX Subcommittee meeting. The results of a literature search on inhibitor incidence were presented. The ensuing discussion emphasized the need to more accurately ascertain the rate of new inhibitor formation in heavily treated patients with hemophilia A through post-marketing surveillance. The scientific question of whether this was a host or product phenomenon was raised. This problem was further discussed at the FDA Inhibitor Conference in the USA in November 2003. As a follow-up, Dr. DiMichele presented a potential schema for a universal post-licensure data collection system for all FVIII products at this subcommittee meeting. Her presentation included the following proposals:

- An international database for globally distributed products, and national/multi-national databases for products with more limited distribution.

- PTP’s defined by pre-licensure clinical trials, and observed for a period defined by exposure days, not time.

- Two data sets: 1) a minimum data set (MDS) defined by regulatory agencies with industry input; focus on product immunogenicity and the goal of providing an ongoing assessment of a product risk/benefit ratio; and 2) a comprehensive data set (CDS) defined by clinical investigators and scientists with a focus on ascertaining the role of host/treatment interaction in PTP inhibitor formation.

- Both databases should not be industry-driven, but rather compiled and interpreted by physicians and scientists.

- The FVIII-IX subcommittee (PTP Inhibitor Working Group?) could be a possible clearinghouse for the data generated by the MDS, in a role that would include coordination/analysis of the national databases. Proposed funding would come from industry and national governments, where applicable.

- Although the CDS would be largely grant funded in this schema, a reporting relationship between clinical investigators/scientists working on this issue and the FVIII-IX Subcommittee would be optimal.

Dr. DiMichele’s proposal received a positive response from the audience. At the same time, however, concerns were raised with regard to the feasibility of such a complex program.

**Proposal to refine the low response inhibitor definition: J.Gill, presented by D.M. DiMichele**

26
In 2000, the Factor VIII-IX Subcommittee of the Scientific and Standardization Committee of the ISTH developed standardized definitions to classify the variable clinical course of inhibitors. High response inhibitors were defined as those > 5 BU/ml currently or at any time in the past; and low response inhibitors as those persistently ≤ 5 BU/ml despite repeated challenge with substitution factor replacement. An analysis of the long-term follow-up data of the Recombinate PUP study suggests that the low response inhibitor category should be further refined to separate out those patients with inhibitors persistently ≤ 5 BU who have a short, benign course that does not require therapeutic intervention. Dr. Gill’s proposal was that these inhibitors be termed “transient” and be defined as those whose maximal inhibitor titer is ≤ 5 and disappears within 12 months of detection. This group should be analyzed separately when the immunogenicity of new products is being analyzed. In the discussion a more simple definition was proposed depending on whether or not the inhibitor interferes with normal treatment. The issue will be further worked out by Dr. Gill in more detail for presentation to the Subcommittee in 2005.

Panel discussion: Can we predict product-related loss of tolerance?

Animal models of FVIII immunogenicity in the setting of gene transfer: D. Lillicrap

Dr. Lillicrap reviewed animal studies on host immune response to FVIII gene transfer. Variables that influence the potential development of antibodies to the transgene product (FVIII inhibitors) include the type of vector, the dose of vector, the site of vector administration, the site of transgene expression, the level of transgene expression and the type and genetic background of animal in which the studies are being performed. At the current time, viral vector-mediated gene transfer is the most effective means of transgene delivery. The immune responses accompanying the use of the different viral vectors, adenovirus, AAV and lentivirus are very different, and are further complicated by the potential of pre-existing immunity to proteins presented on the incoming vector. Overall, while adverse immune responses to gene transfer remain a critical challenge to the success of this therapeutic approach, the utilization of several different animal models is essential to evaluate the likely occurrence of these events. While so far gene transfer has successfully cured hundreds of hemophilic mice and a few hemophilic dogs, it remains clear that more work will be needed to further control the inhibitor risk.

Transgenic mouse models for FVIII inhibitor formation: J. Voorberg

While inhibitory antibodies develop in approximately 25% in patients with severe hemophilia A following treatment with factor VIII, inhibitor formation in mild and moderate hemophilia A is rare. Several reports have described inhibitor development in patients with mild hemophilia A caused by an Arg593 to Cys mutation. To mimic the immune response in these patients in mice, Dr. Voorberg has created a transgenic mouse model expressing human factor VIII-R593C (hufVIII-R593C mice). Transgenic hufVIII-R593C mice were crossed with factor VIII-deficient mice (E-16 KO mice). Factor VIII-deficient E-16 KO mice develop anti-factor VIII antibodies after five serial intravenous injections with human factor VIII while hufVIII-R593C/E-16 KO mice did not develop an immune response. Apparently, hufVIII-R593C mice are tolerant to human factor VIII which is in agreement with the strongly reduced incidence of factor VIII inhibitors in patients with mild hemophilia A. However, anti-factor VIII antibody development
was induced in hufVIII-R593C/E-16KO mice by multiple subcutaneous injections of factor VIII with an adjuvant. These data indicate that partial loss of tolerance can be induced in this novel mouse model for inhibitor development in hemophilia A. Dr. Voorberg suggested that this model might have future potential as an immunogenicity model for assessing novel FVIII products or gene therapy protocols.

**Dissecting immune response to FVIII products: B. Reipert**

Dr. Reipert reviewed the immunological mechanism of inhibitor formation, including a delicate interplay between antigen presenting cells, B-cells and T-cells as well as co-stimulatory ligands involved in this process. Although we currently know some factors which contribute to the inhibitor risk of Previously Treated Patients such as type of gene mutation, B-cell response (epitopes), other issues still remain poorly understood. These include the development of FVIII-specific memory cells, and “danger” signals that trigger anti-FVIII response. Dr. Reipert is using an animal model to study these immunological response mechanisms, in which hemophilic mice are challenged with human FVIII. Subsequently, lymphocytes are isolated which can be used in stimulation experiments in vitro. By using different FVIII products for T-cell response is should be feasible to study loss of tolerance if “altered” FVIII is used. In the future, this approach may have potential for detecting neoantigens, for instance due to changes in manufacturing procedure of FVIII products.

**Factor VIII assays and standards: Co-Chairs: K. Mertens and J.M. Saint-Remy**

**Overview of ongoing activities: K. Mertens**

Dr. Mertens introduced this part of the program. Various standardisation issues have recently been completed, such as the establishment of new International Standards of FVIII in plasma and concentrate. Others have been addressed in two more informal, separate meetings:

- The Steering Committee on SSC Collaborative Studies on FVIII-IX field studies met at NIBSC on November 13th, 2003. The committee, currently consisting of Drs. S. Raut, T. Barrowcliffe, M. Lee and K. Mertens, has been working on a new design for these studies to make them more systematic and useful. The first results are planned to be reported at the Subcommittee meeting in 2005.

- The Working Party on novel FVIII Assays met in San Diego, on December 5th, 2003. Present were Drs. K. Mann, B. Sorensen (for J. Ingerslev), P. Turecek, D. DiMichele, J. Gill, M. van den Berg, J. Oldenburg, and K. Mertens. After a review of various novel assays, it was decided that some collaborative study would be the next step, and that the thrombin generation assay may be the most promising approach for this purpose. A proposal will be worked out for discussion in the Subcommittee meeting in 2005.

Because these technical issues were already addressed, this year’s standardisation section was devoted to a panel discussion on FVIII assay methodology.
The FVIII assay problem: neither rhyme, nor reason: P. Lollar

Dr. Lollar presented his view on the issue, as published in JTH last year. Discrepancies between one-stage coagulation and chromogenic assays of factor VIII (FVIII) activity and between labeled potency and in vivo recovery of FVIII are two examples of the FVIII assay problem. The chromogenic assay appears to have gained prominence primarily by official decree. Reports that the chromogenic assay is the “correct” assay lack scientific rigor. It has been reported that different FVIII products are comparable because they appear to satisfy the criteria of the parallel line assay. Because of parallelism and because concentrate standards are referenced against a plasma standard, there is no apparent need for concentrate standards or assays of “like versus like”. The FVIII assay problem potentially could be solved by making the one-stage assay the official assay for in vitro and in vivo measurements and by adopting a single FVIII plasma standard for all assays.

WHO approach on International Standards: T.W. Barrowcliffe

Dr. Barrowcliffe summarised the FVIII standardisation strategy followed during the last 25 years. The introduction of WHO International Standards and the principle of “like-versus-like” testing has much reduced interlaboratory variability. For FVIII concentrates the chromogenic assay has proven superior over the 1-stage assay in term of CV values, and for this reason the chromogenic assay is the SSC recommended assay for product potency assessment since 1993. Although Dr. Barrowcliffe acknowledged the simplicity of the use of a single type of assay, he also noted some disadvantages of the 1-stage assay. These mainly relate to the great variability between reagents, which make the 1-stage assay difficult to standardise.

Chromogenic assay: background and design: S. Rosen

Due to its higher precision and lower interference as compared to clotting methods, the chromogenic FVIII method was recommended by the ISTH-SSC in 1993 for assay of FVIII concentrates and adopted as the Ph Eur reference method 1995. These features were also demonstrated in the recent calibration of the Mega-2 standard. In a further characterization of the chromogenic FVIII method, Dr. Rosen has performed a study on assay of FVIII concentrates vs the 6th IS with human and bovine species of FIXa, FX and thrombin or, alternatively, prothrombin and FV. Using fixed concentrations of FIXa, FX, thrombin / prothrombin + FV, calcium chloride, phospholipid, FVIII and one common buffer, no difference in FVIII potency assignments of Octonativ-M, Recombine and ReFacto was obtained when using either only human or only bovine components. The results thus indicate that FVIII does not show a strict species specificity in the chromogenic assay.

FVIII assays: a regulator’s view: R. Seitz

Dr. Seitz reported that in Europe potency assessment of FVIII products requires the chromogenic assay. All labelled Medicinal products marketed in Europe have to comply with European Pharmacopoeia (Eur. Pharm) monographs. According to the new monograph for recombinant FVIII, reference preparations should be calibrated against the International Standard. However,
with a B-domain-deleted recombinant FVIII, discrepant values are obtained with different chromogenic assay kits. Following a collaborative study verifying the discrepancies, the respective reference preparation was relabelled resulting in a fill of the product increased by approx. 20%. The Eur.Pharm expert group 6B looked into details of the assay and concluded that the FXa generation should be stopped at about half the plateau level. It was decided that the chromogenic method will remain the reference method in Europe, although the assay description will be amended with regard to the optimal incubation times.

**Thrombin Generation Assays on post-infusion samples: P. Turecek**

Classification of the severity of haemophilia is generally based on the levels of factor VIII or factor IX levels in the plasma. However these levels do not always match the bleeding tendency of individual patients, especially those with severe deficiencies, i.e. factor VIII or factor IX level <1%. The usual clotting and chromogenic assays have a detection limit of approximately 1-2 %. Thrombin generation assays can assess all activating and inactivating systems of coagulation and therefore, they might be suitable to monitor coagulation factor substitution therapy with plasma-derived or recombinant FVIII concentrates. Dr Turecek reviewed presented a thrombin generation assay which is suitable for detecting treatment-dependent changes in the kinetic of thrombin generation and thus monitoring the pharmacokinetics of inhibitor-bypassing agents during treatment. The same assay can also be used to assess the thrombin generating capacity of severe haemophilia patients before and after treatment. *In vitro* spiking experiments with severe haemophilic plasma samples using plasma-derived or recombinant therapeutic FVIII preparations showed that the thrombin generation assay is especially sensitive in the low factor activity range, even below 0.01 U/ml. Plasma samples from severe haemophilia patients treated with single doses of FVIII showed a measurable thrombin generation even at time points, when no FVIII activity could be detected.

**Pilot collaborative study on clinical samples (C. Negrier, also on behalf of A. Srivastava)**

Dr. Negrier showed some data of a collaborative study, in which thrombin generation assays was assessed in samples collected in India. These assays proved particularly useful in a pharmacokinetic study. The study further identified some technical issues that may be subjects for further standardisation. These include the tissue factor concentration, and the need to prepare cell-free plasma samples.

**General discussion on current SSC guidelines:**

These presentations were followed by a lively general discussion, in which the various speakers and several attendees participated. It was evident that all speakers were in favour of rigorous standardisation, although the proposed solutions were different. Overall, there was no reason to consider the current SSC guidelines for potency assessment (Barrowcliffe, 1993) and for post-infusion assays (Lee et al, 2001) as being inappropriate. At the same time, however, it was felt that the thrombin generation assay, if further standardised, might have the potential of making 1-stage and chromogenic assay obsolete in the future.
Rare Bleeding Disorders: Co-Chairs: D.M. DiMichele and J. Oldenburg

SSC Working Group on Rare Inherited Bleeding Disorders: F. Peyvandi

Dr. Peyvandi’s presentation was the kick-off of this new Working Group, which was established as part of the FVIII-IX Subcommittee in 2003. Rare bleeding disorders (RBDs) are typically orphan diseases, relatively neglected until recently by health care providers, advocacy organizations and pharmaceutical companies. They comprise deficiencies of fibrinogen, prothrombin, factors V, combined V and VIII, VII, X, XI and XIII, that together have a global prevalence of around 3% to 5%. Due to the rarity of each factor deficiency, as a consequence of the relative rarity of these deficiencies, type and severity of bleeding symptoms, underlying molecular defects and actual management of bleeding episodes are not established as well as in haemophilia A and B. Also purified factor concentrates are not as readily available as they are for the haemophilias. Various group spread all over the World deal with RBDs, from clinical, phenotypical and genotypical point of view. The development of an International Registry of RBDs through a co-operative network of Haemophilia Centres around the world will help to fill the gap between data production and their accessibility. Preliminary data obtained from the International Rare Bleeding Disorders Database (RBDD) already present in Hemophilia Centre-Milan-Italy with more than 200 patients affected by each single disorder, indicate the possibility to perform interesting and transversal studies among different centres in order to confirm data obtained by each single Centre. The aim of this registry is to efficiently organize and extract the consistent amount of information on rare bleeding disorders. This would certainly optimise retrospective studies in the field and static reports extracted from the database planned to be made available on the World Wide Web. Another important goal of SSC Working group is the standardisation of laboratory methods for phenotypic diagnosis of RBDs. The last but not the least important goal is to foster the development of orphan drugs for deficiencies with no available therapeutic concentrate, particularly for FV deficiency.

Dr. Peyvandi emphasized that the Working Group on Rare Inherited Coagulation Disorders needs to focus on following issues:

· to develop the International Registry of RCDs
· to standardise laboratory phenotype and genotype diagnosis methods
· to foster the preparation of orphan drugs, particularly for those deficiencies with no available therapeutic concentrate
· to evaluate women’s health in RCDs
· to prepare diagnosis and treatment guidelines (on demand and on prophylaxis)

Rare Bleeding Disorders: Therapeutic Needs: A. Shapiro, presented by D.M. DiMichele

This report was on behalf of the Medical and Scientific Advisory Council (MASAC) of the National Hemophilia Foundation In the United States. A rare disorder is legally defined as a disease or condition that affects fewer than 200,000 Americans. Deficiencies of coagulation factors such as VII, II, X, V, protein C, and plasminogen are so rare as often not to be listed in rare disease registries. These rare deficiencies pose significant treatment issues. Individuals affected with rare deficiencies may have little or no option for treatment due to barriers to
product development including cost of research and development and conduct of clinical trials, both of which often far outweigh potential financial gain due to the limited market. Added to these issues may be regulatory burdens for both the manufacturer and the investigator. Therapeutic modalities may be available in one country but not in another at times due to lack of synchronization of regulatory agencies. As well, some products may have off-label use for some deficiencies, but may not be available in all countries. Off-label use of drugs may lead to difficulty with reimbursement. Thus patients with rare deficiencies suffer from limited options for care, and their standard of care is often lower than that of persons with hemophilia, with subsequent increased risk of morbidity and mortality. Options for pre-licensure studies for products for treatment of rare deficiencies in the United States, such as investigator initiated INDs and use of registry data to support new indications, with suggested minimal aims for such trials, should be explored.

**Orphan Drugs: the FDA perspective (M. Weinstein)**

The Food and Drug Administration strongly encourages the development of orphan drugs. FDA considers designating a drug (or biologic) as an orphan drug if it will be used to treat any disease or condition that affects less than 200,000 persons in the US, or that affects more than 200,000 persons in the US, but for which there is no reasonable expectation that the cost of developing and making the drug available in the US will be recovered from sales in the US. Incentives for orphan drug development include seven years of market exclusivity following approval of the drug, award of grant funding to defray the costs of clinical testing, and a tax credit on certain clinical testing expenses. The approval of an orphan designation request does not alter the standard regulatory requirements and process for obtaining marketing approval. Safety and efficacy of a compound must be established through adequate and well-controlled studies. Clinical trials should be designed to make a valid comparison with a control, to provide a quantitative assessment of the drug effect. Clinical trial designs for orphan drugs take into account the size of the patient population, prior clinical experience with similar products, whether the endpoint is easily recognized, and whether surrogate markers for efficacy exist, among other parameters. Examples of controls include placebo, dose-comparison, no treatment, or active treatment. In some cases normal volunteers could be used to demonstrate the safety of a product if not enough patients are available. Post licensure (Phase IV) data can be used to gain additional information about safety and efficacy, e.g. registry information. Foreign clinical studies are acceptable for support and/or market approval if they are well designed, well conducted, performed by qualified investigators, and are ethically sound. Examples of recent product approvals employing relatively small patient populations in clinical trials include an immunoglobulin (50 patients), and alpha one protease inhibitor (15 patients). In summary, FDA encourages orphan drug development, requires establishing the safety and efficacy of a compound through adequate and well-controlled studies, and will work with the sponsor to design clinical trials appropriate for the patient population.

**Orphan Drugs: the EMEA perspective (R. Seitz)**

There are some medicinal products for rare bleeding disorders (e.g. FXIII, FXI) licensed in European countries. Such products have been licensed more than 20 years ago, when the
requirements for clinical studies were different. There is nowadays a special EC legislation for orphan medicinal products (OMP), providing incentives, such as protocol assistance, waiver of fees, market exclusivity for 10 years, and potentially support of research, development, and availability. Criteria for OMP designation are: serious condition affecting <5 in 10,000, and lack of, or superiority over alternatives. The EMEA is willing to give advice to potential applicants, and there is a Blood Product Working Group (BPWG), providing guidelines on clinical evaluation of blood products, taking account of the rarity of the respective disorders. The question is, which OMP would be needed, and whether the industry would be willing to develop such products.

**Orphan Drugs: manufacturers views on concentrates for treatment**

(1) **LFB (Z. Tellier)**

The French laboratory for Fractionation and Biotechnology (LFB) develops and manufactures plasma therapeutic proteins. In consistency with its public health mission, LFB’s port-folio includes a large number of Coagulation factors. The majority of these are designed for the treatment of rare and very rare bleeding disorders. It is specially the case for the treatment of severe types of von Willebrand disease (WilfactinÔ, a high purity and low FVIII content von Willebrand Factor); the substitutive treatment of congenital afibrinogenemia (ClottagenÔ), of inherited Factor VII deficiency (Facteur VII-LFBÔ) and of inherited Factor XI deficiency (HemolevenÔ). A large program of viral safety and protein yield optimisation is applied and updated on an on going basis leading to safe products and to the possibility of an increased availability for patients suffering from these very rare diseases outside of France.

(2) **ZLB Behring (S. Knaub)**

Hereditary deficiency in fibrinogen (FI) and coagulation Factor XIII (FXIII) are rare bleeding disorders with a prevalence of 0.5 to 1 per million people and 200 to 300 known cases worldwide to date. These disorders are mainly characterized by moderate to severe bleeding; the bleeding events are observed soon after birth or in early childhood. Haemocomplettan P (a fibrinogen concentrate) and Fibrogammin P (a FXIII concentrate) are provided by ZLB Behring as orphan drugs to treat these rare disorders. Both products are made from human plasma. Virus safety is achieved by careful donor selection, testing of plasma donations by NAT/PCR, effective virus elimination and inactivation steps during production and by final product testing. Haemocomplettan P and Fibrogammin P have been on the market for more than 20 years with an excellent track record of efficacy and safety as documented by data from clinical studies, published data as well as data from ZLB Behring’s post- marketing surveillance.

(3) **BPL (P. Feldman)**

BPL supplies factor VII, factor XI and antithrombin concentrates for the treatment of rare bleeding disorders. These products are manufactured and tested using the same exacting quality
standards, facilities and procedures which are used for our other, licensed products. This includes additional testing by the independent WHO laboratory at NIBSC, because all BPL products are manufactured primarily to serve clinical needs in the U.K. Surplus capacity is then supplied to serve non-UK needs, with an emergency capability to deliver within 48 hours. Even greater access to such products, and the development of new ones, requires progress to eliminate perceived constraints. This may be helped by a new product licence category for orphan drugs which would (a) accept limited patient numbers/outcomes for clinical trial; (b) accept generic validation and risk-assessment to rationalise development costs; and (c) establish a legal framework in which products may be marketed within acceptable bounds of liability.

General discussion: treatment of rare bleeding disorders

A lively discussion ensued when Dr. Mannucci issued the challenge to manufacturers to create novel concentrates for other rare disorders such as factor V deficiency and to the regulators to facilitate and allow development and licensure thereof. The regulators response was positive but cautious on what even good collaboration between Europe and US authorities could accomplish. Dr. C Lee referred to the need of such products in the developing countries as well. Manufacturers’ representatives reminded to the fact that costs may be prohibitive given the complexity of doing extensive pre-licensure evaluation. The general impression was that global harmonisation of regulatory policies in dialogue with manufacturers is needed to open the way for developing and licensing products for rare bleeding disorders. Collaboration on all levels will be needed to achieve this goal.

Standardisation issues: assay variability and standards

External Qualify Assurance data: factor V assays: E. Preston and S. Kitchen

Dr. Preston presented data on factor V assays that demonstrated a significant interlaboratory variability. These discrepancies were caused by variability between available reference plasmas. Dr Preston strongly advocated the establishment of an International Standard for factor V in plasma.

International plasma standard for factor V? A. Hubbard)

Dr. Hubbard continued on the issue and emphasised the importance of factor V assays in connection with the diagnosis or exclusion of rare bleeding disorders such as "parahaemophilia" (FV-deficiency) or a combined deficiency of FV and FVIII. Estimation of FV is also included in the quality control testing of virus-inactivated fresh, frozen plasma. There is currently no internationally defined unit for FV and hence determinations rely either on local plasma pools or on a variety of independently calibrated commercial reference plasma preparations. This is not surprising considering the lability and wide normal range of FV in plasma. Upon Dr. Hubbard proposal, the Subcommittee was in favour of calibrating a freeze-dried pooled normal plasma
preparation as the WHO 1st International Standard for Factor V in plasma, in particular if this
calibration would also include the new 3rd SSC Plasma Working Standard.

Dr. Mertens closed the meeting at 12.30, thanking all presenters and participants.
Factor XIII and Fibrinogen Joint Meeting

Chairmen:
Robert Ariëns (Factor XIII)
Nicodermo Weinstock (Fibrinogen)

Co-Chairmen:
Paul Bishop, Akitada Ichinose (Factor XIII)
Jaap Koopman, Susan Lord, Ron McIntosh (Fibrinogen)

Active Members:
Charles Greenberg, Hans Kohler, Laszlo Muszbek, Reiner Seitz (Factor XIII)

The FXIII and fibrinogen subcommittees held a joint meeting this year. There were approximately 30-70 interested researchers present and the presentations were followed by lively discussions. Thanks to recent efforts in the first international collaborative study for FXIII standardisation, a large part of the meeting was dedicated to the presentation of these data.

I. Measurements of FXIII

The meeting was opened with a presentation from Trevor Barrowcliffe (NIBSC, UK) regarding the general principles for standardisation of coagulation factors and inhibitors. Standardisation efforts are normally composed of several stages including preliminary investigation of materials suitable for standardisation, trial fills, stability studies, large-scale fills, international collaborative studies, and the final report for recommendation through the SSC/ISTH to the WHO. Once a standard has met approval by the WHO, continuity of the unit can be assured through either long-term usage of same standard, replacement by similar material, and/or crosschecks versus normal plasma or previous standards. Stability studies can be performed at a higher temperature to accelerate the procedure, but high-temperature degradation studies may overestimate stability. Stability studies are best performed in more than one lab. An important issue for standardisation is to compare ‘like’ with ‘like’, for example use a plasma standard for plasma measurements. Assay methodology can play a role in potential inconsistencies. However, reference methodology is normally not easily definable, though some pre-description for protocol may be useful.

Sanj Raut (NIBSC, UK) presented a report on the first international collaborative study for a FXIII standard. Preliminary studies had shown improved consistency between activity assays when FXIII deficient plasma was used as diluent rather than buffer. Large-scale fills and the questionnaire/recruitment stages were successfully completed to proceed with the international collaborative study. Samples used were X - a 40 fold concentrate of FXIII, Y - the proposed first international standard for FXIII, A – plasma lot 2 and B – plasma lot 3. FXIII deficient plasma was provided as reagent to all participating labs. In total, 23 labs returned data, there were 23 activity and 10 antigen measurements. Potency and variability versus pooled plasma and candidate Y were assessed. There was a good consistency of the measurement of FXIII activity
for candidate Y with a potency of 0.91 u/ml and an inter-laboratory CV of 11.5%. Antigen measurements were mostly consistent with this although showed somewhat greater variability. Data for the FXIII concentrate showed some discrepancy between methods and this will need further consideration. A detailed report has been submitted to all co-chairs, active members of the FXIII SSC and participants of the Collaborative Study for feedback and approval prior to submission to the SSC.

Laszlo Muszbek (Hungary) discussed the measurement of FXIII activity in tissue sealants and concentrates. The activation of FXIII is greatly enhanced by the conversion of fibrinogen into fibrin and its subsequent polymerisation. There are therefore important considerations to be made whether FXIII activity is measured in the presence or absence of polymerising fibrin. Dilution of samples in buffer or FXIII deficient plasma can change the concentration of fibrin(ogen) present and hence activation. As a result of the presence of fibrinogen it was found that FXIII was best measured in plasma as opposed to citrate buffer. In addition FXIII appears to be more stable when diluted in plasma when compared with buffer. When fibrin sealants are diluted 20-40 fold a physiological concentration of FXIII is found.

In the last presentation of this section, Janos Kappelmayer (Hungary) discussed data regarding the assessment of FXIII expression on various cells by flow cytometry. Lymphocytes in M5 leukaemia stained strongly positive to both CD14 and FXIII A-subunit. FXIII A-subunit expression correlated positively with CD14. The expression level of FXIII A-subunit increased significantly from M0 to M0-2 and the highest staining was observed in M4. FXIII A-subunit expression on cells was in increasing order M0, M4, M5, CMML and PLT. It was concluded that FXIII A-subunit is an early marker of haematopoietic development in monocyte lineage.

II. Developments in treatment of FXIII deficiency

Aki Ichinose (Japan) presented an update on the studies on FXIII gene-knockout mice. Transglutamination can be considered as a major post-translational modification in proteins. There are up to 9 different human transglutaminases known to date, and an array of diseases ranging from neurological disorders, bleeding/thrombosis, cancer and hepatic disorders amongst others have been associated with malfunctions of transglutaminases. In order to determine respective functions of FXIII, a mouse model was established in which the FXIII A-subunit and B-subunit genes were targeted respectively. FXIII B-subunit gene knockout was performed by targeting exons 1 and 2. Blotting confirmed that B-subunit was absent in plasma. Although B is a carrier for A, some A-subunit appeared to be still circulating in the plasma of B-knockout mice. Interestingly, no major pathological defect was found for the FXIII B-deficient mice. It may be possible that B knockout mice are normal unless challenged for bleeding. To a certain extent in agreement with this, B-deficiency in humans is normally associated with a milder bleeding disorder than A-deficiency. The A knockout mice showed complete absence of A in plasma as determined by blotting. These mice showed excessive bleeding. Male mice died earlier from bleeding than female mice if the latter were not used for breeding. However, the female mice showed excessive bleeding, with necrosis and bleeding in the uterus and placenta, upon pregnancy. Miscarriage in the A-subunit knockout mice was due to bleeding and not
implantation defects for example. It was concluded that FXIII gene knockout mice provide a good model for human FXIII deficiency.

In the next presentation, Ken Lewis (USA) discussed recent data on the biochemical – physiological analysis of recombinant FXIII A-subunit infusion in humans. Single doses up to 50 U/kg of rFXIIIA appeared safe, with a dose-response of 1.77% per U/kg. Five daily doses were assessed and the relationship of FXIII half-life to doses and B-subunit levels were investigated. Pharmacodynamics showed a much reduced half-life in a FXIII B-deficient subject. In normal subjects, there were differences in the levels of total A-subunit, A2B2 complex, and B-subunit levels in response to rFXIIIA infusion. There was normal affinity of rFXIIIA for B, leading to spontaneous and rapid A2B2 formation. Activity levels increased more than A2B2 levels suggesting saturation of B. The approximate half-lives were determined as 30 hrs for A2, 8.5 days for A2B2 and a surprisingly short 16.7 hrs for B. Overall, rFXIIIA appeared to behave according to expectations based on these pre-clinical and biochemical studies.

III. Fibrinogen interactions

Leonid Medved (USA) presented structural data on molecular interaction between thrombin and fibrinogen E-region. X-ray crystallography was performed on the complex of thrombin and the fibrinogen E-region produced by cleavage with the leech enzyme hementin. Hementin uniquely produces an E-fragment with intact fibrinopeptides A and B. The crystal complex showed two thrombin molecules on either side of the fibrinogen E-region and was resolved at a resolution of 3.6 Å. It was shown that thrombin interacts with the E-region of fibrinogen through exosite I. Superimposing the structures of hementin E-fragment with that of the thrombin exosite I showed that the exosite is only partly involved. The orientation of thrombin on the E-region through exosite I means that the catalytic triad is located to the side of the molecule at a certain distance from the fibrinopeptides. A model was proposed to explain the preferred cleavage of FpA. FpA extends to the catalytic triad of thrombin, whereas FpB demonstrates a more random orientation. After cleavage of FpA, FpB assumes a conformation that orientates it towards the thrombin catalytic triad. Molecular modelling was used to support this theory and the structural changes in molecular confirmation were shown by computed animation.

IV. Clinical implications

Gordon Lowe (UK) discussed the role of fibrinogen in atherothrombotic disease. There is strong support for a consistent association between fibrinogen levels and atherothrombotic disease. The questions that remain include whether fibrinogen is a causal factor for disease and, related to this, whether lowering fibrinogen concentration reduces risk. Several potential mechanisms were discussed. Fibrinogen is involved in fibrin clot formation, fibrin structure/function, platelet aggregation, cell adhesion, erythrocyte aggregation and determines plasma viscosity. It is perhaps the latter that in addition to fibrinogen itself shows the most significant association with disease. Associations have been described between fibrinogen and/or plasma viscosity with carotid intimamedia thickness or claudication. In the latter, bezafibrate reduced fibrinogen by around 14%, along with decreased cholesterol and red cell aggregation, and improved walking distance. Exercise has been shown to lower fibrinogen and to alter plasma viscosity. There is
evidence to suggest that not only increased fibrinogen associates with vascular disease but also alterations in structure/function of fibrinogen, such as effects of oxidation and other post-translational modifications. Fibrinogen levels significantly alter the risk for myocardial infarction or stroke even after adjustment for all known other risk factors. However, associations between genetic polymorphisms that alter gene expression and risk for disease are inconsistent. It was concluded that fibrinogen levels are significantly associated with vascular disease and that this association may be causal (although this requires further investigation), but that at the moment there does not seem to be a clinical utility in measuring fibrinogen, as fibrinogen-lowering drugs are currently not an option for the treatment of vascular disease.

**Hans Kohler** (Switzerland) discussed the clinical relevance of FXIII assays. It was noted that there are several different types of FXIII assays and that there are differences in the definition of activity in some of these. The NEQAS study has shown that with some FXIII assays in some labs there is a high percentage of misclassification of FXIII deficiency with measurements up to 50% of normal, which clearly identifies the need for standardisation. A better characterisation of FXIII deficiency is obtained through the usage of ELISA methods for the separate FXIII subunits. An additional problem is different responses of activity assays to the Val34Leu polymorphism. A poor correlation exists between activity assays that measure either incorporation of a small amine or the generation of ammonia by NADH respectively, but this correlation improves on separate analysis of the Val34Leu genotypes. Assays that are sensitive to the activation step by thrombin (which is affected by Val34Leu) may need to be altered to achieve full activation before assay. It was also noted in the discussion that it is important to include a blank to control for basal NADH oxidation of plasma in assays that are based on the measurement of the amount of ammonia released during the cross-linking reaction.

The joint meeting of the FXIII and fibrinogen SSC’s was concluded at 6.15 pm, after a lively discussion regarding assay methodological and other issues.
Fibrinolysis

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

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

I. TAFI session:

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

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

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

In this TAFI session, Dr. Declerck also announced that, as discussed at the previous meeting, his laboratory (Dr. A. Gils) together with the laboratory of Prof. I. Juhan-Vague has been collecting plasma samples for evaluation of the currently used assays. It is anticipated that by the end of 2004 or early 2005 these samples will be sent for analysis to participating laboratories. The results of these analyses will be presented at the SSC-fibrinolysis meeting in 2005.
II. **Standardization session**

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

III. **III D-dimer**

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

IV. **General discussion and future activities**

Currently used (pro)CPU/TAFI(a) assays will be evaluated by various laboratories (coordination Dr. A. Gils) and the results will be presented at the 2005-meeting.

Consequent to discussions with the Working Group on Secondary Standards and preliminary evaluation by some members of the SSC-fibrinolysis subcommittee the meeting agreed to calibrate the newly prepared SSC Plasma standard lot 3 for t-PA antigen and for PAI-1 antigen and activity. This will be coordinated by Dr. Longstaf and the results will be presented at the 2005-meeting.

D-dimer as well as the standardization, which are further handled for the next SSC subcommittee. In addition, as the new issue in this SSC subcommittee, we will work on plasmin and streptokinase preparation, the detail of which is announced later. Any of the (co-)chairs can be contacted for further suggestions concerning the meeting in 2005.
The meeting was attended by around 60 – 70 people including all (co-)chairs. The meeting was closed at 12:00.
Haemostasis and Malignancy

Chairman: A. Falanga, Italy
Co-chairs: C. W. Francis, USA; A. K. Kakkar, UK; A. Lee, Canada;
M. Prins, The Netherlands; L. Zacharski, USA

The first part of the meeting was devoted to discussing some biological aspects, that require particular attention and better standardized methods and was divided in two sections: Section 1 (Chairled by A. Falanga and CW. Francis) was dedicated to the heterogeneity of tumor tissues as regard to hemostatic properties and to methods to measure Tissue Factor and angiogenesis in human and experimental systems.

L. Zacharski reported on the heterogeneity in mechanisms of coagulation expressed by individual tumor types. Starting from the observation that antithrombotic drugs tested in clinical trials for survival in cancer patients and experimental models gave different results, he showed data demonstrating that tumor tissues can express different patterns of coagulant and fibrinolytic proteins. Therefore tumor cells can respond differently to antithrombotic drugs, depending on the pattern of coagulant/fibrinolytic proteins they express. Recognition of this heterogeneity should be fundamental to the design of future clinical trials with antithrombotics in cancer patients.

N. Key overviewed the methods for tissue factor measurement in circulating blood.

Methods available for TF measurement in circulating blood are:

**Whole blood TF**: TFmRNA, whole blood clotting time, whole blood procoagulant activity.

**TF in isolated cell fractions** (i.e.: monocytes, leukemic cells, platelets): TF antigen by flow cytometry on intact cells; TF procoagulant activity (by coagulometric or chromogenic method) on intact or lysed cells; TF antigen by ELISA on lysed cells.

**Plasma TF**: TF antigen by ELISA, FVIIa assay.

**Microparticles**: TF antigen by flow cytometry; TF procoagulant activity by capture assay.

The preanalytical variables and other methodological problems related to the standardization of these methods were discussed. A proposal was made and was accepted by the subcommittee to start a collaborative *ad hoc* project between different laboratories of participants in this ISTH-SSC. Lastly, to resolve the problem of the lack of reference material against which measured TF:PCA can be standardized, T Barrowcliffe offered to work to preparing an international TF standard.

A. Falanga will prepare a formal proposal.
C. Francis overviewed the methods available to measure tumor angiogenesis and brought up the issue of the lack of well standardized methods to evaluate the response to anti-angiogenic drugs either in preclinical or clinical studies. Particularly the following methods were analysed: microvascular density, imaging modalities, biopsy evaluation, and blood biomarkers. Some data on the predictive value of tumor response to therapy of some of these tests in human malignancy were also shown. However, clearly there is a need for more specific and sensitive methods to measure angiogenesis. Particularly measurement reproducibility is crucial when assessing angiogenesis in clinical trials.

M.B. Donati gave her comments and reflections on the methodological issues presented above.

In the Section 2 of Biological aspects (Chaired by L. Zacharski and AYY. Lee), A. Falanga discussed the issue of the clinical utility of measurements of laboratory markers of hypercoagulability. She overviewed the current data available in the literature on ‘thrombotic markers’ measurements in the setting of cancer patients and pointed out that very little information is useful for the patient management in clinical practice. It is therefore urgent to incorporate studies of biological markers in large prospective randomized clinical trials in order to find some predictive tests of thrombosis. This would help to identify ‘high risk’ categories of patients and correctly plan the appropriate prophylaxis, particularly when additional risk factors are present (i.e. chemotherapy, surgery, etc.).

J. Fareed showed what can we learn on the mechanism of anticoagulant drugs by monitoring surrogate markers of thrombotic activation and inflammation. Particularly he stressed the importance of profiling for these markers at baseline, in patients enrolled in clinical trials of antithrombotic treatments, and after starting therapy. He presented data of the measurements of TF, TFPI, TAFI, TNFalpha, CRP, NO, CD40L, MCP-1 and IL-6 in patients enrolled in a trial of DVT treatment in cancer patients (ONCENOX) compared to normal healthy controls and medical non-cancer patients. This data demonstrate that including substudies to profiling for the surrogate markers of inflammation and hemostatic activation can help to identify the mechanisms of cancer-mediated thrombosis and to understand the inter-relation between thrombosis, cancer and inflammation.

B. Brenner commented on the laboratory test presentations and provided his personal reflections on that.

The second part of the meeting was dedicated to the Clinical Research: methods for clinical investigation (Chaired by A.K. Kakkar and M.H. Prins).

First there was a focus on areas addressed by clinical research. AK Kakkar gave a review of the current valuable data available and drew some preliminary recommendations on the primary prevention of VTE in cancer (surgery, CVC). AYY Lee reviewed the problem of the difficulties currently encountered in the management of antithrombotic therapy in patients with cancer and VTE. From the results of the recent clinical trials, she drew some preliminary recommendations on the topic of initial treatment and long term treatment of VTE in cancer.
Second there was a focus on areas for further investigations. **M. Levine** discussed the current knowledge on thromboprophylaxis during chemo-/hormone-therapies for cancer and what are the future challenges in this area. **M. Prins** critically overviewed the studies available on antithrombotics and cancer survival and gave his suggestions on how to design future studies in this field.

The meeting was concluded with a Session on **Update on ongoing clinical trials and registries**.

**G. Agnelli** showed the results of the ETHICS study evaluating six week of Enoxaparin vs placebo for the prevention of upper limb CVC-associated DVT. Patients enclosed were 385 and 50 of them had positive venographies (16.1%). The results show a trend to reduced DVT in the prophylaxed arm without reaching a statistical significance.

**S. Haas** presented the TOPIC-I and TOPIC-II studies. These two studies evaluate Certoparin against placebo in the prevention of VTE in breast (TOPIC-I) or lung (TOPIC-II) cancer patients receiving chemotherapy. The TOPIC I study has been stopped to the first phase (primary end point was any VTE event) because there was no difference between certoparin–treated group and placebo group. In contrast, TOPIC II shows an efficacy of the LMWH in reducing DVT without increasing bleeding. There was much discussion about different response to prophylaxis by the different tumor types.

**M. Levine** presented a clinical trial of Fragmin prophylaxis (for at least 6 months) in patients operated for malignant glioma eligible for radio- and chemo-therapy treatments (PRODIGE). Ongoing (100 patients enrolled so far).

**G. Agnelli** presented the PROTECHT study for the prevention of venous and arterial VTE with Nadroparin (versus placebo) in patients receiving chemotherapy for cancer of the lung, breast, GI, ovary, head and neck. Ongoing (260 patients enrolled so far).

**L. Zacharsky** presented in place of F. Icli the preliminary data on a pilot study of the effect of LMWH on survival in advanced pancreatic cancer.

**M. Rassmussen** presented the results of the FAME study on prolonged thromboprophylaxis with LMWH dalteparin in major abdominal surgery. The study has enrolled 590 patients and demonstrates the efficacy of prolonged versus standard thromboprophylaxis, differences are statistically significant.

**M. Moia** presented the Italian registry of CVC-related thrombosis in haematological patients (CATHEM). Included > 450 patients. The analysis has been completed and confirms a 3% incidence of symptomatic DVT and gives information on other CVC related thrombotic complications (i.e. arterial thrombosis, superficial thrombophlebitis, CVC-occlusions/malfunctions, infections). Incidence of symptomatic DVT + superficial thrombophlebitis + CVC-occlusions/malfunctions was about 12%.
AK Kakkar presented the Prospective registry of cancer events involving venous thromboembolism (PERCEIVE) which will answer to the following questions:

- establish the clinical incidence of VTE in cancer;
- determine when, in the natural history of different cancer, thrombosis occurs;
- identify other tumor, patient or therapy factors that impact on the thrombotic risk;
- establish the appropriate opportunities for potential antithrombotic interventions.
Lupus Anticoagulants/Phospholipid-Dependent-Antibodies

Chairman: Ph. G. De Groot, The Netherlands
Co-chairs: J. Arnout, Belgium; M. Galli, Italy; S. Machin, UK; V. Pengo, Italy; J. Rand, USA; R. Roubey, USA

Number of attendees: more than 100

Introduction: The chairman opened the meeting and summarized the goals of this meeting. Dr. Pengo distributed a questionnaire with questions regarding antiphospholipid testing in diagnostic laboratoria. The goal of this questionnaire is to reach standardization in testing and the questionnaires were collected at the end of the meeting.

Prospective in LA diagnosis

Dr. Pengo (Italy) discussed his recent finding that by using different Ca\(^{2+}\)-concentrations to activate coagulation it is possible to discriminate between a b2-Glycoprotein I (b2GPI)-dependent lupus Anticoagulants (LAC) and a prothrombin dependent LAC. A reduction in final calcium concentration, from 10mM to 5mM, increased coagulation times in both dilute Russell Viper Venom Time (dRVVT) and dilute Prothrombin Time (dPT) when plasmas of patients with anti-b2GPI antibodies were used. Instead, all LA-positive anti-b2GPI antibody negative patients showed decreased coagulation times from mean. These results are confirmed by running dRVVT of normal plasma spiked with affinity purified IgG anti-b2GPI antibodies. Therefore, when a PL-dependent coagulation test is run twice, at different final calcium concentrations, clinically important anti-b2GPI LA can be identified.

Dr. Arnout (Belgium) have used his panel of monoclonal antibodies directed against b2GPI and prothrombin that exert LA activity to confirm the effects of different Ca\(^{2+}\) concentrations on dRVVT and dPT. He confirmed the original observations of dr. Pengo but showed that the presence of a high level of a anti-prothrombin antibody mediated LAC masks the presence of a b2GPI dependent LAC. Also the presence of an anti-factor VIII, LMWH. OAC etc should be carefully evaluated before the test as proposed by dr. Pengo could be introduced.

Dr. de Groot (the Netherlands) discussed the assay they use to detect a b2GPI–dependent LAC. This assay is based on the use of cardiolipin as confirming agent in a PTT-LA coagulation assay. His group studied the clinical of the detection of b2-glycoprotein I-dependent LAC in a cohort of 198 patients with autoimmune diseases. Presence of b2-glycoprotein I dependent LAC was strongly associated with a history of thrombo-embolic complications, an Odds Ratio of 42.3 (95% confidence interval 194.3 - 9.9) was found. An increased frequency of thrombosis was not found in the 33 patients with LAC independent of anti-b2-glycoprotein I antibodies (Odds Ratio 1.6, 95% confidence interval 3.9 - 0.8). The use of a LAC assay with cardiolipin as confirming agent strongly improves the detection of patients at risk for thrombosis. This suggest that anti-b2-
glycoprotein I antibodies with LAC activity are antibodies responsible for the thrombo-embolic complications in APS.

In a separate study he compared dr. Pengo’s assay with the assay they developed in their own lab. In general a good correlation was found, although some discrepancies were noticed, probably due to the presence of mixtures of antibody populations.

A multicentre study was launched in which the clinical relevance of a b2GPI-dependent LAC will be tested in a cohort of at least 500 patients. This study will be performed in collaboration with and supported by Stago R&D (France)

*Standardisation of the LAC assay*

**Dr Arnout** (Belgium) discussed the development of a new standard for LAC testing. The standard should contain a mixture of patient pooled plasmas, normal pooled plasma spiked with monoclonal antibodies and normal pooled plasma. To make the standard, 10 L of patient plasma is needed. He suggested to collect 100 mL of plasma from 100 patients with high titer LAC. Dr Arnout distributed a form with which participants could enlist donors for this pool. After a long discussion on ethics and selection of patients a protocol and time table was accepted. The plan is to have the standard ready for the next meeting in Sidney. A steering committee (Arnout, de Groot, Mackie, Jennings, Gray, Sie, de Moorloose & Pengo) will guarantee the quality and progress of the procedure.

**Dr. Mackie** (United Kingdom) assessed a new dilute prothrombin time diagnostic assay, ACTICLOTÔ dPTtestâ and ACTICLOT dPTconfirm (AdPT, American Diagnostica Inc, USA) for lupus anticoagulant (LA) detection. The ACTICLOT dPTtestâ is a lipidated recombinant human tissue factor-based clotting assay designed to screen plasmas for LA. The ACTICLOT dPTconfirm test utilizes a high phospholipid-containing reagent to confirm the presence of LA. The clotting times are compared using calculations similar to those employed for DRVVT methods. AdPT was positive patients, 23 oral anticoagulant and 17 new thrombophilia outpatients. Samples were considered LA positive if they complied with International criteria and were positive in at least 2/4 reference methods (APTT ratio using Actin FSL, two DRVVT methods employing either high phospholipid or platelet neutralization confirm reagents, and StaClot LA). Two different lots of AdPT reagent were tested on an ACL300R analyzer and gave similar results in normals and LA patients. The AdPT reagents were stable for at least 96hr and the within run cv for normal and LA positive samples was <5%. Cut-off values for LA were based on AdPT results from normal subjects. 19/23 APA group patients had LA by AdPT (median and range 2.5 and 1.1-5.7 for patient/normal ratio; 1.4 and 0.9-3.7 for test/confirm ratio). All 17 thrombophilia patients were LA negative and AdPT negative. Using the APA and thrombophilia groups, and definite LA positive and negative samples, AdPT showed 83% sensitivity and 100% specificity. When AdPT was coupled with any of the other LA tests, sensitivity varied from 96-100% and specificity 82-100%. When a sensitive APTT reagent was used for screening, with AdPT and a DRVVT as specific tests, 96% sensitivity and 100% specificity were achieved. If three tests were performed, using APTTr mainly as a screening test, specificity was 100%. In oral anticoagulant patients, AdPT exhibited similar problems to other
reagents, suggesting that mixing tests should be performed. The results suggest that AdPT is highly specific and detects the majority of patients with LA, albeit a slightly different spectrum of LA antibodies than seen with DRVVT or APTTr. ACTICLOT dPT used in combination with two recognized LA reagents provided the best diagnostic sensitivity for LA in patient samples studied in 20 healthy normal subjects, 23 known antiphospholipid antibody (APA)

Dr. Jennings (United Kingdom) discussed the effect of pH and buffering on DRVVT results with different commercial Kits. Following the observation that the dRVVT results of normal pooled plasma of NEQUAS-distribution in England showed substantial variation he found that the pH of the plasma strongly influenced the results of LAC testing. When the pH of normal plasma is above 8.0, a dRVVT becomes positive. As the pH of lyophilised plasma after reconstitution can be above 8.0, he advised to use a Hepes buffer in dRVVT-testing.

Prospective in ELISAs

Guido Reber (Switzerland): At the Sapporo consensus meeting [6], anti-β2GPI antibodies (aβ2GPI) were not included in the list of biological criteria defining the antiphospholipid syndrome (APS). At the SSC held in Boston July 2002, a new classification was proposed, which included aβ2GPI either alone or in the presence of lupus anticoagulant. One of the issues raised by adding this test to the biological criteria was the standardisation of these assays. Since 1999, our group has performed three collaborative studies on this topic. One study addressed the performances of homemade assays and the last one looked for the agreement between commercial tests (both were presented at the SSC meeting held in Birmingham July 2003). In summary, the results of our studies showed that the agreement was good for high positive samples, fair to poor for medium positive and poor for low positive. For commercial kits, there was agreement in sample classification (positive or negative) between the ten commercial kits in 12/22 samples for IgG isotype and in 5/22 for IgM isotype. Analysis of the data prompted us to make some proposals to improve the agreement between assays.

1) To perform duplicates

2) Control group for the determination of the upper limit of the reference range (cut-off)

3) Calculation of the cut-off value

Because the distribution of the values is not Gaussian, the cut-off has to be calculated by the method of percentiles and not by adding standard deviations to the mean value. When analysing the distribution of the values of the control group, it must be kept in mind that anti-β2GPI antibodies are found in 2 to 5% of healthy individuals, especially in individuals aged more than 60. Kits' manufacturers are asked to indicate the units values corresponding to the 95%, 97.5% and 99% of the distribution of the control group in addition to the cut-off value they recommend. To fulfil regulation authorities requirements, the proposed cut-off takes into account sensitivity and specificity data obtained from clinical studies.

4) Humanized monoclonal antibodies as reference calibrators
Because there are still no worldwide-accepted anti-β2GPI calibrators, we propose to use humanized MAb dilutions as calibrators to allow comparisons between assay systems. Our group is aware that a MAb, because it is directed to one single epitope, cannot mirror the diversity of the subsets of patients' autoimmune polyclonal antibodies with different binding affinities, generally lower than MAb. MAb may not detect micro heterogeneities in β2GPI preparations because they possibly do not affect their binding, as would be the case for subsets of patients’ antibodies. But MAb are not affected by batch-to-batch variation because their affinity do not change notably with time. This ensures that the stability of assay systems can be checked over many years.

We hope that these proposals will improve the agreement between assay results. Thirteen different commercial firms have agreed to follow this proposal and to include the humanised monoclonal antibodies as standard in their tests.

**Does a panel of aPL tests improve diagnostics?**

**Monica Galli** (Italy): A systematic review of the published articles on the antiphospholipid syndrome showed lupus anticoagulants were a clear risk factor for thrombosis, irrespective of the site and type of thrombosis, the presence of systemic lupus erythematosus, and the methods used to detect them. Anticardiolipin and antib2-glycoprotein I antibodies were possible risk factors of thrombosis, at least in some selected situations, whereas the measurement of antiprothrombin antibodies was not helpful to define the patient’s risk of thrombosis. In most cases, these studies analyzed the relationship with thrombosis of each single antiphospholipid antibody, rather than the combination of different antibodies with each other and/or with the coagulation tests commonly used to detect lupus anticoagulants. Based on these premises, we retrospectively studied a group of 103 well established lupus anticoagulant-positive patients for whom laboratory data about anticardiolipin, antib2-glycoprotein I and antiprothrombin antibodies were available, in order to investigate whether laboratory patterns emerged for their association with thrombosis in the antiphospholipid syndrome. First, anticardiolipin, antib2-glycoprotein I, and antiprothrombin antibodies were analyzed separately. We observed the following statistically significant associations. No association with arterial thrombosis reached significance.

Second, the antibodies were combined in different patterns, which included from 2 to 5 laboratory variables. Matching the variables for the antibody isotype, 60 laboratory patterns were generated. Their analysis with respect to three different types of thrombosis made us available 180 different combinations. Overall, 22 associations reached significance:

<table>
<thead>
<tr>
<th>Type of thrombosis</th>
<th>N. of significant associations/Total N. of associations</th>
</tr>
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<tbody>
<tr>
<td>Any</td>
<td>14/60</td>
</tr>
<tr>
<td>Venous</td>
<td>8/60</td>
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</table>
In all but 2 cases, anticardiolipin antibodies > 40 units (G or any isotype) were present in the laboratory patterns that reached significance. Antib2-glycoprotein I antibodies (G or any isotype) were present in 11 significant patterns, and antiprothrombin antibodies (M or any isotype) in 7 cases. KCT Ratio of 1:1 mixture > 1.5 was present in 7 patterns which were significantly associated with thrombosis, dRVVT Ratio of 1:1 mixture > 1.5 in other 7 cases, and both KCT and dRVVT Ratios > 1.5 in one case. Increasing the number of variables of the laboratory patterns did not increase the Odds Ratio towards thrombosis. Figures 1 and 2 show the OR with 95% CI of the laboratory patterns which reached statistical significance.

**Conclusions**

The analysis of the antibody patterns of a large cohort of lupus anticoagulant-positive patients confirmed that the presence of medium to high titres of IgG anticardiolipin antibodies, either alone or in various combinations, increases their risk of thrombosis. Conversely, the role of the other laboratory variables, irrespective of whether they were considered separately or combined, is less clear.

The way the results of the various antiphospholipid antibodies are reported may partly explain these findings. Unlike anticardiolipin antibodies, antib2-glycoprotein I and antiprothrombin antibodies were expressed in a qualitative fashion, which does not allow establishing clinically relevant cut offs. With respect to coagulation tests, dRVVT and KCT ratios did not show any correlation with the patients’ history of thrombosis. This is apparently in contrast with our previous observation that the dRVVT profile rather than the KCT profile was associated with thrombosis in lupus anticoagulant-positive patients. However, we must underscore that the two studies had different designs, and that in the present study we considered the ratio of the 1:1 coagulation times of the mixture of patient’s with normal plasma, in order to rule possible interference of oral anticoagulant treatment.
Vittorio Pengo (Italy) performed a comparable study. Over a 6-year period, 618 consecutive patients (55% of whom with previous documented thromboembolic events) were referred to their clinic for Antiphospholipid antibody detection. LA was detected according to internationally accepted recommendations. Enzyme-Linked-Immunosorbent Assays (ELISA) detect aCL and Anti-human b2GPI antibodies. Patients’ records were reviewed for the presence of previous thromboembolic events or obstetrical complications and each patient received a physical examination. LA (Odds Ratio 2.9, Confidence Interval 1.1-7.6) and ab2GPI (Odds Ratio 2.3, Confidence Interval 0.9-5.8) but not aCL (Odds Ratio 0.7, Confidence Interval 0.3-1.6) as individual test positively are significantly associated to thromboembolic events. The rates of thromboembolic events or obstetric complications were 94% (34/36) in patients with a triple positivity, 58% (18/31) in those aCL-and ab2GPI-positive but LA-negative, 50% (4/8) in patients in which ab2GPI antibodies was the single positive assay, 35% (7/20) in patients in which medium-high titre aCL was the single positive test, while no one of 5 patients in which Lupus Anticoagulant was the single positive test has had previous thromboembolic events. When results were tested in a logistic regression analysis, only the complete positive antiphospholipid profile was independently associated with thromboembolic events (OR 14.8, CI 3.5-62.2). ACL and ab2GPI antibody positive but LA negative profile showed an association with thromboembolic events without reaching statistic significance. The mean level of IgG Anti-human b2-glycoprotein I antibodies was statistically higher in triple positive profile and might account for positive Lupus Anticoagulant. These results show that LA and IgG ab2GPI antibodies as individual positive test but specially the combined positivity of all three tests in a single patient may be considerable for identifying patients with antiphospholipid syndrome (APS).

Dr. Machin (United Kingdom) commented on these observations and showed that in the patient population of his hospital a substantially number of APS patients had only a positive anti cardiolipin.

After an intense discussion agreement on headlines became apparent but no agreement could be reached on details. It was proposed that the chairman and cochairs should make a proposal on aPl testing and that this proposal should be proposed at the next meeting in Sidney. Hopefully, in Sidney an agreement of new criteria on aPL testing could be reached.

Relevance of other antibodies

Dr. Rand (USA): gave an overview on annexin A5 and its possible role in APS. He discussed an assay he developed in his lab that was based on the inhibition of a clotting test by annexin A5. In
the presence of aPL, the inhibitory effect of annexin A5 becomes less. The ‘shortening’ of the Annexin A5 mediated prolongation of the clotting test correlated with the presence of aPl, although there is an overlap between controls and patients. More studies are needed before this assay can be introduced as a aPL specific test.

**Dr. de Groot** (the Netherlands) investigated together with dr. Mackie the prevalence of IgG or IgM annexin A5 (A5) antibodies. An ELISA was performed with purified recombinant A5 coated onto the plate in two different laboratories on patients with various autoimmune diseases, mainly the antiphospholipid syndrome (APS). In laboratory A, 2/36 patients were positive for anti-A5 IgG, both patients had APS and had complications during pregnancy. 6/36 patients had anti-A5 IgM antibodies, 4/6 patients had a history of pregnancy loss. There was no association between thrombosis and A5 antibodies. In laboratory B, 26/198 patients were positive for IgM A5 antibodies with an odds ratio of 2.7 (significant) for thrombosis and 1.3 (non-significant) for pregnancy loss. 53 patients were positive for IgG antibodies with an odds ratio of 1.5 (non-significant) for thrombosis and 2.2 (non-significant) for pregnancy loss. In this laboratory the A5 antigen levels were also measured and were non-significantly lower in patients with IgM and/or IgG anti-A5 (7.9 ± 0.7 ng/ml plasma) than in patients without anti-A5 (9.8 ± 0.9 ng/ml plasma). In conclusion, the measurement of annexin A5 antibodies seems to have no additional value for the detection of patient at risk for pregnancy loss. The relation with a history of thrombosis needs further studies.

**Dr. Mackie** (United Kingdom) investigated together with dr. de root the prevalence of IgG or IgM antiprotein S antibodies. A prototype commercial ELISA method for protein S antibodies was assessed in two different laboratories. The assay used microplates coated with highly purified human protein S, with bovine serum albumin in the blocking and wash buffers. Peroxidase conjugated monoclonal anti-human IgG or IgM were used for detection and humanised murine monoclonal anti-protein S for calibration. Good agreement was obtained between duplicate wells and replicate calibrant dilution curves on the same plate. The optical density of calibrant varied between plates and was therefore used to normalise the results. Each laboratory established separate normal ranges using 36-40 healthy normal subjects each. Laboratory A studied 14 serial samples from a 20-year-old female with thrombotic necrosis of the extremities. Six of these samples were IgG anti-protein S (aPS) positive and the results correlated with protein S activity, antigen and neutralising activity. aPS disappeared with treatment and clinical improvement. Each laboratory studied separate samples from patients with SLE, PAPS, lupus-like disease, pregnancy complications, acquired inhibitors and controls. Patients were judged APA positive if they had positive results for lupus anticoagulant, anti-cardiolipin or anti-B2GPI assays. Laboratory A studied 118 such samples, of which 18/86 APA positive samples and 1/32 APA negative samples had aPS (14 IgG, 5 IgM). Laboratory B studied 198 samples, of which 64/128 APA positive samples had aPS (54 IgG, 3 IgM, 7 dual positive); and 17/70 APA negative samples had aPS (14 IgG, 1 IgM, 2 dual positive). Overall, 38% of APA positive patients had aPS and 18% of APA negative patients; the presence of aPS thus having a high association with other APA (FET, p=0.0002). There were no significant associations of aPS with pregnancy loss or thrombosis, although further, prospective studies are required.

**Treatment of APS patients**
Guido Finazzi (Italy) discussed the optimal intensity of oral anticoagulation for the prevention of recurrent thrombosis in patients with the antiphospholipid antibody syndrome is uncertain. Retrospective studies have suggested that only doses of warfarin adjusted to achieve an international normalized ratio (INR) of more than 3.0 are effective, whereas a recent randomised clinical trial comparing high (INR range 3.0 to 4.0) vs. moderate (INR 2.0 to 3.0) intensities of anticoagulation failed to confirm this assumption. They conducted a randomized trial in which patients with persistent lupus anticoagulant and/or moderate to high levels of anticardiolipin antibodies and previous thrombosis were given either high-intensity (INR range 3.0-4.5, target 3.5) or standard intensity (INR range 2.0-3.0, target 2.5) warfarin therapy. We sought to determine whether intensive anticoagulation was superior to standard treatment in preventing symptomatic recurrent thromboembolism without increasing the rate of bleeding complications. The WAPS trial was launched in the framework of the SSC Subcommittee on antiphospholipid antibodies/lupus anticoagulants and started in July 1997. A total of 109 patients were recruited in the trial and followed for a median of 3.6 years. Mean INR during follow-up was 3.2 (SD 0.6) and 2.5 (SD 0.3) (p<0.0001) in the high- and standard-intensity groups respectively. Recurrent thrombosis was observed in 6 of 54 patients (11.1 percent) assigned to receive high-intensity warfarin and in 3 of 55 (5.5%) assigned to receive conventional treatment (hazard ratio for the high intensity group, 1.97; 95 percent confidence interval 0.49-7.89). Major and minor bleeding occurred in 15 patients (2 major) (27.8%) assigned to receive high-intensity warfarin and 8 (3 major) (14.6%) assigned to receive conventional treatment (hazard ratio 2.18; 95 percent confidence interval 0.92-5.15). High-intensity warfarin was not superior to standard treatment in preventing recurrent thrombosis in patients with the antiphospholipid syndrome and was associated with an apour results with those of a similar trial recently published support the recommendation that conventional thromboprophylaxis with warfarin targeted at INR 2.5 is usually appropriate for these patients.

The results of this meeting in Venice are:

1. A collaborative study will be started on the value of testing b2Glycoprotein I specific LAC (de Groot / Woodhams). Results will be presented in Sidney.

2. A standard will be developed for LAC testing (Arnout) The first results will be presented in Sidney.

3. A proposal for better standardisation for the b2GPI ELISA has been discussed (Reber). 13 different commercial firms have already agreed to follow these rules and to include the humanised monoclonal antibodies as standard in their tests. The proposal will be published.

4. A consensus is in reach for new criteria for aPL testing. A proposal will be formulated and presented in Sidney (chair and co-chairs).

5. The European WAPS study is finished (Finazzi). It is concluded that it is not necessary to treat patients with APS with high intensity warfarin. These results will be published. It is recommended to treat patients with warfarin targeted at an INR of 2.5. Further analysis of the observery arm of the study will follow.
Perinatal/Pediatric Haemostasis

Chairman: P. Massicotte, Canada
Co-chairs: B. Brenner, Israel; G. Kenet, Israel; P. Mathew, USA; P. Monagle, Australia; W. Muntean, Austria; U. Nowak-Göttl, Germany; N. Schlegel, France

Congenital Heart Disease

1. Cardiopulmonary Bypass (CPB) and Thrombosis: Stroke – G deVeber / A. Chan / P. Massicotte

There are minimal amount of literature in the topic of stroke in children post-CPB. There is only one abstract that estimates the incidence to be 0.4%. Approximately 50% of these children suffered from significant neurological morbidity. Other studies estimated the incidence of neurologic abnormalities (including stroke) to be 25% in children undergoing cardiac surgery. The studies are limited by methodologic issues ie. small, retrospective and not valid testing (neurocognitive in neonates). There have been studies in children to prevent neurologic injury secondary to CPB. Most studies suffer methodologic flaws.

Recommendations: The subcommittee recommends a literature review to be submitted by October 2004 with the recommendations that more studies are urgently needed.

2. Thromboprophylaxis of mechanical heart valves – M. Bauman / P. Massicotte/ A. Chan

Children receiving warfarin as primary prophylaxis for mechanical heart valves who require interventional procedures (cardiac catheterization) must have reversal of warfarin pre-procedure. Many centres hospitalize children the night before the procedure to administer intravenous heparin. The use of unfractionated heparin subcutaneously the night before the procedure was described. The children received age appropriate UFH dosing (weight kg X u/kg/hr X number of hours of anticoagulation coverage required) and a APTT the morning before the procedure was normal in 99% of children. There were no adverse events in this cohort (bleeding or thrombosis). This appears to be a safe option for anticoagulation reversal in this population and allows children to remain home until the time of the procedure.

Comments from international colleagues described other reversal methods such as the use of low molecular weight heparin (LMWH) the night before the procedure.

Recommendations: The committee suggested that further lab studies including anti-factor Xa and anti-factor IIa levels were needed to provide more data re UFH clearance.

3. Oral anticoagulant therapy in infants younger than 12 months of age. - M. Bonduel

Warfarin, acenocoumarol and phenprocoumon are the VitK antagonists used pediatric patients with thrombotic complications in different countries according to their experience and familiarity with these drugs. The studies of warfarin and acenocoumarol highlight the difficulty of their use in infants < 12 months of age. This age group required increased doses to achieve and
maintain target INRs, as well as more frequent testing, and adjustments of loading dose to achieve the target INR faster with no overshooting.

Increased alpha 2 macroglobulin levels and decreased thrombin generation were described in the pediatric population compared to adults receiving warfarin. Therefore the intensity of the oral anticoagulant therapy may vary in children respect to adult patients

**Recommendations:** The committee suggested that further studies of warfarin, acenocoumarol and phenprocoumon requirements are needed. The factors that could interfere in the action of OA agents in this age-group should be identified (i.e., Vit K use, diet, drugs, cytochrome p450 reductase levels and genotype).

**Risk factors for Venous Thrombosis/Thromboprophylaxis**

1. **Central Venous Lines: Thromboprophylaxis – L. Mitchell/S. Revel Vilk**

   Central venous lines appear to be a strong risk factor for the development of thrombosis in children. The incidence has been estimated at least 20% through prospective cohort studies and is dependent on the method of diagnosis. The outcomes include death and morbidity of post-thrombotic syndrome and recurrence. A proposal for the subcommittee to recommend a study to investigate the safety and efficacy of thromboprophylaxis was suggested.

   **Recommendations:** International colleagues felt that there was not enough data re outcomes and that certain populations of children may have varying risk with the development of thrombosis related to CVLs. Therefore, the subcommittee was reluctant to state that a thromboprophylaxis study is recommended. However, it was agreed that a communication from the subcommittee should include that risk assessment of different disease cohorts of children should be carried out. This would be followed by recommendations for a study in those cohorts at highest risk of developing VTE with adverse outcomes.

2. **Factor VIII levels and lipoprotein (a) Standardisation normal values and risk cutoffs for thrombosis in children – U. Nowak Gottl/ E. Grabowski**

   Lipoprotein (a) and persistently elevated FVIII levels have been shown to be a risk factor for venous thromboembolism in children. However, the assays for lipoprotein (a) and FVIII require standardization in order to make studies comparable.

   **Recommendations:** The subcommittee agreed that discussion should be carried out re a study with central laboratory testing. There was agreement that more studies to determine the relationship of FVIII to the development of VTE in children are necessary.

**Treatment of Thrombosis**

1. **Treatment of venous thrombosis – E. Chalmers/ H. Van Ommen**
The studies on the treatment of VTE in children using LMWH were reviewed. There is only one randomized clinical trial (RCT) estimating the safety and efficacy of LMWH for treatment of VTE in children. Discussion revealed differing treatment practices internationally.

**Recommendations**: The subcommittee agreed to set up an expert group to design a survey to determine the treatment practices. This will be a properly designed survey that will be completed and presented in Australia 2005.

2. *Effects of hirudin and heparin in neonatal plasma* - **Baier/ W. Muntean**

There have been in vitro studies carried out using cord plasma spiked with heparin and hirudin. Results suggest that neonates respond differently than adults to thrombotic stimuli and the response depends on the strength of the stimulus. Neonates may require different concentrations of anticoagulants than adults to achieve the same effect.

**Antiphospholipid Antibodies**

*Risk of thrombosis in children with APLA – C. Male*

There is good evidence of a high risk of thrombosis in children with SLE and APLA. The association of APLA with TE found in pediatric cohorts are stronger than those found in adult studies. Few children with SLE who are negative for APLA develop TE. Lupus anticoagulant is a stronger predictor of the risk of TE than anticardiolipin antibodies, anti beta 2 glycoprotein antibodies and anti prothrombin antibodies.

In children without underlying SLE case reports describe associations of APLA and severe thrombotic complications (primary APLA syndrome). Currently, it is unknown what the risk of TE is in well children with APLA. Increased prevalence of APLA are found in children who suffered from stroke compared to controls. However, recent evidence suggests, in general, APLA presence is not associated with an increased risk of recurrent stroke.

**Recommendations**: The subcommittee recommends that the data on stroke and APLA in children be published in a Position paper with the recommendations that more studies are urgently needed in this area especially in primary prophylaxis of patients with APLA and SLE.

**Monitoring of Anticoagulation**

1. *Anti-factor Xa monitoring LMWH therapy in children* – **W. Muntean**

No definite conclusions about the benefit of anti Xa monitoring and association of levels with bleeding can be made. Whether target levels recommended for adults are adequate for infants and children and whether anti factor Xa monitoring in pediatric patients can be made on the basis of the limited data.
**Recommendations:** There will be a subgroup within the committee to discuss how to assess risk of bleeding in children receiving LMWH. This group will be the same as the treatment subgroup.

**Treatment of Bleeding**

1. **FVIIa use in non hemophiliac children - P. Mathew/Bomgaars**

There is little data on the use of FVIIa in nonhemophiliac children other than case reports and small case series. A cohort study in children with liver disease and coagulopathy was proposed to determine the pharmacokinetics and estimate the safety and efficacy using surrogate markers.

**Recommendations:** A subgroup to make recommendations re studying the use of FVIIa in children will be set up. This may result in carrying out studies or an international registry.

**Antiplatelet Therapy**

1. **ASA resistance, Testing for resistance and Dosing - M. Rand/ M. Albisetti**

There is no standard definition for ASA resistance. In terms of a laboratory definition ASA resistance has been considered as the failure of ASA to produce an expected inhibitory response on one or more lab measures of thromboxane-dependent platelet activation/aggregation. The lab investigations include metabolites of thromboxane A2 or thromboxane A2 dependent responses. A small study in children with arterial ischemic stroke has found a 25% lab determined non-response to ASA therapy. Larger prospective studies are required to explore the ASA resistance and the predictive value of platelet activation parameters.

**Recommendations:** There will be discussions with Drs. Michelson and Rao (Chair and Co-chair of the Working Group on Aspirin Resistance of the Platelet Physiology Subcommittee) re the definition of ASA resistance.

**Predictors of Bleeding**

1. **Coagulation tests as predictors of Bleeding in CPB: New ways to predict bleeding /When should we test – G. Kenet/ N. Schlegel**

There are various new lab techniques available to monitor coagulation. However, it is unclear which of these techniques will be of clinical use in children.

**Recommendations:** It was agreed that the subcommittee should continue to focus on this area.
Collaborative study on a proposed international genetic reference panel for FV Leiden – E Gray

Collaborative study results on a panel of 3 genetic reference gDNA for FV Leiden were presented. Forty-one laboratories employing a total of 32 different methods participated in the study. Error rate was only 0.7%. There was no correlation with incorrect results and particular preparations. The panel behaved similarly to the laboratories own in house controls with known genotypes. It is therefore recommended that this panel of reference gDNA should be established as the 1st International Genetic Reference Panel for FV Leiden. Twenty-five out of the 41 participants, the chair and all the co-chairs of the Plasma Coagulation Inhibitors SSC have agreed with this proposal. The remaining 16 participants have yet to return their comments.

Approval from SSC required before submission to ECBS of the WHO.

Call for participants for collaborative study to validate a proposed international genetic reference panel for Prothrombin G20210A mutation –E Gray

A collaborative study to assess a panel of genetic reference gDNA for Prothrombin G20210A will be initiated in late 2004. Invitation to laboratories wishing to participate was announced.

Progress on the working group on thrombin generation tests- A Lawrie and E Gray

A strategy and proposed activities of the working group was presented. A nucleus working group (NWG) has been established. The main objective of the working group is to investigate, standardize and validate methodologies for the quantitation of results to facilitate good within and between laboratory agreement. The proposed activities of the group in the coming year include distribution of a user survey to establish current working practices and investigate the optimal conditions for preparation of a reference plasma. The results of these activities will be reported in the next SSC meeting.

At least 4 other subcommittees (FVIII/FIX, Control of Anticoagulation, FXIII and Fibrinogen and Women’s Health Issues) that are interested in thrombin generation tests, it has been agreed that these subcommittees will work co-operatively with the NWG for their specific applications. The core activities of the Working Group will be reported in the Plasma Coagulation Inhibitors subcommittee.
Standards for Protein C and activated Protein C – E Gray

Two collaborative studies will be initiated in 2004-2005 to replace the current international standard for plasma Protein C and to establish a new international standard for Protein C and activated Protein C concentrates. There was a call for participants.

Endothelial Protein C Receptor (EPCR)

Overview – C H Toh

Dr Toh presented an overview on the functions of EPCR and the in vivo physiological relevance of EPCR was also discussed. At cell surfaces, EPCR promotes Protein C activation by up to 80% and also acts as a co-factor in APC-mediated cell survival and anti-apoptotic signaling. In addition, a truncated soluble form of EPCR is present in normal circulation which increases significantly in sepsis and systemic lupus erythematosus. This is mediated through metalloproteinase cleavage that is activated by thrombin and pro-inflammatory cytokines.

A haplotype of the EPCR gene is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis – S Gandrille

Dr Gandrille described a link between the A3 haplotype of the EPCR gene and an increase in sEPCR level in healthy subjects. Genotype analysis of patients in the PATHROS (Paris thrombophilia) study also showed that subjects carrying the A3 haplotype had an increased risk of thrombosis. It was concluded that the A3 haplotype, which is associated with elevated plasma sEPCR levels, is a candidate risk factor for venous thrombosis.

sEPCR levels and EPCR genotype in European whites and Indian Asians with type 2 diabetes - H Ireland

Dr Ireland presented analysis of 3 clinical studies (HIFMECA, NPHS II and EDSC) and shown that sEPCR levels were strongly associated with EPCR Ser219Gly. There was an increased frequency of the Gly allele in Indian Asians and an increased thrombin generation (F1.2) across the genotype groups in healthy individuals and in patients with type 2 diabetes. It was concluded that increased thrombin generation is likely to be contributing to the increased CHD-risk associated with the Gly allele.

Antithrombin

Antithrombin standardisation – C Jackson

Dr Jackson reported preliminary data from measurement of antithrombin and heparin in plasma. Antithrombin was measured from 0 to 4 µM with linear dependence on antithrombin concentration. Two low molecular weight heparins were also measured in plasma over a 50-fold
concentration range also bracketing the commonly observed concentrations of these heparins employed therapeutically. The bias created by assay of antithrombin in plasma because of inactivation of FXa and thrombin by α-1 antitrypsin and α-2 macroglobulin was discussed. An introduction to metrological approaches and the WHO approaches was presented with the intent of illustrating how the common goal of standardisation both similar in many respect but still different in some.

**SI and IU issues related to antithrombin – T Barrowcliffe**

Dr Barrowcliffe described the differences in approaches on standardisation by the WHO and metrological principles. For coagulation factors and inhibitors, the WHO principles of assaying “like against like” and using multiple method have produced reference standards that allow good intra- and inter-laboratory agreement, while the SI approach of using a single reference method and reference material gave high variability and bias in results in some instances.
Plasma Kallikrein-Kinin System  
(Formerly Contact Activation)

Chairman: R. A. DeLa Cadena, USA.  
Co-Chairs; D. Gailani, USA; M. J. Gallimore, UK; K. R. McCrae, USA; H. Saito, Japan; 
A. H. Schmaier, USA

Approximately 20 people attended the meeting during the morning session. The content of the meeting was arranged according to the objectives of the SSC, namely standardization of plasma, methods, animal models and clinical trials. There was a business-like meeting at the end of the presentations to discuss the items identified as a result of the program presentations. Such items are underlined in the minutes and grouped at the end. The items were then discussed during the business component of the agenda and represented the backbone of a strategic plan that was formulated during the business-like session and that will be implemented by the next Chair and Co-Chairs as well as Members.

We began the session with studies on standardized plasma presented by Dr. Jones with the following findings. Lot 3 has now become available but due to the limited availability NIBSC have been investigating the possibility of producing a working standard. There are no calibrated plasma preparations standardized against International reference plasma available for the measurement of FXII, FXI, PK and HK.

Plasmas assayed:

- SSC/ISTH lot 3
- NIBSC plasmas (siliconized and non-siliconized).

By the use of chromogenic assays, antigenic assays and one stage clotting assays the following plasma kallikrein-kinin system factors were evaluated, FXII, PK, HK and FXI. A fresh frozen plasma pool prepared from 50 blood donors (25 male & 25 female) was used as reference plasma. The findings of Dr. Jones indicated that the SSC/ISTH secondary coagulation standard lot 3 would appear to be a suitable reference material for the determination of Factor XII, XI, PK and HK in coagulation laboratories. In addition, the NIBSC plasma preparations, as presently produced, may be suitable for Factor XII but unsuitable for Factor XI, PK and HK, due to degradation/absorption losses.

The session continued with a presentation by Dr. Hubbard. Dr. Hubbard invited the audience to join him in addressing the following questions in relationship to the plasma kallikrein-kinin system (PKKS):

a) What standards are needed?

b) How to define suitable candidate preparations?
c) What to calibrate against?

The standards needed are: FXI, FXII, HK and PK. The FXI standard is scheduled for 2004/05 and the person in charge of this task is Dr Elaine Gray. Dr Hubbard indicated that Elaine Gray is anxious to work with the PKKS SSC to add other factors to this standard. It was the consensus of the group to start with FXII and once the task is completed to move on with PK and HK. Such task will require the following elements to be present:

1. Sufficient laboratories to warrant / enable calibration
2. Survey of commercial reference standards

Dr. Hubbard continued to present more data indicating that the comparison of FXII reference plasmas (N=6) by clotting activity compared to local normal pooled plasma was not different across the board thus raising the question of the need for such standard and therefore requested guidance of this SSC. Members of the SSC pointed out that FXII activity varied by 23% from the lowest to highest values amongst the reference plasmas, showing that a standard is needed.

Dr. Hubbard mentioned that suitable candidates include: SSC Plasma Standard Lot#3 which has the least activation and represents a property of SSC/ISTH and NIBSC preparations (+/- silicon), which have varying degrees of activation but are readily available. Dr. Hubbard indicated that determining the meaning of "level of activation" is an important issue quoting as an example the experience gained from FVII. Directly related to this issue was the need for guidelines (protocols) for appropriate collection of plasma samples for making pools to assay Plasma Kallikrein-Kinin System factors. He encouraged the SSC to participate with Elaine Gray and to make an effort to attend the Working Group on Plasma Coagulation Standards to make the SSC expertise available.

Dr. Hubbard presented a graph which illustrated that the slope between FXII activity in freeze-dried plasma NIBSC preparations when compared to SSC Lot #3 were not different in clotting activity despite the known activation in NIBSC preparations. Thus, there is a need to draft a consensus protocol for preparation of fresh local normal pooled plasma (with minimal activation) to be used to calibrate against in order to define an International Unit.

Dr. Antovic presented background and data on a new assay, Overall Hemostatic Potential, or OHP. The assay is based on spectrophotometric registration of fibrin generation and fibrinolysis in citrated plasma samples with addition of small amounts of thrombin and tissue type plasminogen activator (t-PA). Dr. Antovic applied his new assay with the following protocol:

- 15 patients (FXII 0.01 – 0.47 U mL-1) (7 M & 8 F)
4 women recurrent miscarriages
2 women occasional easy bruising – from history data without objective verification
1 man with DVT (from history data)
8 patients with routine coagulation investigation after finding prolonged aPTT
9 patients have an F XII concentration below 0.01 U/ ml (homozygous) while 6 are heterozygous

- 30 healthy controls
- Dr. Antovic’s results are summarized in the table below:

Dr. Antovic concluded, based on the above table, that the OHP assay may be a method of choice for distinguishing hypercoagulable conditions associated with FXII deficiency and hypo-
coagulable conditions. Findings of decreased OFP correspond with previous finding of impaired fibrinolysis in FXII deficiency. The OHP assay may be a possible diagnostic tool for distinguishing patients with similarly low FXII levels. It seems that it could be used as a predictor of thrombotic risk. However, the study included a small number of patients and thus, future studies should be performed with a larger number of patients.

Dr. Shariat-Madar presented a study evaluating the function of the PKKS in an animal model. Background data was initially provided indicating that prolylcarboxypeptidase (PRCP) is a PK activator and that recombinant PRCP (rPRCP) degrades angiotensin II. Thus, an interaction between the PKKS and the rennin-angiotensin system (RAS), leading to vasodilation and vasoconstriction as indicated by the model below.
With this premise (model below), Dr. Shariat-Madar used the Rose Bengal model of arterial thrombosis in mice, with the following results: the BK-B2 receptor KO mouse is protected from thrombosis. This last result was also used to validate the findings from a non-KO mouse for the BK-B2 receptor and the HOE-140 compound, an antagonist of the BK-B2 receptor. This protection was associated with the plasma phenotypic changes shown in the table below.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control</th>
<th>BKB2R/-</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1</td>
<td>7.56±0.98</td>
<td>10.7±0.92</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>FXI</td>
<td>0.39±0.05</td>
<td>0.23±0.03</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>PK</td>
<td>0.23±0.015</td>
<td>0.29±0.012</td>
<td>p&lt;0.006</td>
</tr>
</tbody>
</table>
In addition, nitric oxide and PGI2 were significantly elevated in the KO mouse when compared to control animals, leading to vasodilation and thus providing a mechanism for the protective effect seen in the KO mouse.

Dr. Mackie presented results on the in vitro effect of DX-88 on Thromboelastography in blood samples from patients undergoing cardiac bypass. DX-88 is a 60 amino acid recombinant protein produced in yeast, a variant of the 1st Kunitz domain of TFPI (FVIIa binding site), differing at 7 residues with high affinity, specific inhibitor of human plasma kallikrein. DX-88 is approximately 1000 times more potent than aprotinin against PKK. In addition, there is no inhibition of C1s or C1r, with very weak activity against plasmin and FXIa. Dr. Mackie presented the results of this inhibitor in a Trial: DX-88 in CPB for CABG. The trial has the following characteristics: Phase I/II, randomized, double blinded, placebo controlled study with 3 sites (Atlanta Veteran’s, Duke, Emory).

The protocol has an ascending dose (30, 60, 120mg) (n=14 each):
- 1/3 dose administered iv at anaesthetic induction
- 1/3 dose in CPB pump prime fluid
- 1/3 dose by constant infusion during CPB

Compared to placebo, DX-88 reduced the volume of blood and blood products transfused by approximately 50%.

Dr. Mackie’s in vitro studies consisted on the following protocol:

19 cardiopulmonary bypass (CPB) patients blood samples including pre-bypass and during bypass (at re-warming phase) and post-CPB, 1hr after protamine.

11 patients received aprotinin.

In vitro addition of buffer or DX-88

Clot Formation & Strength investigated by TEG

<table>
<thead>
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</tr>
</tbody>
</table>
As expected, PKK factor levels decreased during bypass and did not appear to be protected by aprotinin. The results of his study are summarized in the table below:

<table>
<thead>
<tr>
<th>Contact Factor Levels</th>
<th>Aprotinin (n=10)</th>
<th>No Aprotinin (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre CPB</td>
<td>CPB</td>
</tr>
<tr>
<td>FII (U/ml)</td>
<td>0.66</td>
<td>0.24**</td>
</tr>
<tr>
<td>FXII (U/ml)</td>
<td>0.71</td>
<td>0.44**</td>
</tr>
<tr>
<td>FXIIa (U/L)</td>
<td>8.04</td>
<td>22.02**</td>
</tr>
</tbody>
</table>

*Median values, *p<0.05, **p<0.01

Patients tended to have shorter ‘r’ times, greater alpha angles and MA values before surgery compared to normal subjects, which may be due to acute phase reactants or the stress of surgery.

A dose dependent prolongation of the TEG ‘r’ time by DX-88 was observed in normal subjects, and patients before, during and after CPB. The presence of aprotinin and DX-88 caused a synergistic increase in ‘r’ time. A decreased alpha angle was observed suggesting that the rate of thrombin generation could be reduced.

Dr. Mackie proceeded to explain the results of the Thromboelastography by using representative examples performed on samples from CPB patients, showing that the responsiveness to DX-88 was increased when PKK factor levels were decreased each of the groups comprising the clinical trial. Noteworthy to mention was the clinical finding with the DX-88 protocol a 50% reduction in blood loss during surgical procedure was observed. Potential explanations for the reduction in blood loss associated with DX-88 that finding are listed below but require further investigation:

- Preservation of Coagulation Factor Levels?
Dr. Gallimore gave an elegant presentation related as well to the use of Thromboelastography.

Dr. Gallimore first provided an explanation of the principal of the assay. The purpose of his study was to study the effect of activation of Plasma Kallikrein-Kinin System proteins on urokinase induced fibrino-lysis with this method (UKIFTEG). The conclusions from his study are listed below:

- The use of this assay under the current experimental conditions (urokinase induced fibrinolysis, UKITFTEG) represents a new model to study coagulation and fibrinolysis in whole blood and thus allowing the evaluation of the contribution of cellular components such as platelets, monocytes and neutrophils.
• Activation of the PKKS by the use of celite markedly reduces the coagulation time and produces an increase in fibrinolysis.
• Addition of FXIIa produced a concentration dependent reduction in coagulation time and increase in fibrinolysis.
• Addition of FXIa produced a concentration dependent reduction in the coagulation time and had a biphasic effect on fibrinolysis.

The final 30 minutes of the SCC meeting were devoted to discussion of a strategic plan that will be presented in writing in the very near future to be implemented in the same fashion.

• The standards needed are: FXI, FXII, HK and PK. This will be achieved by synchronizing efforts with Dr. Gray as to include FXII initially and subsequently other PKKS proteins.

• Sufficient laboratories to warrant / enable calibration. The Lot #3 seems the suitable one and at least 12 laboratories will be identified to participate. I survey will be immediately formulated and send to members to request willingness to participate in the task. Networking of Chair, Co-Chairs and Members will be instrumental to enlist the 12 laboratories (or more).

• The need for guidelines (protocols) for appropriate collection of plasma samples for making pools to assay Plasma Kallikrein-Kinin System factors. This will be achieved by formulating a protocol (s) for collection of blood to produce pooled fresh normal plasma.

• Dr. Booth suggested because of the fibrinolytic activities of the PKKS that closer links between the PKKS SSC and the Fibrinolysis SSC be effected in the future.

Respectfully prepared and presented by Dr. DeLa Cadena
Platelet Immunology

Chairman: T Warkentin, Canada
Co-Chairs: JB Bussel, USA; BH Chong, Australia; D. Cines, USA; A. Greinacher, Germany; V. Kiefel, Germany; H. Kroll, Germany; M.F. Murphy, UK; G.P. Visentin, USA

Co-Chairs (in attendance): J. Bussel, B. Chong, A. Greinacher, V. Kiefel, H. Kroll, M. Murphy

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

Autoimmune Thrombocytopenia (Chairs: J. Bussel, V. Kiefel)

Paul Imbach (Basel, Switzerland), as a guest of the committee, addressed the topic, “PARC-ITP Study.” He began by summarizing some information learned from the the Intercontinental Childhood ITP Study Group, e.g., on the natural history of acute ITP. He discussed some issues on pathogenesis (e.g., could single base changes in regulatory regions of cytokines lead to altered immune response in ITP?), laboratory testing (limited sensitivity and specificities of current assays), treatment consensus (e.g., role of corticosteroids, IVIg, or anti-D for initial treatment; lack of consensus on treatment of refractory patients), and future research directions. Historical developments of the ICIS were listed, including registry I (about 2000 children enrolled, registry now closed), registry II (remains open for registered centers), splenectomy registry, and, most recently, the Pediatric and Adult Intercontinental Registry on Chronic ITP (PARC-ITP). Information gleaned from ICIS included gender imbalance in childhood ITP, variable rates of hospitalization for ITP around the world, and choice of therapies (about 1/3 each for no treatment, corticosteroids, and IVIgG, with a minority receiving combined corticosteroids/IVIgG). The PARC-ITP registry was summarized, including various planned substudies (“trees in the PARC”) were summaries, such as investigating genetic polymorphisms, patients with severe bleeding, health-related quality of life, refractory ITP, secondary ITP, etc.

Drew Provan (London, UK) spoke on the “European Haematology Association, Working Group: Platelets, which represents the first subgroup within the EHA. This Working Group is chaired by Francesco Rodeghiero, and co-chaired by Drew Provan, with executive members including J. Bussel, P. Fenaux, B. Godeau, P. Imbach, and M. Ruggeri. The goals are to share expertise, identify priorities and create appropriate research networks, perform observational clinical and laboratory studies, and therapeutic studies. Funding for joint projects will be sought.

Contributing groups extend beyond the EHA, and include the USA, Canada, and Asia (within ICIS). Meetings will occur yearly at EHA and ASH. A website is being developed (http://www.tcpeha.org). Initial projects and topics for study include: terminology consensus, type IIb von Willebrand disease, PARC-ITP registry, genomic studies, H. pylori studies, Evan’s syndrome, quality-of-life, therapeutic questions, and epidemiologic studies. The first planned substudy is a genomic study within PARC, with plans to include 400-500 patients. Individual
patients’ SNP profiles will be determined. Various genes to be investigated in first and second
generation studies were listed.

**J. Bussel (New York, USA) and V. Kiefel (Rostock, Germany)** addressed the challenging issue
of standardizing diagnostic approaches to ITP from clinical and laboratory aspects, respectively.
Several of the challenging aspects of ITP diagnosis were listed. The possible use of a ‘gold
standard’ of response to treatment (e.g., IVIgG, anti-D, rituximab) in defining ITP was
suggested. **A proposed project of the Platelet Immunology SSC is to prepare a report on a
combined clinical-laboratory approach to the standardized diagnosis of ITP.** A suggested
approach to this topic is to select members of a working committee to take on this project, which
would determine topics to be addressed (e.g., childhood vs adult ITP, primary vs secondary ITP,
etc.), and then attempt to define a specific clinical diagnosis that should be easy to follow.
Finally, a study should be proposed to confirm/refine the algorithm, including laboratory studies.
Highlights of the presentation of Dr. Kiefel included the emerging consensus that "platelet-
associated IgG" assays are not specific enough to have any diagnostic usefulness, and that gp-
specific platelet antibody assays have reasonable sensitivity (40-55%) and very high specificity
(about 95%). Suggestions for enhancing diagnostic utility included using platelet eluates for
platelet binding studies, and using platelets rather than serum/plasma for identifying platelet
antibodies.

**Alloimmune Thrombocytopenia (Chairs: R. Aster, M. Murphy)**

**H. Kroll (Giessen, Germany)** speaking on behalf of S. Santoso (Giessen, Germany) provided an
“Update on Status of Platelet Alloantigens and Alloantibodies” (details of the known platelet
alloantigens can be found at [http://www.ebi.ac.uk/ipd/hpa](http://www.ebi.ac.uk/ipd/hpa)). He discussed the responsibilities of
repository laboratories. Various low frequency platelet alloantigens have recently been
implicated in cases of neonatal alloimmune thrombocytopenia (NAT) beyond those sites where
the alloantigen systems were first described, suggesting the importance of methodologies being
available for investigating even low frequency alloantigens. Two newly-described alloantigens
were listed (Br^c a third allele of HPA-5; and val33 as a third HPA-1 allele). As an addendum, R.
Aster (Milwaukee, USA) presented their group’s experience in identifying platelet protein
polymorphisms from 17 fathers of 328 families with unresolved possible neonatal alloimmune
thrombocytopenia. The HPA-9a (Max) polymorphism was suggested to be the third most
important cause of NAT after HPA-1a and HPA-5b. Potential new alloantigens were shown (Sta,
Bec, Aus, Kno, and Br^c).

**V. Kiefel (Rostock, Germany)** presented interesting clinical data on several patients with severe
unexpected NAT who responded to “incompatible” platelets. These observations challenged the
dogma that compatible platelets (whether obtained from mother or from typed platelet donors)
should always be preferred in such situations, given that random, incompatible platelets can be
obtained much more quickly (e.g., maternal platelets require viral testing, there are technical
requirements to remove maternal plasma, maternal platelets are often “preactivated” and provide
low yields, etc.). It was suggested that rapid absorption of alloantibody to the transfused platelets
might help explain their efficacy in this situation. A lively discussion ensued, with several
members of the audience reporting their anecdotal experience that incompatible platelets can be
effective in this situation. It was suggested that a collaborative multicentre observational study be performed to document the outcomes with this treatment approach.

Drug-Induced Thrombocytopenia (Chair: H. Kroll)

R. Aster (Milwaukee, USA) discussed “Thrombocytopenia caused by GPIIb/IIIa Inhibitors: syndromes, definitions, laboratory testing.” The frequencies of thrombocytopenia among the various such agents were listed, which ranged from 2-6% for roxifiban (usually occurred after a week or two of use, although sometimes acutely), 0.67% for xemilofiban, 0.67% for orbofiban, 0.4-1.3% for eptifibatide, 0.2-0.4% for tirofiban 0.2-0.4%, and about 1% for abciximab (but 12% in reexposure situations). Most often, the syndrome begins within 2-6 h of a first exposure to the drug, with bleeding highly variable, and observed in about half the patients. Platelet recovery occurs within 2-5 days, and patients typically respond to platelet transfusions. Other syndromes include “delayed thrombocytopenia” (Curtis et al. 2004), which occurs 5-7 days after giving abciximab (and probably roxifiban). It was noted that both abciximab and roxifiban remain associated with circulating platelets for 1-2 weeks post-infusion. Another syndrome is “pseudothrombocytopenia”, which usually is observed using EDTA anticoagulant. Laboratory features of patients with abciximab-induced thrombocytopenia include the presence of antibodies that bind to abciximab-coated platelets (most easily studied using flow cytometry), which are found in greater quantities that those in normal individuals. Notably, adding Fab fragments from normal immunoglobulin can distinguish “normal” antibodies from those associated with abciximab-induced thrombocytopenia, since only the former antibodies are inhibited by Fab fragments. Another technique is to use Mab 7E3 to coat the platelets, as sera from patients with abciximab-induced thrombocytopenia react much stronger than “normal” antibodies (assessed as ratio of 7E3 to AP3 binding). Unresolved issues of the target epitope(s) for gpIIb/IIIa antagonist-induced thrombocytopenia antibodies were addressed. Clearly, different epitopes are produced by different drugs, given studies of cross-reactivity, different reactivities in the presence of varying calcium concentrations, etc. It appears that the antibodies do not react against the gpIIb/IIIa antagonists themselves, but rather to epitope(s) formed on the gpIIb/IIIa complex itself. Nevertheless, the conformational changes induced are to some extent ligand-specific (each “tweaks” the gpIIb/IIIa complex in a slightly different way). A caveat is that no antibodies can be demonstrated in about half the patients who have acute thrombocytopenia after a first exposure to the gpIIb/IIIa antagonist. A proposed project of the Platelet Immunology SSC is to prepare a report on gpIIb/IIIa antagonist-induced immune thrombocytopenia, with an aim to describe a classification of the various thrombocytopenic syndromes caused by these drugs, including definitions and laboratory testing methods.

B. Chong (Sydney, Australia) and T. Warkentin (Hamilton, Canada), in a presentation “Scoring System for Heparin-Induced Thrombocytopenia”, summarized the development and evolution of clinical scoring systems for estimating the likelihood of HIT. Common features of the scoring systems were emphasized, including assessment of Thrombocytopenia, Temporal features of the thrombocytopenia, Thrombosis (or other sequelae of HIT), and the presence of oTher explanations for thrombocytopenia (the “4 T’s”). It was noted that most scoring systems have classified these features on a scale from 0 to 2. It was proposed that the scoring systems
be amalgamated into a single system that can be adopted by the SSC, with a goal to standardizing further progress in the evaluation of diagnostic tests and therapies for HIT.

T. Warkentin (Hamilton, Canada) and Y. Gruel (Tours, France) presented data relating to the “Role of IgG, IgA, and IgM anti-PF4/Heparin Antibodies” in the pathogenesis of HIT. In the Canadian presentation, the results of serologic assessment (GTI assay for anti-PF4/H; IgG, IgM, and IgA classes of anti-PF4/H; and serotonin release assay [SRA]) of over 600 patients exposed either to unfractionated heparin or low-molecular-weight heparin post-orthopedic surgery was presented. All 14 patients with clinical HIT had similar serologic features (generally, strong reactivities in the GTI, IgG anti-PF4/H, and SRA). Only a minority of these patients (about 20-40%, respectively) evinced positive reactivities of IgM and IgA class antibodies. Remarkably, among non-HIT patients with serologic evidence of anti-PF4/H reactivities, there was no difference in the IgM and IgA class reactivities compared with the HIT patients, even though there was significantly less reactivity by GTI, IgG anti-PF4/H, and SRA. These findings cast doubt on whether IgM and IgA class antibodies effect a pathogenic role in HIT. In the French presentation, results of a study (Blood 2004 in press) were presented indicating a role for the 158V polymorphism of the Fc gamma III receptor gene (which results in increased affinity of IgG1 and IgG3 class antibodies for phagocytic cells bearing these receptors) in predisposing to HIT. The workers compared the distribution of the 158V/158F alleles in 3 post-cardiac surgery groups: (a) non-seroconverters (n=86); (b) subclinical seroconverters (HIT antibodies but not HIT; n=84), and (c) cardiac surgery patients with HIT (n=102). A significantly higher 158V allele frequency was found in patients with HIT compared with seroconverters who did not develop HIT (0.416 v 0.339; p=0.02). This study therefore suggests that thrombocytopenia in HIT could be a balance between antibody-induced platelet activation and antibody-induced platelet clearance via phagocytosis. This study also provides indirect support for an important role of IgG class antibodies in the pathogenesis of HIT.

In the final presentation, A. Greinacher (Greifswald, Germany) presented an overview of “Drug-Induced Immune Thrombocytopenic Purpura (D-ITP)” (excluding HIT and gpIIb/IIIa antagonist-induced thrombocytopenia), namely drug-induced autoimmune thrombocytopenia and typical drug-dependent immune thrombocytopenic purpura. The rare syndrome of drug-induced autoimmune thrombocytopenia has been best characterized by gold, with the interesting feature of often evincing reactivity against gpV. Clinical observations of the presenter that alphamethyldopa might also cause an apparent autoimmune syndrome were also presented. Regarding the more frequent D-ITP syndrome, a laboratory approach to diagnosis was presented, including: use of appropriate controls, use of appropriate methodologies (e.g., flow cytometry, MAIPA), and use of drug metabolites. It was proposed that standard diagnostic criteria be developed by the SSC, including a systematic laboratory approach.
The Platelet Physiology Subcommittee discussed aspirin resistance as the major topic at this year’s session. Although there are a large number of publications on various aspects of aspirin resistance, there does not appear to be a well-defined definition of this entity. Moreover, issues such as clinical relevance and biochemical mechanisms are far from clarified. In the first part of the subcommittee meeting, the focus was on aspirin resistance. There were five presentations. They were targeted to address the issues of definition, clinical relevance and mechanisms of aspirin resistance. Dr. Alan Michelson (USA) provided an overview of the current status, and he summarized some of the studies published to date. Dr. Kandice Kottke-Marchant (USA) discussed the studies performed at the Cleveland clinic focusing on the clinical relevance of aspirin resistance. Dr. Thomas Kunicki (USA) discussed recent studies on aspirin resistance in subjects with arterial diseases using the platelet function analyzer. Dr. Fabio Pulcinelli (Italy) discussed their studies with respect to aspirin resistance in patients with ischemic heart disease. Dr. Marco Cattaneo (Italy) discussed the issue of definition of aspirin resistance.

Following these presentations, there was a vigorous discussion on various aspects of aspirin resistance. A Working Group on Aspirin Resistance was established with Dr. Alan Michelson as the Chairperson and Dr. A. Koneti Rao as the Co-Chair. The Working Group will generate a document that summarizes the current status as well as a recommendation to be submitted for publication in Journal of Thrombosis and Haemostasis.

Clopidogrel is widely used as an antiplatelet agent with or without aspirin. Dr. Paul Gurbel (USA) summarized their studies on clopidogrel resistance.

Working Party on Congenital Platelet Function Defects

Under aegis of this Working Party on Platelet Function Analyzer, Dr. Catherine Hayward presented a report on the advantages and shortcomings of the evaluation of platelet function disorders using the Platelet Function Analyzer (PFA). In addition, she presented to the subcommittee studies on the patterns of practice in clinical laboratory testing of platelet disorders, a compilation of the results of a North American survey. This survey included approximately 50 laboratories in North America involved in platelet function testing using the optical aggregometry.

As a follow-up from last year’s meeting of the Working Group on Platelet Function Defects, Dr. Alan Nurden presented his efforts in establishing a network for studying patients with inherited disorders of platelet function in France, as a model for others to follow.
Congenital Thrombocytopenia Registry

This registry was intended to encompass patients with non-immune thrombocytopenias. Dr. Bussel presented his efforts in establishing an ongoing registry on patients with type II von Willebrand disease.

Working Party on Platelet Genomics and Proteomics

Dr. Steve Watson presented a report on behalf of this Working Party, summarizing aspects of the potential and the pitfalls in the application of proteomics to platelets. He summarized the contents of a document prepared for eventual submission for publication in the Journal of Thrombosis and Haemostasis.
Predictive Haemostatic Variables In Cardiovascular Disease

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

The session was co-chaired by Drs Iacoviello and Lowe, Dr Grant had sent his apologies.

**Dr. Lowe** gave an overview of haemostatic variables in prediction of cardiovascular (arterial) events – principally coronary heart disease (CHD) events in meta-analyses of prospective studies. Currently, fibrinogen, von Willebrand factor antigen, fibrin D-dimer, tissue plasminogen activator antigen, and possibly factor V Leiden and prothrombin 20120 mutations, showed significant associations in such analyses. Further data are required for other haemostatic variables including factors VII, VIII and IX; PAI-1, and other activation markers. The Fibrinogen Studies Collaboration (FSC) is analyzing individual data from 200,000 persons in prospective studies of plasma fibrinogen; this should clarify the shape of the dose-response curve, the effect of assay type, and the additional predictive value of fibrinogen to that of classical CHD risk predictors. The Emerging Risk Factors Collaboration (developing from the FSC) aims to perform similar collaborative meta-analyses on other circulating potential risk predictors, starting with C-reactive protein, lipoprotein (a) and albumin. Action: Dr Lowe to draft brief report of overview; and to liaise between Subcommittee and Emerging Risk Factors Collaboration on future analyses of haemostatic variables.

**Dr. G. Palareti** (Italy) reviewed the predictive value of haemostatic variables for recurrence of venous thrombosis. Currently, D-dimer, factor VIII and factor IX appeared of potential clinical value; further studies were required. Dr Palareti outlined the ongoing multicentre, Italian PROLONG study of D-dimer, assayed one month after discontinuing oral anticoagulation, in prediction of the need for prolonged therapy.

**Dr. D. Fitzgerald** (Ireland) reviewed the potential for platelet proteomic studies to identify markers of cardiovascular risk. Many platelet proteins had roles in inflammation, rather than haemostasis. There remained many methodological challenges, including quantification of proteins. Recently-developed antibody arrays allowed some quantification of platelet cytokines and growth factors.

**Dr. A. Carter** (UK) reviewed the potential for plasma proteomic profiles to identify cardiovascular risk. There are about 500 proteins which function in plasma, and another 50,000 which are markers of cell secretion, death or damage. There is a wide dynamic range (10 to power 10) of currently measured plasma proteins (from albumin to interleukin-6), and 10 to power 7 for haemostatic proteins. The Human Proteome Organisation (HUPO) Plasma Protein Project is assessing issues including variants, population distributions, and the effects of age, sex, lifestyle, drugs and diseases. Current activities include assessment of technology platforms, pre-analytical variables, and methods for depletion of the 6 most abundant plasma proteins. Different methods are required for classic plasma proteins, tissue damage markers, tumour markers and cytokines. Complex bioinformatics is required for analyses of 2D electrophoresis data. There is a need for standards to run in epidemiological studies. Dr Carter presented some illustrative data.
on clot proteomic analyses. Action: Drs Carter to liaise with Dr Fitzgerald to draft brief report of overviews; and to liaise between Subcommittee and HUPO-Plasma Protein Project for haemostatic variables.

**Dr. Iacoviello** presented new perspectives in studying the associations between genetics and cardiovascular disease. We have studied until now only individual polymorphisms in single genes. However, the picture is much more complex.

Several polymorphisms have been discovered in each single gene and, since the linkage disequilibrium principle has been invalidated, we should in principle measure all the identified polymorphisms in our studies. However, an analytical method has been recently developed to identify combinations of polymorphisms that can be studied in haplotypes to cover about 80% of the genetic information contained in a gene.

Linkage analysis in large families, by using a wide genome scanning approach, can also be useful for further approaches. The latter implies the identification of genomic areas associated with QTL and, through QTL, with thrombosis. Action: De. Iacoviello to draft brief report of overview.

Numbers attending session were estimated as 60-80.
Von Willebrand Factor

Chairman: A. B. Federici, Italy
Co-chairs: G. Castaman, Italy; J. Eikenboom, The Netherlands; E. Favaloro, Australia;
A. Goodeve, UK; P. A. Kouides, USA; D. Lillicrap, Canada; C. Mazurier, France;
R. Montgomery, USA; R. Schneppenheim, Germany

Summary of Approvals and Working Parties:

1. Continuation of WP on VWF Assays in VWF in VWD Diagnosis: The lyophilized samples are ready to be sent out to labs and the survey will start by September 2004 (A.B. Federici, C.A. Lee, R.R. Montgomery)
2. Continuation of the WP on Standardization of Multimeric Analysis, with more laboratories (U. Budde and C. Mazurier)
3. A new WP on the use of suitable reagents for VWF:CB (collagen binding assay) has been approved (L. De Marco, E. Favaloro and A. Hubbard)
4. Continuation of the WP on VWD classification has been approved with presentation of a final report during the next ISTH-SSC meeting in Sydney (E. Sadler & the panel of VWD experts)
5. The WP on Standardization of methods for mutation and expression studies will continue (A.Goodeve, D. Lillicrap, J Eikenboom, R. Schneppenheim)
6. A WP on development of new improved assays for ADAMTS-13 has been proposed, as a continuation of the previous inter-laboratory assay standardization study (J.E. Sadler & R. Schneppenheim)
7. A WP on requirements for shear-stress related VWF assays to be used in clinical diagnosis of VWD and drugs interfering with VWF-platelet interactions have been proposed (Y. Ikeda & Z.M. Ruggeri)
8. An updated version of International Registry on Acquired Von Willebrand Syndrome has been proposed to be organized on line in a specific Web site (A.B. Federici, U. Budde, H. Mohri, J.H. Rand)

Two co-chairs, Emmanuel Favaloro and Peter A. Kouides were not present. The VWF Subcommittee was attended by about 190 attendees at the first session and by about 140 at the second session. They actively participated in the discussion of the various topics provided in the program. The following represents the minutes of the program.

1) WP on VWF Assays (Claudine Mazurier, France)

Christine Lee presented an update of the Working Party on VWF assays in VWD diagnosis (http://www.vwfassays-in-vwd.com/). The main achievement since SSC Birmingham July 2003 is the plasmapheresis and preparation of lyophilised plasma samples from 2 donors and 6 VWD patients. Preliminary results showed that no changes in VWF:Ag, VWF:RCo and multimers were induced by the lyophilization.
Giancarlo Castaman then presented his experience with LIA test for VWF:Ag. This test based on latex technology was used in the context of the European MCMDM-1VWD study on type 1 VWD. The VWF:Ag values are: 112.4±40.9IU/dl in 1049 controls and 38.3±23.3IU/dl in 127 patients. In comparison with ELISA this high precision turbidimetry test is 2 to 4 times cheaper and adapted to emergency situation.

Ulrich Budde reported on the Working Party on Standardization of VWF multimers. He presented the data from six laboratories having tested 5 VWF concentrates and plasma samples of 2 VWD patients. Using or not transfer, radioactivity, enzymes or luminescence for revelation, the patterns were consistent. For quantification the multimers were classified in the following categories: LMW: multimers 1 to 5, IMW: multimers 6 to 10 and HMW: multimers>10-mers. For a given sample the range of the values was rather wide from one lab to another but the rank of the samples based on their HMW content was consistent in the majority of labs.

Don Gabriel presented a EQELS (Electrophoretic Quasi Elastic Light Scattering) method for multimeric analysis. The effect of temperature, ionic strength and hydrophobicity was studied on partially purified VWF preparation. Results obtained with plasma of a type 3 and a type 2B VWD patient were also presented. The results obtained within 1 hour showed excellent reproducibility.

Ronald Kotischke summarized the data of a collaborative study on automated VWF:RCo assay organized by the German society on Transfusion Medicine and Hemotherapy (DGTI). Thirteen labs not only from Germany but also from Austria, France, Italy and UK, tested 8 reconstituted and frozen concentrates (5 plasma-derived VIII/VWF, 2 plasma-derived VWF and 1 recombinant VWF product). The CV were ranging between 10.9 and 23.5 %.

2) VWF collagen binding versus VWF:RCo activities (Giancarlo Castaman, Italy)

Augusto B. Federici presented data on the relationships between VWF:CB and VWF:RCo in a sample population from the MCMDM-1 VWD study. It appeared that about 10 % of subjects had both VWFCB/Ag and VWF:RCo/Ag ratios below 0.6. All these subjects had VWF gene mutations clustered in the D’, D3 and A1 domains of VWF.

Luigi De Marco presented preliminary data on a new VWF:CB assay using human collagen VI. This test is able to exploit both A1 and A3 domains of VWF. The test has been so far used for flowing experiments and some data should be available after testing in patients for the next subcommittee meeting in Sidney.

Anthony Hubbard presented data on a possible standard for VWF:CB. He suggested that no potency assignment could have been made as to VWF:CB activity due to extreme heterogeneity obtained among different labs and the uncertainties of the results obtained by using collagen type I or III.

Tobias Suiter presented data on VWF:CB and VWF:RCo in several VWF concentrates.
Conclusions The general feeling was that some guidelines on the use of VWF:CB should be provided by the Subcommittee prior to its definite use in clinical practice. At the present, the role of time-honoured tests for VWD diagnosis remains fundamental. A WP on the use of VWF:CB has been proposed to be organized by Luigi De Marco, Emmanuel Favaloro and Anthony Hubbard.

3) Working Party on VWD Classification (Jeroen Eikenboom, The Netherlands)

The session started with a presentation of the proposal for a new VWD classification by Evan Sadler. The Subcommittee on VWF approved the current classification of VWD at the meeting of the SSC in New York, on July 4, 1993, and it was published the next year. The 1994 classification was based mainly on differences in pathophysiology, and was intended to correlate with clinical behavior, response to therapy and the need for genetic counseling.

A working party was authorized at the 2003 SSC meeting in Birmingham to consider revising the current VWD classification. This working party will continue through the 2005 ISTH meeting in Sydney, to allow the incorporation of results from ongoing Canadian and European studies of VWD type I. An interim report was made to the Subcommittee in Venice. Members of the working party are: Ulrich Budde, Jeroen Eikenboom, Augusto Federici, Emmanuel J Favaloro, Frank Hill, Lars Holmberg, Jørgen Ingerslev, Christine Lee, David Lillicrap, Pier Mannuccio Mannucci, Claudine Mazurier, Dominique Meyer, Robert R. Montgomery, William L. Nichols, Masato Nishino, Ian Peake, Francesco Rodeghiero, Zaverio M. Ruggeri, J. Evan Sadler (chair), Reinhard Schneppenheim, and Alok Srivastava.

Concepts and suggestions for a revised VWD classification were presented and discussed. There was broad agreement that the general principles outlined in the 1994 classification should be retained. In particular, the classification is intended primarily to guide VWD patient treatment and genetic counseling, and therefore must be clinically relevant. In addition, the classification should be simple, with a minimum number of formal categories, and implementation should depend mainly on laboratory tests that are widely available. The classification should be separated conceptually from specific laboratory testing protocols, so that the development of new assay methods will not render it obsolete. During the following year, the working party will resolve some issues relating to the boundaries between some VWD variants, and the annotation of certain subtypes. Final recommendations for VWD classification will be presented and discussed in Sydney, with the expectation that the VWF subcommittee will then vote on their approval. If so approved, a revised VWD classification will be published.

Alberto Tosetto presented data on Bleeding scores in VWD. The bleeding score was retrospectively evaluated in two multicenter studies. The bleeding score may be useful to identify subjects with a possible bleeding diathesis and to quantify the severity of the affected individuals. The bleeding score showed a good correlation with unaffected, affected, and index cases. It was also correlated with the VWF:RCo level (lower levels, higher bleeding scores). The score could be useful for biological correlations. However, prospective evaluation is needed. Implementation of the bleeding score in the VWD classifications has to be discussed.
Margareta Blombäck illustrated the problem of diagnostic criteria for VWD by the presentation of a case report.

Conclusions The revised classification for VWD will be further debated during the next year and the results from ongoing multicenter studies on type 1 VWD will be implemented. Final recommendations will be presented at the SSC in Sydney 2005. The role of the bleeding score has to be evaluated in a prospective study.

4) Methods for genetic analysis of VWF defects (Anne Goodeve, UK)

Anne Goodeve presented an update of VWF gene defects from the ISTH VWF website, http://www.shef.ac.uk/vwf. A total of 307 mutations are listed. Mutations comprise type 1, n=14; type 2A, n=71; 2B, n=52; 2M, n=18; 2N, n=37 and type 3, n=85. 80% of the mutations are missense. Whereas type 3 mutations are located throughout the VWF gene, type 2 mutation distribution reflects the functions that they disturb. The summary has been added to the VWF website.

David Lillicrap discussed the use of the terms polymorphism or mutation to describe two sequence alterations in the VWF gene newly recognized as associated with increased risk of low VWF and bleeding, but which demonstrate incomplete penetrance. Y1584C and R924Q were previously referred to as polymorphisms as they are found at a frequency of >1% in the normal population. However, both are enriched in the type 1 VWD population. Both result in enhanced intracellular retention. The findings suggest no simple distinction between the terms mutation and polymorphism.

Jeroen Eikenboom discussed the relative merits of SNP and STR polymorphisms for linkage analysis within VWD families. STR polymorphisms benefit for enhanced informativity and therefore are useful within single pedigrees. However, their mutability renders them less useful for population based studies, and SNPs may be preferred for such studies. Polymorphisms can then be utilized with parametric analysis to determine inheritance mode or in non-parametric analysis to examine quantitative trait loci (QTL).

Reinhard Schneppenheim described mutation scanning techniques that have been utilized to seek sequence variation in the VWF gene; CSGE, DHPLC, CCMA, SSCA and DNA sequencing have all been used. The EU MCMMDM-1VWD study enabled comparisons of sensitivity of various methodologies to be made. DNA sequencing was the most sensitive technique, with a single false negative result of 60 individuals analysed. Other techniques were up to 90% sensitive. The variety of techniques used to express VWF in vitro were also described, and the importance of expression of both homozygous and heterozygous recombinant mutant VWF in comparison with wild type VWF was emphasized.

5) ADAMTS-13 Assays (Reinhard Schneppenheim, Germany)

Armando Tripodi reported in detail the results of the large international laboratory assay standardization of the different assays available until now. He described the blind design of the
study showing the type of samples with different levels of ADAMTS-13 sent to the participating labs. Eleven international expert laboratories for ADAMTS13 participated in this study with five different methods: a) Multimer analysis of plasma or recombinant VWF as substrate incubated with patient’s plasma and quantitative evaluation (2); IRMA (1); Immunoblotting of recombinant VWF A1-A3-fragment as substrate incubated with patient’s plasma and quantitative evaluation (1); Collagen binding assay (VWF:CB) of plasma or recombinant VWF as substrate incubated with patient’s plasma (5); Ristocetin-Cofactor assay (VWF:RCo) of plasma VWF as substrate incubated with patient’s plasma (1); Cone and platelet aggregometer test after incubation of VWF with patient’s plasma (1)

Results were as follows: In general the performance of tests was of considerable variance even between comparable methods. Test linearity was excellent for VWF:RCo assay, multimer analysis and VWF:CB (not in all cases of the latter). Reproducibility was excellent for VWF:RCo assay, and VWF:CB with a CV < 10 % (not in all cases of the latter). Identification of severe deficiency was excellent for multimer analysis, VWF:CB, VWF:RCo and IRMA. Discrimination between 0 and 10 % activity was excellent for RCo, multimer analysis and in one center for CBA. Overall the best suitable methods were VWF:RCo assay, multimer analysis and VWF:CB.

Derrick Bowen presented the data on the effects of ABO blood group and of specific VWF polymorphisms in relationship with ADAMTS-13 effect, discussing the results of his recent publications in JTH and Blood. He described enhanced proteolysis of VWF in the order of 0, B, A, AB with bg 0-VWF as being the most susceptible. He also presented data on enhanced proteolysis in patients with the mutation Y1584C. Most patients had blood group 0. Enhanced proteolysis was detected by decreased VWF:CB. Enhanced proteolysis is considered a pathogenic mechanism that may have implications for therapy, i.e. with DDAVP.

The issue of standardization of assays for ADAMTS-13 was then discussed by Flora Peyvandi, J.Evan Sadler and Reinhard Schneppenheim who presented preliminary data on new assays developed in their laboratories.

Flora Peyvandi reported on an ongoing project for the development of new ADAMTS13 assays both quantitative as well as functional. Her group produced 12 monoclonal antibodies directed mainly towards the carboxyterminal of ADAMTS13. These monoclonals are now tested for their usefulness in an ADAMTS13-ELISA. A functional assay shall be developed on the basis of a small fragment of VWF containing the proteolytic site flanked by a fluorochrome and a quencher. The method would detect the activity of the protease directly by measuring the increasing fluorescence.

Evan Sadler presented experiments on the proteolysis of VWF fragments containing the proteolytic site flanked by antigenic tags. He could show that fragments lacking the VWF A1-domain are more easily proteolysed than fragments including it. However, if the A1-domain was present, proteolysis could be enhanced by GpIb. He also introduced a VWD type 2A mutation into the A2-domain that further increased proteolysis. He concluded that functional ADAMTS13
assays that employ VWF fragments with the A2 domain may be a suitable method. Such assays may, however, not reliably reflect the situation in vivo.

Reinhard Schneppenheim presented data on the interaction between recombinant ADAMTS13 and diverse VWD type 2A mutants, with a recombinant fragment comprising the A1-A2-A3 domain as substrate flanked by antigenic tags. He could differentiate between mutants with enhanced proteolysis, characterized by cleavage in the absence of urea, those with normal proteolysis compared to the wildtype fragment (cleavage only in the presence of urea) and a mutant fragment that was not cleaved neither without nor with urea. He considered the method as suitable for the study of mutants providing both enhanced or decreased resistance against proteolysis. The limitation of the method is, however, its performance as a static assay without the influence of shear.

The session was concluded with the statement that fast, sensitive and reliable assays to measure ADAMTS13:Ag and ADAMTS13 functional activity, respectively, are highly desirable. It was emphasized that there is still a deficit in good routine assays to detect ADAMTS13 antibodies in patients with acquired TTP, a condition that requires specific therapy. There was a general agreement in the audience to continue the efforts to develop additional and more standardized assays for ADAMTS-13 evaluation. A WP on ADAMTS-13 will be organized by E. Sadler & R. Schneppenheim and will include all the interested experts working in this field.

6) Shear-stress related VWF Assays: new tools of VWF activities ?

(Augusto. B. Federici, Italy)

Zaverio M. Ruggeri discussed the general concepts for the use of shear-stress tests as a measurement of VWF function. He described the most recent data about the basic mechanisms on VWF-platelet interactions in different shear-rates conditions, from relatively low to high shear rates. He is positive about future applications of these methods in a more clinical setting to determine VWF defects.

Dr. Ikeda reported about his experience in Japan on shear-stress assays. In particular he reported the data obtained with these assay in collaboration with cardiologists who evaluated with him the effects of different anti-platelet agents, aspirin, ticlopidine and different monoclonal antibodies against anti-IIb IIIa receptors. His assay can distinguish very well the effects of these different drugs (aspirin versus ticlopidine versus monoclonal antibodies) and therefore it can be useful in the clinical setting to monitor the effects of these drugs.

Catherine Hayward reported her experience with the use of PFA-100 in diagnosis and monitoring treatments of VWD. She reported that the assay is very sensitive but not specific for VWD and therefore she has not introduced the PFA-100 as a standard routine test for primary hemostasis in her coagulation laboratory at the Mc Master University in Canada.

Due to the low costs of VWF panel tests she prefers to perform specific VWF assays instead.
David Varon presented results on VWD cases with the improved version of the Automated Cone and Plate(let) analyzer. His device can show reduced thrombus formation in different types of VWD patients followed in Israel and in Italy, at the Angelo Bianchi Bonomi Hemophilia Thrombosis of Milan.

Conclusion: After a fruitful discussion it was proposed that Dr. Ikeda and Ruggeri will organize a WP on Shear-stress related VWF assays to determine the requirements for such assays to be used in a more clinical setting.

7) Clinical Studies on Management of VWD (D. Lillicrap, Canada)

The session relating to clinical studies in von Willebrand disease (VWD) was introduced by Professor Mannucci, who addressed the issue of mucosal bleeding in VWD. He re-emphasized the fact that we still do not know which measures of VWF function best determine the risk of mucosal bleeding in patients. The optimal treatment of gastrointestinal bleeding (especially from angiodysplastic lesions) and menorrhagia, with VWF concentrates, will require further evaluation of VWF dosing using different potency assessments. Professor Mannucci encouraged the development of proposals to investigate this issue in prospective clinical trials.

Giancarlo Castaman next discussed the preliminary information available from the EU Type 1 VWD Study relating to the correlation between DDAVP-responsiveness and VWF genotype. He showed data to indicate that missense mutations in the propeptide and D4/CK domains of VWF were associated with prolonged elevations of VWF post-DDAVP, whereas, in contrast, missense mutations in the D3 domain were associated with more rapid clearance of VWF post-DDAVP. To continue the DDAVP theme, Stefan Lethagen presented the current status of the DDAVP biological response and clinical assessment study. This study will evaluate the biological response to DDAVP infusion at 4 time points in type 1 and 2 VWD patients, and in those patients with a positive biological response (>3-fold increase in VWF levels and a post-DDAVP level of >0.30 u/mL) a subsequent clinical assessment of efficacy can be made. In addition, there are plans to enroll 40 VWD patients in a more extensive post-DDAVP pharmacokinetic study in which VWF and FVIII levels will be measured at 8 time points up to 24 hours post-therapy. Currently, 29 patients have been enrolled on the biological study. The study is recruiting patients through the web site – http://www.ddavp-in-vwd.com. Finally, in this session, Peter Lenting presented data on the clearance of recombinat normal and type 1 VWD mutant VWF in a mouse model. He showed data to indicate that several of these VWF mutants, including C1130F in the D3 domain, are associated with significant shortening of the circulating mutant VWF half-life. This preliminary data suggests that premature clearance of mutant VWF may play an important role in pathogenesis in some cases of type 1 VWD.

8) Other reports and proposals, concluding remarks (Augusto B. Federici, Italy)

Derrick Bowen presented on behalf as his colleagues of UK Hemophilia Centers the preliminary results of molecular pathology study in VWD type 1.
Jacob H. Rand presented the proposal of an updated version of the International Registry on Acquired von Willebrand Syndrome. The results of previous registry were published in 2000 with data collected until December 1999. The new registry will enroll directly on line with an appropriate web site (www.intreaws.com) all cases with AVWS diagnosed after January 1st, 2000. Aims of this new updated registry on AVWS are also to develop better diagnostic tests for AVWS, to promote prospective studies in specific underlined clinical conditions usually associated with AVWS and to determine clinical responses with new therapeutic approaches (WP organized by A.B. Federici, U. Budde, H. Mohri, JH Rand).

James Bussel was not present during this session but he sent a letter to the Chairman with a proposal of joint study with platelet Subcommittee on 2B VWD frequency on non-autoimmune thrombocytopenia.

Augusto B. Federici presented the concluding remarks of the entire two sessions, summarizing all studies and proposals to be reported next year in Sidney.
Women's Health Issues

Chairman: M. Manco-Johnson, USA

There were approximately 50 persons present at the subcommittee meeting.

Recommendations:

Several activities have been completed and recommendations were forwarded at the meeting.

1. Radiologic exposure during pregnancy: Dr. M. Nijkeuter reported on fetal x-ray exposure from either CT or VQ scanning performed to exclude pulmonary embolus in high-risk pregnant women. It was concluded that fetal exposure is less than background (environmental) radiation from helical CT chest scanning and less than that of VQ scanning. It was recommended that testing for pulmonary embolus not be avoided during pregnancy and that CT is the preferred technique. A recommendations paper was completed and will be submitted within a month following circulation to other committee members for approval.

2. Value of thrombophilia testing for VTE related to pregnancy, oral contraceptive or hormone replacement therapy as well as following various adverse pregnancy outcomes. Dr. I. Greer reviewed data that conclusively implicates thrombophilia in the predisposition of women to thrombosis during pregnancy and hormone therapies. In addition, convincing evidence was presented that thrombophilia predisposes to adverse pregnancy outcomes of preeclampsia, low birth weight, abruptio and fetal loss. However, analyses of several studies did not support that diagnosis of thrombophilia would alter management, nor that prevention of first episodes of venous thrombosis by either universal or selective screening programs would be cost effective. Thus, thrombophilia studies have been important to determine the pathophysiology of these disorders, but not to manage pregnant women. A recommendation manuscript has been developed and will be submitted for publication following circulation for review.

3. Coagulation testing and treatment for women with menorrhagia. This was presented by Dr. C. Phillip. Evidence exists that menorrhagia is an important clinical problem of women, that bleeding disorders are increased in women with menorrhagia, that racial differences exist in the etiology of bleeding disorders of women and that testing is difficult and expensive. A recommendation paper summarizing the state of knowledge and calling for careful studies of coagulation and platelet function in women with menorrhagia, the effectiveness of current coagulation evaluations to determine the etiology of menorrhagia and the efficacy of therapy and cost-effectiveness of screening will be submitted within the next two months following further review and refinement of the initial paper.
4. Scoring systems for severity of thrombophilia and pregnancy complications. The existing literature regarding the contribution of thrombophilia to adverse pregnancy outcome is difficult to interpret due to the lack of standard definitions for pregnancy outcomes as well as thrombophilic disorders. Dr. B. Brenner reviewed the existing literature and developed an innovative system of scoring maternal, fetal, gestational, and genetic factors for application to future studies. A paper describing the rationale and proposed scoring system will be submitted within the next three months and applied to individual and group activities. Next year’s meeting will describe experience using the scoring system.

5. Conditions for drawing blood samples to determine hormonal effects on coagulation in women. Dr. M. Blomback has reported on this project for the past two meetings. Her recommendations have been finalized. The initial recommendations paper will be rewritten and submitted for publication within the next three months.

Registries:

1. Pregnancy complications in women with prosthetic heart valves. Dr. A. James presented the finalized version of the registry which will be put on the ISTH web page for a committee activity this year.

2. Ovarian hyperstimulation syndrome. Dr. B Konkle presented the finalized version of the registry data forms for the incidence of the ovarian hyperstimulation syndrome and rate and outcome of thrombosis caused by hormonal stimulation for infertility. This registry will be put on the ISTH web page and entered as a committee group activity.

3. Pregnancy outcome in antithrombin deficient women. Dr. J Conard presented the final version of this registry which she piloted in France during the last year. This registry will also be posted on the ISTH web page for use this year.

Global assays for screening of hormonal effects in women.

Various global assays were presented by Drs. Antovic, Hellgren and He of Sweden including thrombin generation assays, variations of the thromboelastogram and the overall haemostatic potential. A plan was made to conduct a validation study. Dr. Manco-Johnson will prepare aliquots of unknown plasmas and distribute them to participating laboratories for local performance. The analysis will assess the sensitivity of the assays to bleeding and clotting disorders.

Gender-influenced recurrence risk for venous thrombosis.

Dr. Eichinger presented intriguing evidence that women have a lower risk for recurrence of venous thromboembolism that is not influenced by use of oral contraceptives at the time of initial
clot onset. Younger women exhibit a lower recurrence rate than women beyond the age of 45 years. A discussion of protective effects of hormones on recurrence risk for venous thromboembolism will be continued next year.

The meeting was concluded at 12:35.

Respectfully submitted,

Marilyn J. Manco-Johnson
Working Group on Vascular Biology

Chairman: P. J. Newman, USA

SSC Organizing Committee: Michael C. Berndt, Australia; John Griffin, USA; Irène Juhan-Vague, France; Klaus T. Preissner, Germany

Detection and characterization of (circulating) microparticles

Membrane microparticles (MPs) constitute relevant hallmarks of cell activation or damage, whilst the cells they stem from remain sequestered in tissues or are promptly submitted to phagocytic clearance. MPs participate in transcellular exchange of biological information. They can disseminate potent bioactive effectors, including blood-borne tissue factor (TF), the main cellular initiator of the clotting cascade, and procoagulant phosphatidylserine, pro-inflammatory or apoptogenic mediators, with a central role for the P-selectin pathway in the amplification of the generation of MPs harboring TF. Because they are pathogenic markers, MPs are pharmacological targets of great interest in the therapeutic approach of vascular disease. However, assessment of their clinical relevance is hampered by methodological limitations.

Owing to the more and more recognized significance of MPs in cardiovascular disorders and other pathologies (infection, immunity, cancer, metabolic disorders, …), or even in more fundamental processes, such as development for instance, one of the goals of the Working Group on Vascular Biology is to set up a network aimed at standardizing MP detection and characterization by using immunological and functional procoagulant assays. To illustrate the importance of MPs and the need for standards, it may be added that a recent PubMed search yielded ~750 hits, with exponential increase and publication in high impact factor journals.

The founding meeting of the WG-VB was held in Birmingham at the ISTH 2003 Congress (see Dr. P.J. Newman’s corresponding report), where it was decided to set up a questionnaire aimed at identifying practices and needs in the field. This questionnaire, elaborated by Françoise Dignat-George and Jean-Marie Freyssinet, was circulated through a listserv message to all ISTH members. Twelve full replies were received with valuable information for organizing the Venice session (see attached program).

The attendance was high, with ~ 100 participants (~ 40 at 6:15 pm!). Each of the speakers gave a clear view of his own approach of the problem, and had at least 5 specific questions to answer.

Dr. Jean-Marie Freyssinet opened the session with an overview of the issue with respect to the various activities and potentials of MPs related to the cells and nature of stimulation at their origin, and their character of pathogenic markers.

Dr. Françoise Dignat-George summarized the replies to the questionnaire, emphasizing heterogeneity of the approaches (flow cytometry, solid-phase capture assay enabling assessment of the associated procoagulant potential, functional assays), at either pre-analytical (delay, whole
blood, platelet-free plasma, anticoagulant, centrifugation conditions) or analytical stages (numeration, procoagulant activities, immunological and functional determinations). It clearly appeared that these phases need to be standardized in order to make sure investigators are considering the same membrane fragments as MPs.

**Dr. Rienk Nieuwland** presented his view of the structure-functions (or composition-activities) of MPs, and showed that although MPs from healthy subjects can promote coagulation reactions, those from patients are more efficient with a role for tissue factor as confirmed in vivo using an animal model.

**Dr. Johan Heemskerk** investigated the role of MPs in thrombin generation triggered by low tissue factor, relying on procoagulant phosphatidylserine. He also reported that MPs can form in citrated platelet-rich plasma in the absence of coagulation, and that MPs remain circulating one hour after platelet transfusion.

**Dr. Alan Michelson** focused his presentation on platelet MPs after having made a clear distinction from exosomes, which are smaller vesicles (≤ 0.1 µm) playing a role in the immune response. Platelet MPs were shown to be detectable in whole blood by flow cytometry, which may reduce pre-analytical artefacts.

**Dr. David Varon** has assessed platelet MPs for ability to induce angiogenesis, and they indeed do, the process being abolished by blocking VEGF, and to a lesser extent bFGF and PDGF, suggesting that MPs transport these growth factors. The pro-angiogenic role of MPs is mediated by PI3-kinase and p-38 kinase.

**Dr. Nigel Key** addressed the important issue of tissue factor de-encryption and provided evidence that MP-associated tissue factor activity is detectable in all normal individuals. The measurement of such an activity in patients was discussed with respect to pre-analytical, assay (antibody specificity) and metrology (reference for tissue factor) issues.

**Dr. Bruce Furie** presented a challenging new flow cytometric approach of MPs, not based on light scattering parameters usually at the lowest background limits of most of conventional instruments. Impedence flow cytometry allows a better discrimination of MPs, especially when using well-characterized reagents (antibodies).

In an additional short communication, **Dr. Eric Grabowski** emphasized that flow should be considered in MP-cell interactions.

At the end, a general consensus was reached that MPs are true pathogenic markers of prime interest and of course worth standardizing. The pre-analytical and analytical phases were discussed in depth, with particular emphasis on the definition of shed MPs as submicron fragments different from other membraneous cell-derived entities, e.g. exosomes, released organelles, or larger apoptotic bodies. Instrumentation (flow cytometer capacities and limitations) and reagents (antibody specificity and enzyme and cofactor quality for functional assays) were also considered as primary sources of inter-laboratory variability.
Information gained during this lively session was indeed useful to define new and important issues to be integrated in an extended version of the first questionnaire, which will be prepared by Françoise Dignat-George and Jean-Marie Freyssinet and circulated via the ISTH or accessible from the website. Such a detailed survey should prove of prime interest to enter the second phase of standardization with specific tasks to be discussed in Sydney, in order the WG-VB can fulfill its objective.

Respectfully submitted by Jean-Marie Freyssinet
Working Group on Coagulation Secondary Standards

Chairman: Jane Lenahan

The annual meeting of this committee was held on Thursday, 17 June 2004 with 50 attendees.

The chairman opened the meeting with a summary of the major activities carried out during the past year: a contract was signed with Technoclone (purchased the coagulation portion of Immuno) to manufacture Lot 3 of our Secondary Coagulation Plasma Standard. The decision to give the contract to Technoclone was to keep continuity with what has been a very satisfactory product. 54,000 vials (1 ml.) were delivered to the NIBSC in December 2003 to the attention of Dr. Anthony Hubbard. Dr. Hubbard has started the stability studies and has a list of experts representing most countries that are members of the ISTH to carry out the labeling. The labeling will be completed for our meeting in 2005.

Dr. Hubbard reported on the inventory and stability of Lot 2. There are 18,861 vials remaining; approximately 9000 vials are dispatched yearly and the stability is good.

Dr. Hubbard also discussed the need to label for VWF:CB.

Dr. Barrowcliffe reported on the use of the SSC plasmas in the international collaborative study to establish a WHO plasma standard for Factor XIII. The proposed value for the WHO plasma was determined by an assay against fresh normal plasma pools in the participating laboratories and is expected to be confirmed by WHO in November 2004.

Dr. Kitchen presented a publication on the calibration of Lot 2 which will be submitted to the Journal of Thrombosis and Haemostasis.

Dr. Hubbard presented studies carried out by Dr. Declerk and the Subcommittee on Fibrinolysis on tPA and PAI-1.

From these reports and earlier discussions, the Working Group suggested adding VWF:CB and Factors V, XI and XIII to the list of analytes when calibrating SSC Plasma Lot 3. The decision on PAI-1, tPA, and Factors XII and IX will be discussed at our 2005 meeting after more studies have been carried out.

Dr. Gray again stressed the use of the SSC plasma by the Joint Committee for the Traceability in Laboratory Medicine. We thank her for this positive information.

Thanks to our committee members we had a productive year and anticipate another one in 2005.