

1995 MINUTES
PLASMA COAGULATION INHIBITORS

Sunday, 11 June, 1995

8:00 a.m. to 12:00 a.m.

Hall D

Jerusalem International Conference Center

Chair: T. Koide (Japan)

Co-chairs: R.M. Bertina (The Netherlands); B. Dahlbäck (Sweden); S. Iwanaga (Japan); D. Lane (UK)

The program consisted of the following two parts:

- I. Protein C and Related Subjects; and
- II. Anticoagulant Serpins.

I. Protein C and Related Subjects

The meeting began with a presentation by Dr. A.R. Hubbard on "The Calibration of the SSC Plasma Standard for Protein C and Protein S." He presented results from 12 laboratories and was able to assign potency for each standard, PC 0.98 iu/ml (cv 6.0%); PS (total) 94.7 iu/ml (cv 4.8%); PS (free) has not yet been finalized but will be shortly.

Next, Dr. R.M. Bertina summarized the status of "Standardization of the APC-Resistance Test." The many problems were reviewed. There are tests based upon different principles, APTT, PT, Factor Xa clotting and diluted RVVT. How should the normal range be made (excluding factor V R506Q individuals) and what concentrates of APC be used? Should normal ranges for males and females be different?

Dr. Tripodi informed the meeting that plasma from normal and factor V R506Q individuals has been collected for a proposed multicenter study of APC-resistance methods, which is about to be initiated. The protocol of the study was reviewed.

Dr. S. Rosén also presented a report on "APC- Resistance Test: Assay Performance and Influence of Sample Handling." Local plasmas and instruments are very important in determining response of APC-resistance assays. Care in sample handling is very important. Using factor V deficient plasma highly specific assay for the factor V R506Q can be devised. A new chromogenic substrate assay using factor V deficient plasma has been devised and, again, it gives very high specificity for the mutation.

The data on nine patients with severe PC deficiency was presented by Dr. H. P. Schwarz. The most complication-free treatment is PC (70 u/kg) twice a week (in acute phase) and oral anticoagulants. However, in spite of this, blindness is a major outcome of severe PC deficiency. The reason for onset of blindness is uncertain.

Then, Dr. B. Dahlbäck presented "Protein S Deficiency of Type I and Type III Are Phenotypic Variants of One Genetic Disease: Proposal of a Revised Nomenclature." A study of 18 families

with PS deficiency showed that the same families exhibit type I and III phenotypes. Two subtypes are now suggested. Type I, low free PS, normal or low total PS, and low functional activity; type II, low functional but normal free PS.

Following the talk, Dr. Martine Aiach made a short communication, "The Protein S Ser 460 to Pro Mutation (Heerlen Polymorphism) Is Associated with Type III Deficiency," against Dr. B. Dahlbäck's proposal. Dr. Bertina suggested a working party might be convened to discuss further classification.

"Database for Protein C and Protein S Gene Mutations." The recently published PC mutation database was summarized by Dr. P.H. Reitsma.

Dr. Sophie Gandrille also presented some additional data as "Mutations of Protein S Gene in 119 French Patients." Seventy-one percent of type I deficiency was successfully identified, while only 45% of type II deficiency was successfully identified.

Anticoagulant Serpins

Dr. D. A. Lane made a brief presentation on "The SSC Plasma Standard for Antithrombin." This standard was worked on by ten laboratories and assigned potency for standard as 0.96 iu/ml (antigen assay) and 0.92 iu/ml (activity measurement).

Dr. H. van Boven presented the data of "Natural History of Dutch Families with Inherited Antithrombin Deficiency." First, she showed several newly identified mutations of Type 1 ATIII deficiency including those caused by a missense mutation in a region other than a so-called pleiotropic effects mutation. She also showed a few Dutch families with combined deficiency of ATIII and factor V (factor V Leiden) whose risk factor for thrombosis are 92% in contrast to 54% for ATIII deficiency alone.

Finally, Dr. T. Koide presented the results from a study on "Expression and Characterization of Three Recombinant ATIII Mutants which Caused Pleiotropic Effects-Type ATIII Deficiency," in which he showed that three recombinant ATIII mutants (Oslo-type, Kyoto-type & Utah-type), which were grouped as "pleiotropic effects-type" mutants, have different properties from each other in secretion, intracellular degradation, abilities in heparin-binding and in the complex formation with thrombin. As a conclusion of his study, he suggested that a current subtype of "pleiotropic effects-type" mutants may be reconsidered if they should be classified into the same group.

Presentation by Dr. D.M. Tollefsen, "Insight into the Mechanism of Action of Heparin Cofactor II," was canceled.

1996 MINUTES
PLASMA COAGULATION INHIBITORS SUBCOMMITTEE

Saturday, 22 June, 1996, 13.00-17.00
Room Rossini, Fira Palace Hotel
Barcelona, Spain

Chair: T. Koide (Japan)
Co-Chairs: R Bertina (Netherlands), F Church (USA), S Iwanaga (Japan),
D Lane (UK), P Sandset (Norway).
S Bajaj (USA), B Dahlback (Sweden) could not attend.

1. From this year's meeting, our subcommittee included topics on tissue factor pathway inhibitor (TFPI) which have been discussed at the independent subcommittee meeting. Accordingly, Drs. Per M. Sandset and S. Paul Bajaj joined us as new co-chairs. In addition, Dr. Frank C. Church joined us as a new co-chair specializing in a serpins section.
2. We completed two activities in the past year. One is a database of mutations of protein C, and this has been already published in *Thromb. Haemost.* as "Protein C Deficiency: A Database of Mutations, 1995 Update." The other one is an inter-laboratory study on the assay of tissue factor pathway inhibitor.
3. We had about 250 attendees and the meeting room was always full.
4. This annual meeting of plasma coagulation inhibitors consisted of two parts and a total of 12 papers have been presented. The first part was APC-Resistance Test and Related Subjects. In this session, eight papers were presented, and five of them were focused on the APC resistance test to establish a standard of the assay and the diagnosis in the near future. S. Rosén (Sweden) and J. J. Jorquera (Spain) reported a modified APC resistance assay including factor V deficiency plasma with a heparin antagonist (V-DEF Plasma) and showed advantages and limitations of the modified APC-resistance test. A. Tripodi (Italy) reported the results of the multicenter study on the performance of different home-made and commercial APC-resistance methods to detect factor V mutation and suggested that the best discrimination could be observed not for the APTT assay but for the method based on factor Xa-clotting assay. M. M. Samama (France) reported the usefulness of the modified APC resistance test in the diagnosis of thrombophilia with factor V mutation and also suggested the superiority of factor Xa- initiated clotting assay. Lastly, J. P. Miletich (USA) made a very interesting report on APC-resistance in self-described ethnic groups in the US . He and his co-workers tested 2,438 post-menopausal US women and found a big imbalance in frequency of the factor V Leiden allele which is the major genetic cause for the APC-resistance. He showed that it is most common in women reporting as White, followed by Hispanic, Black, and Asian/Pacific Islander at significantly lower frequencies. In the latter part of this session, three papers were presented. R.A. Marlar (USA) and M. David (Canada) gave the initial report of the working party on the clinical aspects and the treatment of homozygous protein C and protein S deficiencies . They will continue their work for the report at the next meeting. Then, S. Gandrille (France) introduced the database of mutations of protein S deficiency, comprising 126 entries. Lastly, T. Koide (Japan) introduced a newly identified

vitamin K-dependent protein with anticoagulant property and high species-specificity. Second part of the meeting was Serpins and Tissue Factor Pathway Inhibitor (TFPI). D.A. Lane (UK) announced the completion of antithrombin mutation database: 2nd (1996) update which is being submitted as an official SSC Communication in Thrombosis & Haemostasis. He also proposed that the future update of the database will be made on Internet, and this proposal was accepted at the meeting. Next, F.C. Church (USA) talked about a comparison of three heparin-binding serpins: antithrombin, heparin cofactor II and protein C inhibitor. He showed the common part and different part of the mechanism in the interaction of heparin and three well-known heparin-dependent plasma coagulation inhibitors. The last two papers were made on TFPI. J.-B. Hansen (Norway) talked on the role of lipoprotein-associated TFPI, and showed that LDL-bound TFPI lack anticoagulant function due to carboxy terminal truncation and that the anticoagulant function of TFPI is restricted to its free form in plasma. Lastly, P. M. Sandset (Norway) made the final report on the interlaboratory study on the assay of TFPI, and at the end of his talk he asked the audience if there was a need for another study and it was approved. The meeting ended 30 min. behind the scheduled time as usual.

5. One proposal of an organization of the second international working group on Standardization of Antithrombin Concentrate was made by Dr. Elaine Gray (National Inst for Biol. Standard & Control, Fax +44-707-646730).

1997 Minutes

PLASMA COAGULATION INHIBITORS

Saturday, 7 June, 1997, 8:00 a.m. - 4:30 p.m.
Raffaello, Fortezza da Basso-Palazzo, Florence, Italy

Chairperson: T. Koide (Japan)

**Co-chairpersons: M. Aiach (France), R.M. Bertina (The Netherlands),
F.C. Church (U.S.A), B. Dahlback (Sweden), H. Kato (Japan)
and D.Lane (U.K.)**

1. The Subcommittee completed two activities in the past year which resulted in Official Communications. One is a database of mutations of antithrombin published in *Thrombosis and Haemostasis* 77, 197-211, as "Antithrombin Mutation Database 2nd (1997) Update." The other, "Protein S Deficiency: A Database of Mutations," is currently in press in *Thrombosis and Haemostasis*.
2. We had a full-day meeting on Saturday with about 450 attendees and the meeting room was always full to overflowing. The meeting adjourned at 4:40 p.m.
3. 3. This year's meeting consisted of six sessions and a total of 17 papers were presented.

The contents of the session are as follows:

Protein S Deficiency

Six papers were presented and the first two were about the assay of free protein S in plasma. The next four papers were on the genetic and phenotypic characterizations of type I and type III protein S deficiency, paying attention to their nomenclature and classification, in particular.

Dr. B. Dahlback (Sweden) introduced a new method of assay of free protein S that is fast, reproducible and highly specific for free protein S. The principle of this method is called ELSA (Enzyme-linked Ligand Sorbent Assay), which utilizes C4BP as immobilized ligand for catching free protein S in plasma.

Next, Dr. J. Amiral (France) reported the results of the measurement of free and total protein S and protein S activity in 520 healthy individuals. He emphasized that cholesterol and BMI (body mass index) are major parameters affecting the total protein S assay, that gender and cholesterol are parameters affecting the free protein S assay, and that gender and BMI are parameters affecting protein S activity. He also emphasized that the lower threshold for the diagnosis of hereditary protein S deficiency should be carefully determined in subpopulations of normal subjects which include males, females, and females using oral contraceptives.

The subsequent four papers dealt with protein S gene mutations.

Dr. R.E. Simmonds (U.K.) examined a large protein S-deficient kindred (122 germline individuals including 44 affected) and identified Gly295 to Val mutation in three family members. He concluded that the type I (low total protein S antigen and low free protein S antigen) and type III (normal total protein S antigen and low free protein S antigen) protein S deficiencies are phenotypic variants of the same genetic disorder and arose because of an age-related increase of total protein S antigen levels.

Dr. T. Yamazaki (Japan) also issued the same conclusion from a study on two protein S deficient families in Japan.

Dr. N. Sala (Spain) also reported results of the genetic analysis of families with type I and/or type III protein A deficiency which demonstrated the complexity underlying type III deficiency. It concluded that while allelic heterogeneity in the protein S (PROS1) gene is the main cause of type I protein S deficiency, type III or free protein S deficiency is likely to be a genetically heterogeneous or complex disease. Free protein S deficiency results either from a mutation in a single major gene like PROS1, or it results from the interaction of different factors, among which the protein S Heerlen allele seems to play a role.

In the last paper, Dr. R. Bertina (Netherlands) discussed protein S Heerlen allele. He concluded that, in spite of reduced levels of free protein S in individuals with protein S Heerlen allele, this allele is not associated with a risk factor for thrombosis. At the end of the session, the subcommittee chairman proposed to organize a new working party on protein S deficiency which would concern assay of free protein S and nomenclature of type I and type III deficiencies.

Protein C Deficiency

Dr. R.A. Marlar stated that a report of the Working Party on the Clinical Aspects and Treatment of Homozygous Protein C and Protein S Deficiencies is being prepared for submission to *Thrombosis and Haemostasis* as an official SSC communication.

Antithrombin

Dr. E. Gray (U.K.) reported the result of the Collaborative Study for the Second International Standard for Antithrombin Concentrate. An international collaborative study including 18 laboratories in ten countries was organized. The proposed Second International Standard, 96/520, was calibrated against the First International Standard for Antithrombin, Concentrate, 88/548, and also compared against the Second International Standard for Antithrombin, Plasma, 93/768, by both functional assays and antigen methods. As a result, based on the means of all assays against the First International Standard for Antithrombin, Concentrate, the overall respective functional and antigenic potencies for the candidate preparation, 96/520, were shown to be 4.7 IU/ampoule and 5.1 IU/ampoule. This result will be published soon as an official publication of the subcommittee.

Dr. S.C. Bock (U.S.) reported that antithrombin-beta, the quantitatively minor isoform in blood, may account for a substantial portion of antithrombin activity in the vessel wall.

Dr. V. Picard (France) reviewed a database of anti-serpin antibodies. He suggested that anti-serpin antibodies are particularly useful in structure-function studies of serpins since they can specifically react with a given conformation, showing several examples of monoclonal antibodies against either antithrombin, C1-inhibitor or PAI-1.

Thrombomodulin gene mutations

Thrombomodulin (TM) is an integral endothelial cell membrane protein that functions as a cofactor in the thrombin-mediated activation of the protein C anticoagulant pathway. It has been suggested that an impaired TM cofactor function also could constitute a pro-thrombotic abnormality leading to thromboembolic disease. Dr. A.K. Ohlin (Sweden) presented the data of TM gene mutation in a patient with venous thromboembolic disease and showed that a defect in the TM gene leads to familial thrombophilia. Dr. H. Ireland (U.K.) also discussed TM gene mutations associated with myocardial infarction and suggested that mutations in the promoter region of the thrombomodulin gene may constitute a risk for arterial thrombosis.

TFPI (Tissue Factor Pathway Inhibitor)

Two papers on TFPI were presented. First, Dr. S.P. Bajaj (U.S.) discussed correlation of the plasma levels of TFPI with various diseases. TFPI is present as free form and lipoprotein-associated form in plasma and also as endothelial cell-associated form on vascular walls. Dr. H. Kato (Japan) introduced the newly developed EIA system which uses polyclonal and monoclonal antibodies against recombinant TFPI to measure free form TFPI and total TFPI in plasma. He also compared two commercially

available kits for the measurement of TFPI now available from Kaketsuken in Japan and from American Diagnostic Corporation. He showed that total TFPI was highly correlated ($r=0.87$) between the two kits; however, the correlation of free form TFPI was not correlated well between the two kits ($r=0.60$).

APC-Resistance

APC-resistance has been discussed at the last three meetings of the subcommittee; therefore, at this year's meeting only three papers were presented. The final one by Dr. A. Tripodi was a summary of the past discussion and a proposal for a Working Party on the Standardization of the APC-Resistance Test. At the end, the chairman suggested possible members of the working party. The preceding presentations were "Cost-benefit analysis for screening of APC-resistance" by Dr. W. Schramm (Germany) and "Factor V 506Q: Prevalence, thrombotic risk and risk modifiers" by Dr. J.P. Miletich (U.S.)

Dr. Schramm discussed the risk of venous thromboembolic events in oral contraceptive users with and without APC-resistance. He showed that the incidence was 17.3 and 1.8,

respectively, per 10,000 person-year, whereas, the incidence of venous thromboembolic events in oral contraceptive non-users with and without APC-resistance were 7.2 and 1.6, respectively, per 10,000 person-year. From this survey, he suggested that although testing of new oral contraceptive users, with and without exclusion by family history of venous thromboembolic events, seems to be less cost-effective than previously reported, screening does seem to be a rational use of scarce health care resources.

Dr. Miletich discussed prevalence, thrombotic risk and risk modifiers of factor V 506Q allele, which is the only genetically evidenced cause for APC-resistance, from a huge survey including 2,312 men from the Physicians' Health Study and 2,439 women from the Women's Health Study. From the Physicians' Health Study, he concluded that heterozygosity for the mutated allele does not detectably alter the probability of heart attack or stroke but does increase the chance for first-event venous thromboembolism about four-fold. He also showed that the mutated allele was found with similar frequency among men and women but is significantly different among ethnic groups, i.e., the carrier frequency was 5.3%, 2.2%, 1.2%, 0.5% and 1.3% in Caucasian, Hispanic, African, Asian, and Native Americans, respectively. Finally, he surprised the attendees by showing that the calculated number of heterozygous carriers is estimated to be more than 11 million among Americans and more than 423,000 are likely to be women who use oral contraceptives.

4. Two new working parties were organized during the meeting:
 - a. Working Party on the Standardization of the APC-Resistance Test, APC-Resistance Assay Method, Expression of the Results, Diagnosis of Factor V Leiden, and Other Causes of APC-Resistance
 - b. Working Party on Protein S Deficiency: Assay Method of Free Protein S in Plasma and Nomenclature of Types I and III
5. The following reports will be issued soon as official SSC communications of the subcommittee:
 - a. Report on the International Collaborative Study for the Second International Standard for Antithrombin Concentrate
 - b. Report of the Working Party on the Clinical Aspects and the Treatment of Homozygous Protein C and Protein S Deficiencies

1999 Minutes

PLASMA COAGULATION INHIBITORS

Sunday, 15 August 1999

8:00 to 12:00 PM

Room 39

Washington Convention Center

Washington, DC

Chair: M. Aiach, France

Co-chairs: F. Church, USA; H. Kato, Japan; D. Lane, UK;

K. Suzuki, Japan

Dr. M. Aiach presented an update of the protein S database, 204 mutations (140 unique events). There are only 3 large deletions, 99 missense mutations, as well as frameshift mutations, etc. There are mainly type I/III deficiencies, very few type II, all of the latter mutations in N terminal region. It was decided to look into the possibility of publishing the update as a SSC communication in *Thrombosis and Haemostasis* and also to explore the possibility of preparing a website for the database.

Dr. I. Jennings discussed the practical issues of APCR/Factor V Leiden tests, under the UK laboratory quality control scheme, NEQAS. In this routine quality control exercise many laboratories could not reproducibly detect heterozygotes with functional assays or even with PCR. Forty-seven centers (mainly UK) were sent fresh whole blood. Different extraction techniques were used with different primer sets for Factor V Leiden and PT 20210A. Finally, different endpoints (SSCP, restriction) were used. In the first study of only the APCR clotting test, five out of 47 tests resulted in the incorrect diagnosis of Factor V Leiden. A later study included genetic testing for FV Leiden and PT 20210A. Once again, there were many incorrect diagnoses. A further survey underway includes polymorphisms of the MTHFR gene, and there are still reporting errors. The conclusion is that proficiency testing for these common polymorphisms is very important in the routine laboratory context. There was some discussion whether identifying these polymorphisms is clinically valuable.

Dr. J. Emmerich summarized results of a meta-analysis of eight case control studies of venous thrombosis concerned with interaction of Factor V Leiden and PT 20210A. There were 2310 cases in total and 3206 controls. An overall OR for thrombosis for Factor V Leiden was 5.01, while that for PT 20210A was 3.88. Both mutations combined gave an OR 24.1. Age of first

onset was significantly younger, 36 compared to 40.5 years. The OR for PE was approximately 1.0 for both Factor V Leiden and PT 20210A. Synergism was demonstrated between oral contraceptives for both Factor V Leiden and PT 20210A. It is planned to submit this as a SSC communication for publication in *Thrombosis and Haemostasis*.

NEW RISK FACTORS

Dr. A.K. Ohlin presented her results on association of thrombomodulin gene mutations and venous thromboembolism. Seven hundred patients (Swedish/USA/France) have had their thrombomodulin gene screened. Thirteen cases were found to be heterozygous for a mutation. A start has been made characterizing gene mutations by in vitro transfection but further work is required. Dr. G. Kunz presented results of thrombomodulin mutations in arterial disease. In a case control study of 104 patients, six different mutations were identified. Three promoter polymorphisms have been evaluated by reporter-gene analysis and one, —33G to A, had reduced reporter activity. A coding sequence mutation Ala25Thr was found in two individuals and evaluated in a large case control study of myocardial infarction (MI). The results suggest Ala25Thr is a risk, ~2 fold, for MI. There is also evidence that an insertion/frameshift mutation in the coding sequence results in reduced expression of thrombomodulin in vitro and in vivo. It was concluded that thrombomodulin gene mutations may be important in MI but more work is required to clarify this.

Dr. R. Simmonds summarized the polymorphic nature of the EPCR gene. This gene is a candidate risk factor for venous and arterial thrombosis. The sequence of the EPCR gene has been completed. There are four exons coding for the receptor spanning 6kbp. Four common polymorphisms have been identified in healthy normals and their population frequencies determined, as a prelude to clinical studies. Dr. E. Faioni also addressed this issue with two clinical studies. Two hundred and two survivors of MI/190 controls were used as well as 209 patients with DVT/402 controls. A 23bp insertion was identified and its consequences evaluated in the clinical studies. Adjusted OR of 2.5 for MI and 2.2 for DVT were obtained, but there were wide CIs. This is the first indication that mutation of the EPCR may have a role in disease.

Dr. F.C. Church discussed nomenclature issues in SERPINS. First, he reviewed the recent advances in SERPINS, basic and clinical. An international committee has been set up chaired by Gary Silverman to develop a logical classification system, to link websites, and to form a SERPINS society. There are approximately 400 known SERPINS. These will be grouped as plants, insects, nematodes, and certain types of factors, e.g., PAI-1. Dr. Church asked for people with an interest in this area to contact him during the meeting.

Dr. F. Bernardi discussed the HR2 Factor V gene allele. Approximately 10% of subjects are carriers and are widely distributed. Polymorphisms between exon 8-25 are linked and form the HR2 allele. This seems to be associated with increased risk of thrombosis, OR around two, but this has been inconsistent. A large study of Italian patients suggests the HR2 might be a risk factor for coronary artery disease. The functionally important change is that HR2 alters APCR. The isoforms of factor differing in glycosylation, Factor Va₁ and Factor Va₂, seem to be altered in their relative distribution in the HR2 haplotype and this could explain the effect on function.

Dr. M. Murata discussed the polymorphisms related to coagulation genes in the Japanese population. Factor V Leiden, PT20210A, and PLA2 are almost absent in the Japanese population, but there are other possibly important ones, particularly G1b α and Factor XII.

PLASMA COAGULATION INHIBITORS

16 June 2000

13:30 to 17:30

Auditorium II

Maastricht Meeting and Convention Center

Chairman: M. Aiach--France

Co-chairmen: F. Bernardi--Italy; F. Church--USA; H. Kato--Japan;

D. Lane--UK; K. Suzuki--Japan

1. Protein S: Genotype and phenotype

After the presentation of Tomio Yamazaki from the group of Bjorn Dahläck and other papers recently published, it appears that type III protein S deficiencies, characterized by low free protein S levels and subnormal total protein S levels, are due in most cases to a protein S mutation moderately affecting the gene expression. Thus type I and type III are respectively severe and mild-protein S deficient phenotypes. The Ser 460 Pro mutation (protein S Herleen) also results in type III phenotype, although in this case the mild decrease in circulating protein S level is not due to a decreased gene expression. The reduced stability of the mutant protein suggests an increased turnover.

Sophie Gandrille took advantage of a new monoclonal antibody recognizing an epitope encompassing gene 61 to 72 to show that 10 to 20% of circulating protein S is cleaved after Arg 60. This might influence the assays measuring activated protein C cofactor activity.

Piet Meijer reported a Dutch study of the variability of protein S concentrations among different laboratories. Three types of calibrators were used, all made from human plasma. In some cases, universal pooled plasma from healthy donors (excluding women receiving oral contraceptive treatment) yielded the lower variability. It would be interesting to test recombinant uncleaved protein S as a calibrator.

2. Factor V gene and new mechanisms of APC resistance

The R2 polymorphism was described in Ferrara by the group of Francesco Bernardi. Elisabeth Castoldi reviewed the biochemical consequences of the different polymorphisms associated to the R2 haplotype (hré OR fv Ferrara) and Elena Faioni reviewed the association of HR2 with APC resistance, low FV levels and deep venous thrombosis (DVT). Overall HR2 contributes to APC resistance and might increase the risk of DVT when associated with other genetic risk factors such as FV Leiden.

3. Tissue factor pathway inhibitors (TFPI)

Theo Lindhout gave a review on the recent advances on TFPI mechanism of action.

Several monoclonal based assays are now available to measure total and free circulating TFPI, such as those presented by Hisao Kato and by Marc Grimaux. The clinical significance of the variations in TFPI levels remains to be extensively studied. Pierre Morange reported associations of TFPI levels with cardiovascular risk factors in a large population.

Since during the past years, most subjects discussed by our subcommittee were extended to all modifications of the coagulation balance (such as APC resistance), we propose to change the name of the

" Plasma Coagulation Inhibitors " subcommittee which could now be the " Thrombophilic Risk Factors " subcommittee.

PLASMA COAGULATION INHIBITORS

7 July 2001

08:00 to 12:00

Room Bordeaux

Palais des Congrès

Chairman: F. Church--USA

Co-chairmen: M. Aiach--France; F. Bernardi--Italy; H. Kato--Japan; D. Lane--UK; K. Suzuki--Japan

The meeting this year was sub-divided into 5 different categories: (1) Protein S genotype, phenotype and deficiency; (2) Factor V gene and mechanisms of APC resistance, protein C deficiency and prothrombin G20210A; (3) Serpins (serine protease inhibitors); (4) Thrombophilic risk factors; and (5) Assay performance results for testing for familial thrombophilia.

1. **Protein S genotype, phenotype and deficiency- consisted of talks by Drs. Walker, Soria, Bates and Faioni.** Dr. Walker described her work of the West Scotland Donor Study (~3800 people), and it was stated initially that identifying protein S deficiencies has always been a difficult problem. She found that men had more total protein S than women, and a trend for more free protein S than women. They also found in women that with increasing age there was more total protein S, and when adjusting for age, post-menopause women had the same amount of protein S. Dr. Soria described work on the GAIT Project focusing on protein S, and his work focused on identifying candidate genes for hemostatic defects, and his work found a linkage with the gene region D1S194 to the factor V gene. Genome-wide linkage analysis found that C4B-binding protein was near, and they concluded that 1q32 could influence protein S levels and that this may be related to expression of C4b-BP. Dr. Bates talked about levels of evidence and the link with hemostatic disorders, asking questions about: a) methodology, accuracy, verification and variability?; b) evidence that the abnormality is associated with venous thrombosis?; c) potential confounders?; d) magnitude of abnormal significance; and e) link between abnormality and venous thrombosis and does it make biologic sense? She used warfarin therapy, pregnancy and antiphospholipid antibodies to study their link with protein S deficiency and disease phenotype. Her studies found no clear evidence with these 3 different states (inherited or acquired) and venous thromboembolic disease linked to protein S deficiency. A primary problem seems to be a lack of statistical power in these analyses. Without a doubt her studies are suggesting that we need more powerful tools to appreciate protein S deficiency, and again it reinforces the notion that describing protein S deficiency is difficult and is problematic for clinicians and laboratory workers. Dr. Faoini presented data exploring the possibility that a dimorphism, A2148G (Pro 626) influence protein S levels - she studied the effect of phenotype, effect of risk, and effects on diagnosis. She used the PROSIT patient group, and she found that Pro 626 modulates free protein S in healthy women independent of a protein S alpha gene mutation; and that

Pro 626 had little effect on total protein S levels in healthy or protein S-deficient individuals.

2. **Factor V gene and mechanisms of APC resistance, protein C deficiency and prothrombin G20210A consisted of talks from Drs. Spek, Bovill, Bernardi, and Magdelaine.** Dr. Spek reviewed data about protein C polymorphisms of the promoter region, and the 3 studies found an overall risk that ranged from 1.00-2.00; and the results suggest that protein C promoter polymorphisms do not increase thrombotic risk in factor V Leiden patients, and that homozygous or compound heterozygous states of protein C deficiency have no association between clinical severity and polymorphisms of the promoter region. It is not clear how promoter polymorphisms in protein C affect protein C antigen levels. Additional studies found no clear candidate for different transcription factor binding sites, but there is a moderate thrombotic risk associated with these promoter polymorphisms, but there is no biological explanation yet for the differences in plasma levels. Dr. Bovill described a large pedigree (>700 live individuals) in Northern Vermont U.S.A., Quebec Canada and now some studies in collaboration with others in Europe. They are characterizing the founder effect of the 3363 C mutation gene insertion found in this family, and he has obtained funds to fully sequence the genome of 300 family members. Dr. Bernardi presented an overview of the R2 factor V, and reported that up to 10% of western-European persons are carriers of the 2 polymorphisms found in R2 factor V. The studies described to date are not in agreement in that some report decrease in factor V levels, some do not, and others report an increase in APC resistance, while some do not. The risk in some studies reaches 2.0 while in others it is <1.4. There is much evidence to further describe the molecular basis for this phenotype, correlation of biochemistry versus in vivo finding, and the basis for the discrepancies between clinical phenotype and laboratory values. Dr. Bernardi suggests the formation of a Working Group in this SSC to further define the clinical and biological significance of R2 factor V. The audience and the Chair/co-Chairís were in agreement with this suggestion. Dr. Magdelaine described an interesting single case of a double thrombomodulin mutation (Ala 25→ Thr and Ala 455→ Val) with homozygous factor V Leiden. Her results highlight that we should continue to study multi-genetic defects in thrombotic-related genes, since these single thrombomodulin mutations are not strongly associated to thromboembolic disease.
3. **Serpins (Serine protease inhibitors) consisted of talks by Drs. Church, Hayashi, and Rezaie.** Dr. Church reviewed a landmark paper of Dr. Kenneth Brinkhous (in memory about) from 1939, where he described the "heparin cofactor" responsible for the biologic activity of heparin. Dr. Church also mentioned a paper in press (Gary A. Silverman, Phillip I. Bird, Robin W. Carrell, Paul B. Coughlin, Peter G.W. Gettins, James I. Irving, David A. Lomas, Cliff J. Luke, Richard W. Moyer, Philip A. Pemberton, Eileen Remold-O'Donnell, Guy S. Salvesen, James Travis, and James C. Whisstock, 2001, *"The serpins are an expanding superfamily of structurally similar but functionally diverse proteins: Evolution, mechanism of inhibition, novel functions, and a revised nomenclature"*, published in J.Biol. Chem., published July 2, 2001 as 10.1074/jbc.R100016200) where the suggestions for nomenclature of serpins, new and old, are summarized. Dr. Hayashi described his work with protein C inhibitor in renal cell carcinoma (RCC), and he

reported that in RCC there was an absence of protein C inhibitor antigen, with no change in urokinase levels. Adding back protein C inhibitor to a RCC cell line resulted in the inhibition of invasion; thus, suggesting that protein C inhibitor might function to regulate tumor cell invasion. Dr. Rezaie summarized his work about vitronectin interacting with plasminogen activator inhibitor type-1, which then becomes a potent inhibitor of activated protein C. This work suggests that the potential pro-fibrinolytic effect of activated protein C might be due to a process involving PAI-1/vitronectin inhibiting activated protein C.

4. **Thrombophilic risk factors included talks by Dr. Miyata and Dr. Moll.** Dr. Miyata described the prevalence of protein C, antithrombin and plasminogen deficiency in the general population of Japan. The study used blood donors/patients seeking normal check-ups from the ages of 30-90. This work identified that 0.2%, 0.18% and ~4% of the population had protein C, antithrombin and plasminogen deficiency, respectively. The odds ratio for patients with deep vein thrombosis, compared to Western man, was higher for protein C and antithrombin deficiency, yet the Japanese have an occurrence of 0% with factor V Leiden. Dr. Moll proposed an International Collaboration Group through this SSC to study "combined thrombophilia" focusing on three rare compound thrombophilias: homozygous Factor V Leiden/heterozygous Factor II G20210A; heterozygous Factor V Leiden/ homozygous Factor II G20210A; and homozygous Factor V Leiden/homozygous Factor II G20210A. The proposal is based on the occurrence of these defects ranging from 1/1,000,000 3/100,000,000 for these combined thrombophilias. It was proposed that an international collaboration be established, genetic labs contacted to inquire about their database; and patients could respond to a questionnaire posted on the Internet. Discussion from the audience was enthusiastic and the co-Chairs found the idea worthy of further discussion, except Dr. Aiach raised a point about should we include other combined thrombophilic defects along with these suggestions. Dr. Moll commented that he thought this was complicated enough to do as proposed.
5. **Assay performance results for testing for familial thrombophilia consisted of talks by Dr. Jennings and Dr. Siegert.** Dr. Jennings reviewed the laboratory pitfalls of their experience in the "UK NEQAS/EQUALS Experience", and he went on to describe the frequency of how often incorrect diagnosis was made for factor V Leiden, antithrombin, protein C, and protein S. He summarized most of the finding as differences in assay methods, assay kits used, reference plasmas, methodology details, and expression of the results using normal ranges. Dr. Siegert reported on the influence of low molecular weight heparin (LMWH) on various assays including protein S, RVVDT, and TFPI. In all cases, LMWH had a global effect on clotting assay measurements, which differs from unfractionated heparin, especially when LMWH is used at >1 U/mL. These results caution us that even when neutralizing LMWH with protamine sulfate, there may be differences in the properties of the plasma samples when analyzed.

In conclusion, Dr. Church thanked the co-chairís for help in organizing this SSC meeting and for help in chairing individual sessions, he thanked the speakers for keeping within the given time constraints of this many presentations, he thanked the attendants provided by the congress for their help with the speakers, he reiterated the call for formation of 2 Working Groups (from Drs. Bernardi and Moll), he thanked the audience for their participation in asking questions, and encouraged them to communicate with the subcommittee chairman. The meeting was then adjourned by Dr. Church. Au revoir  til next year!

(It was estimated by those in attendance that ~300 people were in this session)

Plasma Coagulation Inhibitors

July 20, 2002

8:00 to 12:00

Plaza Room

Boston Park Plaza Hotel

Chair- F. Church, USA

Co-chairs: M. Aiach, France; F. Bernardi, Italy; H. Kato, Japan; D. Lane, UK;
K. Suzuki, Japan

Business:

The meeting was chaired by Subcommittee CoChairs, Frank Church and Koji Suzuki (the other co-chairs were unable to attend due to conflicts) . It was attended by approximately 50-60 persons. There were four sessions organized this year, R2 Factor V, Antithrombin reference standard, quality assessment for screening thrombophilia, and a review on serpin nomenclature and evolution.

Scientific Program:

Dr. Castoldi presented two talks, the first an overview of the FV R2 haplotype and gave the historical perspective and the evidence that the risk between venous and arterial thrombosis with this is not conclusive. She did suggest that heterozygotes with FV R2 and Factor V Leiden may be predisposed to venous thrombosis, but again, this is not totally conclusive. Her second talk centered around the ability of Factor V to serve as a cofactor for APC-protein S inactivation of Factor VIII and APC inactivation of Factor Va, both of which could contribute to the action of this interesting FV variant. She found that FV R2 was associated with altered cofactor function with APC inactivating factor Va.

Dr. Castaman described a metanalysis of existing data comparing the risk factor for factor V R2 with thrombosis. While there is no conclusive evidence that this allele predisposes strongly to venous thrombosis, there appears to be some small risk, and that other studies should be performed with a larger population base and a better defined/detailed clinical description of venous thromboembolic events.

Drs. Jackson and Esnouf spent the next period of time offering advice and guidelines about the newly proposed recombinant standard of antithrombin, and they divided their time to an overview of the question and the reference method to analyze the antithrombin standard by Jackson and the guidelines for the reference material described by Esnouf. There was a lot of conversation generated by their plan, and it is not clear where we stand in testing the reference standard, yet we heard today that a company has offered 2 grams of purified protein.

The third session was occupied with testing analyses and evaluation of different laboratories and their ability to test protein C, protein S, and antithrombin. These were given by Drs. Meijer, Biguzzi, and Jennings. Dr. Meijer summarized his studies of a wide-ranged testing comparing antithrombin, protein C and protein S. He described the use of a simple linear regression model

just published that supports his comparison within the laboratories. The goal was assessment of long-term analytical performance of field methods. There was some widespread variation of CV within the various lab for antithrombin, protein C and protein S. His story was summarized by saying that we need to improve assay variability between the various labs that perform such tests, and there is much research going on to sort out where the large variability occurs. His second talk centered around 11 different labs and their ability to assay only for protein S, pointing to different methods, different calculations, different reagent standards and assays. It was clear that "handling" of samples was a key issue that caused the variance within the assays. Dr. Biguzzi summarized the work on PROSIT, and she provided the first set of preliminary data studies from this group of interesting protein S patients/families in Italy. Fourteen (14) of the probands out of 52 studied had no mutations. The others had one or more mutations (frameshift, nonsense, or missense), and as this work continues they are studying the *in vitro* biochemistry and activity properties of these variants, secretion and activity. This promises to provide new and interesting details about protein S mutations. The final talk was from Dr. Jennings, who summarized a large set of interesting data gathered in the UK (but not from labs all located in the UK). His study focused on protein S activity, free and total protein assays. There was general agreement that the free protein S assay was less precise but more complex, and that direct free protein S levels are more precise to testing. There was also data included that summarized PEG-based versus direct-free protein S methods, and surprisingly, the data set suggested that the PEG-based assay had a better CV and might be a better assay, however, evaluations are still ongoing. Finally, he talked about pre-dilution of a sample being a problem from testing.

The final session was an overview of nomenclature for the serpin superfamily, given by Dr. Silverman. He provided the rationale for the new nomenclature, basing it primarily on need: too many names that are confusing, too many other names being signified for other serpins. Thus, a committee (not from the SSC) was convened and the nomenclature discussed and adopted. A paper was published in the J. Biol. Chem. in 2001 giving the details to this naming system (Silverman G.A., P.I. Bird, R.W. Carrell, F.C. Church, etc. 2001. The serpins are an expanding superfamily revised nomenclature. J. Biol. Chem. 276: 33293-33296.). We are now approaching over 700 different serpins from many different species, not just mammals, and the naming system will be useful as more genomes are sequenced. In no way is he suggesting that the nomenclature will displace the existing names of serpins; antithrombin will remain as is, yet it now has a genetic description based on this nomenclature that will identify it to all.

Drs. Suzuki and Church thanked all of those in attendance and closed the meeting.

Plasma Coagulation Inhibitors

July 12, 2003

09:00 to 13:00

Hall 9

The International Convention Center, Birmingham

Chair- F. Church, USA

Co-chairs: F. Bernardi, Italy; C. Jackson, USA; D. Lane, UK; K. Suzuki, Japan

The meeting this year was held to a very large audience, perhaps the largest ever for this subcommittee, and we had some lively conversations and dialog during the talks, and around the coffeepot. We divided our meeting into 4 sessions: Endogenous Thrombin Potential (ETP); Heritability of Plasma Levels of Coagulation Inhibitors from Family and Twin Studies; Anticoagulant Proteins- Characterization and Clinical Use in Disease Therapy; and Other Topics of Interest to SSC on Plasma Coagulation Inhibitors.

Dr. Hemker presented the rationale behind the EPT in that thrombin begets thrombin and the complexity of platelet-poor and platelet-rich plasma for devising assays. There was much conversation dealing with this presentation, especially related to tissue factor sensitivity, effect of thrombomodulin, standardization of the assay, and the influence of circulating tissue factor/phospholipid particles. Dr. Giesen then focused his presentation on an automated form of the ETP, using a fluorophore that thrombin cleaves, and the need for an internal "calibrator" to take into account the color of the plasma and to help standardize the assay. Dr. Tans then focused his talk on the use of the EPT in assessing APC resistance and the thrombotic effects of oral contraceptives. The role of α 2-macroglobulin to bind thrombin was addressed and issues related to measuring free versus bound thrombin were mentioned. He summarized recent work with transexuals to highlight the different effect of male and female hormones on APC resistance, and devoted some time to the notion that ethinyl estrodial is prothrombotic and that the progestagen in oral contraceptives is antithrombotic. Dr. van Hylckama Vlieg summarized her work with EPT on the Leiden Thrombophilia Study, focusing again on APC resistance. Her observations focused on the EPT being related to APC resistance with and without the occurrence of Factor V Leiden, and that APC resistance was increased in women on oral contraceptives. Dr. de Visser then directly compared the traditional APTT assay to the ETP, again focusing on APC resistance. A ratio comparing activity plus and minus the addition of APC to both plasmas was compared using over 200 patient samples and 200 control samples. Her work highlighted differences in sensitivities to the APTT to prothrombin and factor VIII and to free TFPI and free protein S for the ETP. Dr. Kakkar finished this session with a summary of his work comparing heparin and low-molecular weight heparin in combination with oral anticoagulants using various clinical parameters and assays, including the ETP. At the end of this session, Dr. Elaine Gray spoke briefly about a need and attempt to standardize the ETP, and has suggested that our subcommittee might be able to help implement plans to establish a standard. There are, as would be expected, lab to lab differences, actual methods are slightly different, and it is important to organize a "working group" for this interesting assay, and we would work to have something ready for our next meeting in 2004 in Italy.

The second session was a presentation with Dr. Souto, who had an interesting presentation on the heritability and genomic mapping of modified plasma coagulation proteins. His work is focused on quantifying the genetic trait analysis. He was the GAIT study to highlight some features of a wide genome scan of protein S levels, TAFI, protein C, and antithrombin where data was presented to relate genetic components to plasma levels.

The third session focused on key anticoagulant proteins that are currently being used clinically to treat various thrombotic/inflammatory diseases. Dr. Grinnell began by describing the biology of Drotrecogin alfa (activated) recombinant human activated protein C. He described the effect of APC to modify genes of NF- κ B, and the ability of APC to be much more than an anticoagulant protease. Dr. Joyce then summarized the success of APC in treating severe sepsis, noting the survival rate of patients receiving a single dose, notably the improvement in morbidity, especially related to cardiovascular and respiratory functions. Dr. Heinrichs then summarized the recent antithrombin data for treatment of severe sepsis, and summarized the data showing that antithrombin, like APC, has both antithrombotic and anti-inflammatory actions. He described the KyberSept study, where high doses of antithrombin was given with either unfractionated heparin or low molecular weight heparin. In these studies, there was no benefit shown for the use of antithrombin; however, when the groups were analyzed for those without heparin, there was a significant reduction in death. There was much discussion that suggested that it was too early to conclude that antithrombin was not effective, that additional studies should be performed, especially in light of the information found for antithrombin in the absence of heparin. Even suggestions that combination of proteins, APC and antithrombin and TFPI be considered. Dr. Levi summarized his work on all of the above areas, including APC, antithrombin and tissue factor pathway inhibitor (TFPI). The TFPI data was recently made public, and its use in severe sepsis seemed to mimic the antithrombin data, that there was no major reduction in death for patients treated with TFPI in the presence of additional heparin. Like antithrombin, the TFPI patients who did not receive heparin had a more successful course during their severe sepsis. The final conclusion was that there is significant cross-talk between coagulation and inflammation.

The final session was devoted to other issues of our sub-committee, and Dr. Gray summarized initial studies to prepare a reference genetic panel of genomic DNA for factor V Leiden. She presented numerous methods that have been suggested to prepare the samples, and a survey of users preferred gDNA (genomic DNA). Dr. Jackson updated us on his progress for the SSC Working Group Report on Antithrombin, where numerous scientists and clinicians from around the world have agreed to study and test this protein, using kinetic methods of analyses. The final speaker, Dr. Moll, was delayed in transit to Birmingham, but he stresses the need to continue the International Collaboration Group to study "Combined Thrombophilia", and urges members of the SSC and ISTH to contact him with patient information for collaborative work.

After running over by 40 minutes due to the stimulating discussions generated by the first 3 sessions, we adjourned our meeting. All sub-committee co-chairs were in attendance, and were all active participants in chairing sessions and interacting with the audience in attendance.

Plasma Coagulation Inhibitors

June 18, 2004

14:15 to 18:15

Cipressi Room

Fondazione Giorgio Cini

Chair: F. Church, USA

Co-chairs: F. Bernardi, Italy; E. Gray, UK; C.J. Jackson, USA; D. Lane, UK;

K. Suzuki, Japan

Attendance: 50 - 60

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 μ M 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 Coagulation Inhibitors

6 August 2005

16:00 to 19:30

The Ballroom 1

Sydney Convention and Exhibition Centre

Chair: E. Gray, UK

Co-chairs: F. Bernardi, Italy; F. Church, USA; C.J. Jackson, USA; D. Lane, UK; K. Suzuki, Japan; H. Whinna, USA

WHO International Standards: Chair: F Bernardi

International Genetic Reference Panel for Prothrombin G20210A Mutation. E Gray

An international collaborative study to validate the WHO 1st International Genetic Reference Panel for Prothrombin G20210A Mutation was presented. The panel included 3 preparations of human gDNA: wild type, homozygote and heterozygote for G20210A mutation. The study involved 45 laboratories from 23 different countries, employing a total of 22 different methods against in-house known patient or commercially available controls. The majority of the participants correctly genotyped (error rate 0.7%) and therefore confirmed the validity of the panel. It was therefore recommended that the panel (05/130) should be considered by the SSC and the ECBS of the Who to be established as the international genetic reference panel for prothrombin G20210A mutation. The subcommittee approved the recommendation.

International Standard for Protein C, Plasma and Concentrate. E Gray

E Gray announced the forthcoming international collaborative study to replace the 1st International Standard for Protein C, Plasma. The study will also serve to establish the 1st International Standard for Protein C, Concentrate. The same exercise will also calibrate SSC secondary plasma standard Lot #3 for Protein C activity and antigen. The samples and protocol will be dispatched to the participants in August 2005 and the results of the study will be presented at the next SSC meeting in June 2006.

International Standard for Protein S, Plasma. AR Hubbard

The current WHO 1st International Standard for Protein S, Plasma was established in 1995 and the stocks have fallen to approximately 700 ampoules. It is therefore necessary to calibrate a replacement preparation. The candidate WHO 2nd IS Protein S Plasma (03/228) has been prepared from a pool of 24 plasma units from normal healthy donors. The variability of the liquid fill into ampoules was extremely low with a CV of only 0.09%; the mean fill weight was 1.0063 g and the residual moisture was only 0.064 %. Preliminary tests have indicated a level of 0.92 IU total Protein S antigen per ml. These results indicate that the preparation (03/228) is suitable for calibration as the Proposed 2nd IS Protein S Plasma. The calibration exercise will involve comparison with the current WHO 1st IS and with locally collected normal plasma pools in order to check on the continuity of the International Unit and the possible drift of the IU from

the original calibration of the WHO 1st IS. As with the WHO 1st IS three parameters will be measured: total antigen, free antigen and function. The collaborative study is planned to commence in October/November 2005 with a view to submission to WHO and establishment in November 2006.

Protein S Multimers Chair: F Church and F Bernardi

Another look at protein S monomers/multimers and their direct anticoagulant activity. MJ Heeb

Plasma protein S has activated protein C-independent, direct anticoagulant activity (PS-direct). It was reported that monomeric purified protein S has only weak PS-direct, that multimeric purified protein S has good PS-direct in the presence of limiting phospholipids (0.1 μM), but that plasma contains only monomeric protein S, leading to the possible conclusion that either plasma has no PS-direct or that active purified protein S containing multimers has artifactual PS-direct. Dr Heeb showed that conventionally-purified protein S prepared with MonoQ-Sepharose had poor PS-direct and poor phospholipid affinity. Monomers, dimers, trimers and higher forms of affinity-purified protein S were identified by analytical ultracentrifugation. Multimers were not dissociated by Ca^{2+} or promoted by EDTA alone, but may be concentration-dependent.

On a mass basis, monomers and multimers separated from affinity-purified protein S had the same specific PS-direct in the presence of saturating phospholipids (25 μM) and the same ability to compete with prothrombinase components for limiting phospholipids (2 μM). she concluded that Protein S monomers and multimers were detected in citrated plasma that had PS-direct, and in whole and fractionated hirudin-plasma. Thus, protein S multimers are naturally-occurring and plasma PS-direct is represented by affinity-purified protein S but not by some conventionally-purified protein S.

Should we bother about protein S multimers? T Hackeng

Dr Hackeng addressed the history and current status of protein S multimers and concluded that these multimers are in vitro artefacts that can seriously affect results of experiments and conclusions drawn. The APC-independent activity observed in model systems using purified proteins at low phospholipid concentrations could be completely tracked back to the presence of protein S multimers. The multimers were not present in plasma although APC-independent activity of protein S was observed in plasma. This activity therefore must follow a different mechanism than the APC-independent activity of purified protein S in model systems using purified proteins.

Global Coagulation/ Haemostatic Tests. Chair: H Whinna

Pre-clinical validation of the Calibrated Automated thrombogram (CAT). HMM Spronk

Dr Spronk presented data on setting reference ranges for thrombin generation test performed on the CAT. He compared results obtained from blood samples collected by half and full draw and found that there was no significant difference between these samples. Reference ranges for

female and male were presented. He found that there is no need for reference range for different age groups, but there was a correlation between age and endogenous thrombin potential (ETP).

Recombinant tissue factor (rTF) and tissue plasminogen activator (t-PA) used in a new global assay to determine the combined effect of coagulation and fibrinolysis in plasma. S He

A global assay involving the addition of r-TF and tPA to platelet poor plasma was discussed. Overall coagulation potential (OCP) was obtained by the addition of rTF to platelet poor plasma (PPP), while the addition of t-PA gave results on overall fibrinolytic potential (OFP). The balance of OCP and OFP yielded the overall haemostatic potential (OHP). Results on application of this assay to haemophilic plasmas and patient samples (coronary heart disease and DVT) were discussed.

The Nijmegen Haemostasis Assay (NHA). W van Heerde

Dr van Heerde presented an assay that measures thrombin and plasmin generation simultaneously in a single well, using two different fluorogenic substrates. To validate the NHA several conditions were tested. Titration of TF varied the thrombin generation lagtime, the total thrombin generation (thrombin potential) and the plasmin generation lagtime. Total plasmin generation was not affected. Corn trypsin inhibitor did not interfere in the NHA indicating that initiation via the intrinsic pathway does not occur. Dilution of cephalin resulted in a diminished thrombin potential. Hirudin completely blocks thrombin and plasmin generation suggesting the requirement of fibrin for plasmin generation. Addition of carboxy-peptidase inhibitor diminishes clot lysis time indicating thrombin-activatable-fibrinolysis inhibitor (TAFI) activity. TAFI activity is increased by low thrombomodulin (TM) concentrations. High concentrations of TM and active protein C affect thrombin potential. ϵ -aminocaproic-acid inhibits plasmin generation. Ultimately, increased concentrations of t-PA decrease thrombin generation lagtime suggesting interplay between fibrinolysis and coagulation. Titration of plasmin proved that an increased fibrinolytic activity results in an increased thrombin generation. In conclusion, the NHA allows the detection of coagulation and fibrinolysis and the interplay between both and may be suitable for screening haemostatic disorders.

Thrombin Dynamics Test (TDT) and ROTEM whole blood coagulation analysis potential value for the assessment of inhibitors and thrombophilic states. A Calatzis

The principle of ROTEM was described and it is based on the kinetic analysis of clot formation using whole blood. It allows the assessment of factors affecting thrombin generation, fibrin formation and polymerization as well as fibrinolysis. The principle of TDT was also described and it is an assay for the kinetic measurement of thrombin formation based on platelet poor plasma and optical detection on routine coagulation instrument. Results on the sensitivity of TDT to factor deficiencies and anticoagulants as well as procoagulant treatment were shown.

The possible use of the TDT in monitoring hypercoagulability. B Woodhams

Dr Woodhams reported results from a preliminary study to evaluate the use TDT to discriminate between normal subjects and hypocoagulable samples. Thrombin activity in patient citrated

plasma was monitored using a 'fast' thrombin chromogenic substrate with added Gly-Pro-Arg-HydroxyPro to inhibit fibrin polymerisation. The assay can be performed easily on most automated routine haemostasis analyser. The data showed that there were some overlap between normal ranges and hypercoagulable plasma samples, but was useful for plasma samples from hypocoagulable patients.

Working Party on Thrombin Generation Test (TGT): Results on current practices survey and progress on working party activity. T Henckle

Dr Henckle reported on the activity of the Working Party. The survey results on current TGT practices is now available. The most widely used methods are the non-sampling chromogenic and fluorogenic assays. A mini review on TGT was published by the Working Party. A pilot study on a chromogenic non-sampling method has been initiated and an international collaborative study is now being planned to assess the comparability of current fluorogenic method for TGT.

Reference Materials and Methods for Antithrombin. Chair: E Gray

Structure and activities of the ISTH/IFCC Joint Committee on Standardisation of Coagulation Tests (C-SCT). E Gray

The structure and activities of the C-SCT is still to be decided. Activity such as reference methods and materials for antithrombin will continue to be carried out and reports on the activity of the C-SCT will continue to be presented at this subcommittee.

Assays for detection of antithrombin Type I and Type II deficiencies. S Kitchen

Dr Kitchen presented important differences between results of antithrombin (AT) assays in which human thrombin, bovine thrombin or factor Xa are used as the target enzyme for inhibition. In the case of AT Cambridge II the median results obtained by groups of UK NEQAS participants were 77 IU/dl , 81 IU//dl and 87 IU/dl for bovine thrombin human thrombin and factor Xa respectively. Both AT Cambridge II and AT Denver appear as normal or type I defects if Xa is used, but are phenotypically type II if bovine thrombin is employed. Furthermore the results of AT assays in some subjects are critically influenced by the incubation in the assays. It was concluded that there are a number of issues related to AT assays which must be taken into account in the standardisation of AT testing.

Working Party on reference materials and methods for antithrombin: Pilot study on primary reference methods for antithrombin. E Gray

The remit of the Working Party was reported and they are to develop a primary reference method for measurement of antithrombin with SI traceability, to establish reference materials using the primary reference method and to subsequently establish secondary reference materials for antithrombin type I and II deficiencies. A pilot study has now been set up to identify matrix effects and influence of different concentrations of reactants on the proposed primary reference method.

Plasma Coagulation Inhibitors

Chair: Elaine Gray, UK

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

WHO International Standards

Chair: HC Whinna

Proposed international standards for Protein C. E Gray

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

Proposed International Standard for Protein S, Plasma. T Hubbard

Twenty laboratories from 11 countries have participated in the collaborative study to calibrate the proposed WHO 2nd IS Protein S Plasma (03/228) for total antigen, free antigen and function. Estimates of intra-laboratory variability were acceptably low with geometric coefficients of variation (GCV) below 10 % for 80/96 data sets. Only 5 out of a total of 189 assays were excluded from the analysis. The inter-laboratory variability (GCV) for estimates relative to the WHO 1st IS (range 3.33 - 9.02 %) was lower for each parameter compared to estimates relative to the local normal plasma pools (range 7.88 - 13.16 %). Largest inter-laboratory variability was found for the estimates of free antigen, particularly those incorporating PEG precipitation steps. There were no significant differences between the estimates calculated relative to the WHO 1st

IS and the local normal pools for any of the three parameters and the overall mean estimates were extremely close (total antigen 0.83 vs 0.82; free antigen 0.81 vs 0.81; function 0.77 vs 0.75 respectively). These results are very encouraging in that they have confirmed the original definition of the IU and are also consistent with the stability of the WHO 1st IS and its suitability for the calibration of the proposed WHO 2nd IS. It is proposed that the WHO 2nd IS Protein S Plasma (03/228) be assigned the mean estimates calculated relative to the WHO 1st IS as follows:

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

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

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

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

Reference Materials and Methods for Antithrombin

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

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

Joint session with the Control of Anticoagulation

Global Coagulation/Haemostatic Tests

Chair: HC Whinna, Dr van den Besselaar

Report on the international collaborative study on fluorogenic methods for Thrombin generation test. *E Gray on behalf of the Working Party on Thrombin Generation Tests*

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

with the experts in the FVIII/FIX subcommittee to define TGT protocols for the testing of haemophilia plasma.

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

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

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

Thrombin generation was studied in 55 patients after a first AMI on admission, after 4 days, 3 and 6 months. On admission, patients showed increased thrombin generation: ETP-ratios were elevated compared to healthy persons (1.238, SD:0.264 vs. controls 1.041, SD:0.155, 95%CI:1.015-1.067) and peak values were (1.479, SD:0.344 vs. controls 1.007, SD:0.177, 95%CI:0.977-1.037). Antithrombotic treatment with low molecular weight heparin (LMWH) dose dependently suppressed ETP and peak height ($r^2=0.772$, $p<0.0001$ and $r^2=0.808$, $p<0.0001$, respectively). ETP and peak height remained elevated as compared to normal persons at 3 and 6 months and showed time variation effects in contrast to normals. In conclusion, preclinical studies confirm the reproducibility and stability in time of CAT-analysis and the method is suitable for detecting hypercoagulability in patients after a first AMI.

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

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

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

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

Methods recording dynamics of fibrin formation. *B. Sørensen/J. Ingerslev*

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

Plasma Coagulation Inhibitors

Chair: E. Gray (UK)

Co-Chairs F. Bernardi (Italy), S. Kitchen (UK), H. Whinna (USA)

WHO international Standards

Proposed international standard for protein C, concentrate. E Gray (UK)

In 2006, a collaborative study was carried out to value assigned a replacement international standard for protein C, plasma and a new international standard for protein C, concentrate. The candidate plasma preparation, 02/342 was subsequently established by the Expert Committee on Biological Standardisation (ECBS) of the World Health Organisation (WHO) in October 2006 as the 2nd International Standard for Protein C, Plasma. It was value assigned against the 1st International Standard (IS) for Protein C, plasma (86/622) and the labelled values are 0.85 and 0.84 IU/ampoule for function and antigen respectively.

For the proposed concentrate standard, 04/252, all participants and the SSC subcommittee approved the proposed assigned antigenic value of 14.3 IU/ampoule against the 1st plasma IS. However, based on their in-house experience, one participant did not agree with assigning the candidate with an overall functional potency ie combining the values obtained for chromogenic and clotting assays. As this has implications for the comparability of the proposed candidate with clinical products, further consideration and study was required to clarify this issue and so the recommendation to establish the 1st IS for Protein C, Concentrate was deferred until more discussion and/or data are available.

NIBSC and the participant who raised this concern have now carried out a joint study involving assays of a number of their production batches by chromogenic and clotting methods and have found that there is a distinct discrepancy between the potencies obtained by chromogenic and clotting assays. Taking into consideration that 02/342 has been established as the 2nd IS, the following new proposals and options were presented to the participants of the study and a panel of SSC experts: the candidate concentrate standard, 04/252 to be assigned with potencies relative to the 2nd International Standard for Protein C, Plasma, 02/342 with the two options. The first option is to label with chromogenic and antigenic value and the second option is to label with functional chromogenic and clotting potency and antigen value.

All participants agreed with the proposed assigned value for antigen. All participants agreed with the proposed assigned value for functional chromogenic activity. Five out of the 20 participants also agreed with labelling with functional clotting potency. All SSC experts agreed with the assigned values for antigen and functional chromogenic activity only. Therefore it will be recommended to the ECBS of the WHO to establish 04/252 with an antigenic and a functional chromogenic value of 14.5 and 15.0 IU/ampoule respectively.

Replacement of the 2nd international standard for antithrombin, concentrate. E Gray (UK)

Twenty-one laboratories participated in a collaborative study to establish a replacement for the 2nd International Standard for Antithrombin, Concentrate (96/520). There was excellent agreement between laboratories, as indicated by low inter-laboratory % GCV for the 3 candidate materials which consisted of one recombinant and two plasma derived clinical concentrates. In terms of performance, stability profile and physical characteristics of the candidates, all 3 materials are very similar. However, there is a larger number of ampoules of sample C, 06/166 available. It is therefore proposed that sample C, 06/166 is considered as the 3rd International Standard for Antithrombin, Concentrate, with labelled potencies for both functional (4.4 IU/ampoule) and antigenic (4.5 IU/ampoule) activities. All participants have agreed with this proposal. All participants and SSC experts agreed with this proposal. Therefore it will be recommended to the ECBS of the WHO that 06/166 to be established as the 3rd iS for Antithrombin, Concentrate, Human.

Protein S and protein C

Protein C and protein S assay discrepancies experience from UK NEQAS for Blood Coagulation. I Jennings (UK)

Accurate diagnosis of PC and PS deficiency depends on precise and reliable laboratory methods. UK NEQAS for Blood Coagulation exercises have identified discrepancies in results for PC and PS assays between users of different sources of commercial kit. For PC assays, marked differences (>10%) have been observed over the last 2 years between the two kits most widely used by participants in the programme, both employed by >60 laboratories.

In-house investigations used the SSC secondary plasma standard lot #3 as a calibrator for the two kits, and results for both lyophilised and frozen plasma demonstrated good agreement when this calibrator was used. Data indicated that the potency for one commercial reference plasma was incorrectly assigned by approximately 6%, which contributed to the discrepancy in the NEQAS exercises. A further UK NEQAS exercise in which a patient sample and the SSC secondary plasma standard were distributed demonstrated improved agreement in the patient sample results when results for both methods were cross-calibrated against the SSC plasma standard.

Discrepant results continue to be observed between PS kits, and the importance of locally-determined reference ranges is demonstrated by a >10% error rate in diagnosis of PS deficiency if a local range is not utilised.

Protein C and protein S assay discrepancies - North America experience. R Marlar (USA)

Often there is a lack of correlation between thrombophilia diagnostic test result and correct clinical phenotype. This could be due to numerous interfering substances in the tests or other conditions that may affect the test and interpretations of results. For example, genetic deficiency of PC does not correlate with clinical phenotype (venous thrombosis). Plasma level of protein C or genetic mutation do not always predict clinical phenotype. Age was not found to be a factor for thrombosis and major life events do not affect phenotype. Therefore other non-protein C factors must influence the development of thrombosis. Interfering substances or conditions can cause inaccurate test results. For example, heparin therapy can cause decrease of factors utilizing

or influenced by heparin and warfarin causes decrease of vitamin K-dependent protein levels (and increased Antithrombin levels). The interpretation of protein C and protein S test results should take into account that 1. expression of clinical phenotype is not consistent with genotype, 2. thrombophilia phenotype is a multi-mechanism disorder based on multiple genes & acquired RF, 3. inconsistencies of test results can be due to patient, pre-analytical variables, interfering substances and/or assay problems and 4. cost effective protocol of diagnostic tests should be ordered based on prevalence and understanding.

Global coagulation/haemostatic tests

Progress on the activities of the working party on thrombin generation tests.

E Gray (UK) on behalf of the Working Party on Thrombin Generation Tests

The Working Party (WP) on Thrombin Generation Tests (TGT) was set up in 2004 under the auspice of the Plasma Coagulation Inhibitors subcommittee. The main remit is to investigate, standardise and validate methodologies for the quantitation of results to facilitate good within and between laboratory agreement. In 2004 a survey on current TGT methods was carried out and the WP published a mini review (on line publication www.bloodmed.com The Thrombin Generation Test (TGT) by Lawrie et al.). In 2005 the survey results and a pilot study results within WP on chromogenic non-sub-sampling methods were presented at the SSC. In 2006 a study on “Fluorogenic Methods for Thrombin Generation Tests” was initiated and completed. It is the intention of the WP to publish the data on the findings of the fluorogenic method study in 2007 and also to initiate and complete a study on the feasibility of establishing a reference plasma for thrombin generation tests. The WP also has plans to investigate the application of thrombin generation tests in the study of haemophilic plasma.

Report on the international collaborative study on thrombin generation tests.

E Gray (UK) on behalf of the Working Party on Thrombin Generation Tests

The main aim of this study is to investigate the feasibility of establishing a reference plasma for thrombin generation tests. One hundred and ten labs returned results and in total there was 128 sets of data available for analysis. Six coded freeze-dried samples including 3 candidate normal pooled platelet poor plasmas, the SSC Lot#3 (included for comparative purpose only) and 2 abnormal plasmas were provided. Four commercial methods (CAT, Technothrombin, Dade-Behring-ETP and In-TDT) were used by the participants and results were also obtained for 4 in-house methods. This study confirmed results from the previous study that a reference plasma would improve intra- and inter-laboratory variability. The Working Party therefore recommends the establishment of a reference plasma for Thrombin Generation Tests. Further discussion will be required to determine how the reference plasma should be used. The Working Party will also make a proposal to the SSC to establish the reference plasma as a SSC reference material.

Inter-laboratory evaluation of the TGA Assay. P Meijer (NL)

The first survey on the Technothrombin TGA concluded that the inter-laboratory variation observed depends upon the read-out variable and the level of thrombin generation. There is a difference in quantification of thrombin generation between laboratories and that this difference

is affected by the type of instrument used. This second survey aimed to investigate if there is improvement in the inter-lab variation, are there still quantification differences, does the effect of instrument still exist and could harmonization improve result comparability. Forty-one participants were involved and a set of 8 plasmas including normal and abnormal samples were sent to each lab. The inter-laboratory variability for the measurement of thrombin generation in a normal pooled plasma is comparable in both surveys. The quantification differences between laboratories still exist and that these differences are more systematically higher the higher the thrombin generation. There are small but not statistically significant differences between fluorimeters. There are obvious differences in the inter-laboratory variability between fluorimeters. Harmonisation is only possible if there is a good correlation between two samples. Samples with low to very low thrombin generation did not show a good correlation with a normal sample. Harmonisation of absolute thrombin generation did not improve the inter-laboratory variation. Harmonisation by expressing readouts relative to a reference plasma shows a small improvement of the inter-laboratory variation.

Report on the ECAT Workshop on Thrombin Generation Tests. K Kluff (NL)

Four manufacturers of Thrombin Generation Tests (TGT) Kits took part in a wet workshop to demonstrate their details of their techniques on 9 plasma samples provided by ECAT. The 4 kits were CAT (Thrombinoscope), Technothrombin, TGA (Technoclone), Dade-Behring-ETP (Dade Behring) and In-TDT (Pentapharm). Two variants of two of the kits were also included, so in total there was 6 different methods.

The samples included plain pooled plasma, pooled plasma spiked with hemolysed cell material, alpha-2-macroglobulin (alpha-2-M), argatroban, unfractionated heparin, an ultracentrifuged pooled plasma with low level of microparticle, protein S congenital deficient plasma, a factor VIII congenital deficient patient and a plasma with lupus antibodies.

The absolute data could only be compared when expressed relative to the pooled plasma. It was observed that the 9 plasmas showed quite different TGT profiles and read-out. Method specific effects were also noted. Some of these effects on a single sample were further investigated by ECAT, others will also be follow-up to fully define the differences between the methods which are clearly present. It was identified that ultracentrifugation of a plasma highly reduced thrombin generation in some methods. This was not corrected by exogenous lipids, but only by reconstitution with microparticles (MPs). MPs are a major determinant of some methods. It was observed that increase in alpha-2-M had a strong effect on some read-outs of some methods and further investigation is carried out on this observation. It was observed that argatroban (a direct thrombin inhibitor) reduced the read-out on thrombin activity, but did not properly identified what happened with prothrombin conversion when compared with F1+2 generation. It is recommended that each method should be considered as different. This will provide opportunities for specific applications, with refinement by selecting specific read-outs.

Thrombin generation induced by cancer cells. G Gerotziafas (FR) on behalf of GT Gerotziafas, C Prengel, E Verdy, I Elalamy, J-F Bernaudin

Several lines of evidence show that thrombotic risk is different in patients suffering from different histological types of cancer. Experimental studies have shown that cells from some histological types of cancer express tissue factor (TF) which is implicated to their metastatic and angiogenic potential. However, the influence of cancer cells on blood coagulation has not been adequately studied. The procoagulant potential of pancreatic and breast cancer cells (BXPC3 and MCF7 cell lines respectively) when they are in contact with human platelet-poor plasma (PPP) were evaluated. In addition the procoagulant activity of cancer cells using a specific anti-TF antibody was titrated. The contact of cancer cells with recalcified PPP resulted in acceleration of TG as compared to the control. This effect was manifested by a significant decrease of the lag-time, and time to Peak of thrombin (ttPeak) and by significant increase of the mean rate index (MRI) of the propagation phase of TG as compared to the control experiment. Cancer cells induced a slight increase of thrombin's Peak but they did not significantly influence the endogenous thrombin potential (ETP). Both cell lines when issued from cultures with 40% confluence showed higher procoagulant activity as compared to that manifested by cells from cultures with 90% confluence. BXPC3 had significantly more potent procoagulant activity compared to MCF7 cells. BXPC3 manifested maximum procoagulant activity at the concentration of 2 cells/ml whereas MCF7 manifested maximum effect at the concentration of 200 cells/ml. The incubation of cancer cells with an anti-TF antibody resulted in a concentration dependent inhibition of their procoagulant effect mainly on the lag-time of TG. Significantly higher concentration of the anti-TF antibody was required for 50% inhibition of the effect of BXPC3 on thrombin generation as compared to that required for 50% inhibition of MCF7 procoagulant activity. In conclusion, pancreatic cancer cells (BXPC3) and breast cancer cells (MCF7) accelerate thrombin generation of human plasma in a TF dependent manner. BXPC3 have a significantly more potent procoagulant activity than MCF7 probably due to increased TF expression. The number of cells suspended in plasma and their proliferative status according to the level of confluence, are important determinants for the procoagulant potential of the studied cancer cell lines. Chronometric parameters of thrombogram (lag-time and ttPeak) and the mean rate index of the propagation phase of TG seem to be more sensitive than ETP and thrombin's peak to detect the TF dependent procoagulant potential of cancer cells. A specific anti-TF antibody might serve as calibrator for the evaluation of the TF-dependent procoagulant potential of cancer cells from different histological types of tumors.

Behaviour of different anticoagulants in thrombin generation tests. M Samama (FR)

Three different patterns of thrombograms measured by the CAT were reported. Type 1 was typical of irreversible thrombin inhibitor, hirudin. Hirudin prolonged lag time, but had no effect on peak and ETP. There was an artifact at low dose which was due to mathematical inadequacy in the software to resolve the thrombin generation curve produced by hirudin. Type 2 was typical of reversible thrombin inhibitors such as dabigatran, argatroban, melagatran. These inhibitors all prolonged lag time, decreased velocity and lower peak thrombin and ETP. Type 3 was typical of danaparoid and fondaparinux. These inhibitors had minor influence on lag time, but dramatic decrease in velocity and lowering of peak thrombin. Dermatan sulphate had almost no effect on lag time but decreased peak thrombin and ETP.

With regards to low molecular weight heparins, the importance of antithrombin activity on the thrombograms has been presented. Full detail on the effects of low molecular weight heparins on thrombogram has been published by Gerotziafas et al (JTH, 2007; 5, 955-962)

Thrombin generation in patients with arterial and venous thrombosis.

H Spronk (NL) on behalf of HMH Spronk, AWJH Dielis, AJ ten Cate – Hoek, M Marchetti, R van Oerle, MH Prins, A Falanga, K Hamulyák and H. ten Cate

Thrombin generation (TG) has been shown useful to detect a hypercoagulable state in individuals at risk of venous thrombosis. In the current study we investigated the applicability of TG by means of the Calibrated Automated Thrombogram (CAT) under conditions of hypercoagulability in plasmas from patients suffering from one of the following conditions: acute myocardial infarction (AMI), deep vein thrombosis (DVT), or a chronic myeloproliferative disorder (MPD, such as essential thrombocytosis (ET)).

For the AMI group, mean age was 62 years, 74% was male (n=100). TG was increased and shortened at 0d (lag time (LT) 0.86, ETP 1.07, peak height (PH) 1.19), but decreased and prolonged at 4d (LT 1.43*, ETP 0.99, PH 0.80*) when all patients received heparin/LMWH. Patients with heparin levels >0.05 U/mL at 4d had prolonged LT (1.43), and decreased ETP (0.81) and PH (0.64) compared to patients with levels below 0.05 U/mL (LT 1.04*, ETP 1.26*, PH 1.51*). Heparin concentration correlated with LT, ETP and PH (R=0.37*, -0.82* and -0.87*). Between 4d-3m, TG changed to levels comparable with 0d (no differences between 3m and 6m).

The DVT cohort consisted of 72 males (46%) and the total group had a mean age of 56.1 years (17.5-82.6), with no difference between males and females. TG slightly increased with age. At all visits lag time, ETP and peak height at 1 pM TF were significantly increased in patients compared to healthy subjects. In patients, TG measured at 1 pM TF changed between V1 and V3 with a shortened lag time (-26.1%), increased ETP (+11.2%) and peak height (+13.7%). TG measured at 5 pM showed the same changes (lag time -20.4%, ETP +9.2%, peak height +13.7%). Healthy subjects showed no changes over time. Addition of TM to the 1 pM TF assay reduced ETP in all patients at all visits (between -8.8% and -19.50%) - but considerably less than in normal pool plasma (-48%).

ET-patients had shorter LT (2.10 ± 0.48 vs 2.39 ± 0.33 min*) and time to peak (4.08 ± 0.73 vs 4.50 ± 0.43 min*) compared to controls for TG with 5 pM TF, while no differences were observed in ETP and PH. Similarly, at 1 pM TF, patients showed shorter LT (3.69 ± 0.88 vs 4.56 ± 0.68 min*) and time to peak (6.5 ± 1.4 vs 8.2 ± 1.3 min*). In addition, PH (279 ± 55 vs 244 ± 62 nM*) and slope (106 ± 36 vs 75 ± 35 nM/min*) were increased in MPD. Among MPD, ET patients had higher ETP (1521 ± 219 vs 1290 ± 369 nM.min*), PH (290 ± 49 vs 245 ± 63 nM*), and steeper slope (112 ± 37 vs 87 ± 32 nM/min*) compared to PV patients.

In conclusions, as compared to healthy individuals, the TG shows alterations in time that may indicate systemic hypercoagulability in patients after AMI, DVT, or with ET. The sensitivity of the assay differs per patient population, such that test conditions may have to be adjusted per indication (eg. for DVT, TG assessed with 1 pM TF is more sensitive than 5 pM TF). Finally,

TG is very sensitive to the effects of anticoagulant treatment. Prospective follow up of these patient cohorts should establish the predictive values of TG for recurrent arterial and venous thrombosis.

Plasma Coagulation Inhibitors

4 July 2008
Vienna, Austria

Chair: *Elaine Gray, UK*

Co-Chairs: *Francesco Bernardi, Italy; Herbert C. Whinna, USA; Steve Kitchen, UK; Richard Marlar, USA; Piet Meijer, The Netherlands*

WHO Standards

Replacement of the 2nd International Standard for Antithrombin, Plasma – E Gray

The stock of the current 2nd International Standard for Antithrombin, Plasma is running low and needs to be replaced by the end of 2010. One candidate material will be produced from a pool of at least 30 donations of normal platelet poor plasma from blood transfusion service. As the SSC secondary standard Lot #3 also require a replacement, it is envisaged that the collaborative study will also include the calibration of SSC Lot#4. The collaborative study will be initiated in September 2009 with an ECBS submission date of October 2010.

Report on WHO/ECBS recommendations on the 1st International Standard for Protein C Concentrate – E Gray

In 2006, the WHO/ECBS establishment the 2nd International Standard for Protein C, Plasma. In the same calibration exercise, the SSC secondary standard Lot#3 and a proposed protein C concentrate standard were also assessed. Protein C values were assigned to the SSC secondary standard. For the proposed concentrate standard, all participants agreed with the antigen value, but there was a query over the proposal of assigning a combined chromogenic and clotting functional potency. After further study, the participants and the experts from the Plasma Coagulation Inhibitors subcommittee agreed that the proposed protein C concentrate standard should be value assigned against the 2nd International standard for Protein C Plasma and that the functional potency should be derived from chromogenic assay results only. The ECBS agreed with this proposal and established the 1st International Standard for Protein C, Concentrate in October 2007.

Protein S and Antithrombin

Protein S assays: method-related discrepancies – I Jennings

UK NEQAS thrombophilia screening exercises have revealed marked differences in median results between the three most widely used Protein S activity methods. Results with method X were over a series of exercises an average 17% lower than those with method Z, and 30% lower than method Y. An earlier investigation of protein C activity assay discrepancies had revealed a discrepancy caused by the calibration of a commercial reference plasma. However, with protein S assays, calibration of method X reference plasma did not indicate any calibration issue. Assay of frozen plasma and comparison with free protein S levels showed a greater proportion of results measured lower with method X activity assay than free PS, but the discrepancy was not of the scale seen in the EQA exercises. Lyophilisation of plasma for quality control purposes can introduce artefact with some haemostasis methods, but in house study showed no effect of lyophilisation on the EQA samples employed here. The discrepancy in results therefore remains unexplained at present. Discrepancies in results with different methods will have less clinical impact if locally determined, method specific reference ranges are employed. However, questionnaires from UK NEQAS participants have revealed that 31% employ a locally determined reference range, and 69% of centres using methods x y and z employ literature or manufacturer-sourced reference ranges; approximately 50% of centres report identical lower limits of reference ranges regardless of method employed. Clinical interpretations for EQA samples and potentially clinical samples therefore differ with these different protein S activity kits.

Use of different Antithrombin assays to characterise subjects with antithrombin deficiency – P Cooper

Antithrombin can be measured by functional and antigenic assays, however, antigenic assays may fail to detect subjects with type II antithrombin deficiency and are therefore inappropriate for screening for antithrombin deficiency. The most common functional antithrombin assay is the chromogenic assay, where antithrombin is activated by heparin and then inhibits either thrombin or factor Xa, and residual enzyme is detected when it cleaves a chromogenic substrate. Chromogenic antithrombin assays vary in that either thrombin or factor Xa may be inhibited; heparin and buffer composition also vary as do chromogenic substrate, incubation time and detector settings. Since 1985, it has been known that human thrombin may be significantly inhibited by heparin cofactor II in antithrombin assays with incubation time greater than 30 seconds, resulting in reduced specificity. Assays using bovine thrombin are much less influenced by heparin cofactor II, and factor Xa-based assays are unaffected. Antithrombin level measured with bovine thrombin-based assay is often reduced with antithrombin Cambridge II (Ala384Ser) whereas the level is normal with factor Xa-based assay; this functional deficiency is the most common cause of heritable antithrombin deficiency in the United Kingdom. Sensitivity to type II defects can be increased by measuring antigenic antithrombin level on subjects with borderline antithrombin activity and then comparing this to the activity level, by ratio. Antithrombin Denver (Ser394Leu) is a rarer defect which may be detected by bovine thrombin-based assay, but remains undetected by factor Xa-based assay. Antithrombin Stockholm (Gly392Asp) has also been reported to be detected by thrombin-based assays but not by all factor Xa-based assays. Heparin binding site defects of antithrombin are best detected with short (30 second) incubation of sample with thrombin; short incubation time may also improve detection of HBS defects with factor Xa-based assay, but different manufacturer's kits have different sensitivities to different HBS defects. In addition to short incubation times increasing sensitivity to HBS defects, short incubation time of sample with bovine thrombin increases sensitivity to a type IIRS defect, antithrombin Sheffield/Glasgow (Arg393His). We conclude that antithrombin assays vary in sensitivity to antithrombin deficiency and one type of assay cannot be guaranteed to detect all type II antithrombin defects. Reducing incubation time of enzyme with test dilution can sometimes increase an assay's sensitivity to type II defects; therefore a short (30 second) incubation time is recommended. Phenotype and genetic studies are necessary to define type II antithrombin deficiency.

Global Coagulation Haemostatic Tests

Progress on the activities of the Working Party on Thrombin Generation Tests – E Gray

The Working Party agreed that a reference plasma should be established as NIBSC research reagent and made available as soon as possible. The Party also planned to initiate a new study on FVIII inhibitors plasmas and heparinised plasma in the coming year.

Thrombin Generation: Experience of external quality control surveys – P Meijer

The results from the first external quality assessment surveys for thrombin generation was presented. Three different kits with 5 different methods were used by the participants. Different results were obtained for the read-out parameters evaluated and this was due to the differences in test formats. The inter-laboratory variability was found to be greater than 10%. The different methods had varying sensitivity to different levels of haemostasis factors. The different methods were also found to have different sensitivity to microparticles.

Monitoring Thrombin Generation: Do We Need Addition of Corn Trypsin Inhibitor? - H Spronk, A Dielis, R Oerle, J Govers-Riemslog, K Hamulyák, H Ten Cate

Background: Thrombin generation in calibrated automated thrombography (CAT) could in the future potentially be used as a routine method for measurement of coagulability in clinical diagnostics. Although contact activation does not seem to play a role under physiological conditions in vivo, it has been argued in literature that it might influence in vitro measurements of thrombin generation thereby acting as an unpredictable pre-analytical variable which produces inaccuracy and imprecision. The aim of the current study was to investigate the influence of contact activation on thrombin generation and the exigency of corn trypsin inhibitor (CTI) to abolish contact activation in thrombin generation measurement at low tissue factor (TF) concentrations. Methods: Thrombin generation was performed by using CAT, thereby determining the endogenous thrombin potential (ETP) and the lag time. The presence of activated factor XII (FXIIa) and activated factor XI (FXIa) was measured using ELISAs. Results: Addition of CTI after plasma preparation had no significant influence on thrombin generation triggered with 0.25 pM TF or higher, as demonstrated by unaltered ETP and lag time

values between analysis with and without CTI. Addition of CTI before blood collection reduced the peak height in thrombin generation triggered with 0.5 or 1 pM TF. The ETP and lag time, however, remained unaltered. In contrast, a significant decrease in both ETP and peak height was observed only when the reaction was triggered with 0.5pM TF. Conclusion: This study demonstrated that addition of CTI after plasma separation is not necessary when triggering with TF concentrations of 0.25 pM and higher. Furthermore, it was demonstrated that it is not needed to pre-fill blood collecting tubes with CTI when measuring with TF concentrations of 1pM and higher.

A Protac®-modified Thrombin Generation Assay to identify Individuals at Higher Thrombotic Risk – A Gatt

There is a need for good laboratory prothrombotic markers to identify individuals at high risk of venous thromboembolism. Thrombin generation estimation is an attractive test, however, it is not sensitive enough to changes in the protein C pathway and may lack sensitivity to prothrombotic states. The addition of Protac, a snake venom extract that activates protein C can improve the sensitivity of thrombin generation test. Protac® has a dose dependent inhibitory effect on the endogenous thrombin potential (ETP). Blood from a group of patients with inherited thrombophilia, their first degree family members with no detectable defect and control subjects were tested. ETP and %ETP correlates inversely with protein C, protein S, Activated Protein C Resistance (APCR) and age, whereas there is a positive correlation between the ETP and FVIII, fibrinogen and age and inverse relationship with antithrombin. The Protac-TG assay is sensitive to all the constituents of the PC pathway. The results show why a positive family history of VTE is a risk factor for thrombosis despite negative standard thrombophilia tests. It may also help discriminate between individuals with FVL who have a higher prothrombotic risk than others with the same defect.

Reversible thrombin inhibition in the thrombin generation test - C. Kluft, P. Meijer, R. Kretz, R. Laterveer, J. Burggraaf

The direct thrombin inhibitor, hirudin, induces a prolongation of the lag phase in tissue factor driven thrombin generation tests and shows minimal effect on peak thrombin in the therapeutic range. The lag phase effect is consistently observed also for synthetic reversible direct thrombin inhibitors (rDTIs: bivalirudin, argatroban, melagatran, dabigatran). This is in contrast to effects of rDTIs on peak thrombin which are different among test variants and subject to direct effects on the read-out variable, thrombin activity, by the rDTIs and redistribution of thrombin over inhibitors.

An increase in F 1+2 as a marker of prothrombin conversion happens in the therapeutic range of the rDTIs and presents an additional read-out that requires further study. The lag phase is the presently advised robust effect variable. Thrombin inhibitors are used at high concentrations relative to the inhibition constant for thrombin and suspected to exert actions on other coagulation enzymes as well.

We devised two continuous chromogenic Factor Xa generation assays, starting with a PT reagents (Tissue factor) or an APTT reagents (contact activation). In these assays thrombin participation was excluded by addition of an excess hirudin.

Results in the PT-variant revealed a moderate inhibition of factor Xa by melagatran and dabigatran and a more pronounced one by argatroban. Factor Xa generation was inhibited only at higher concentrations of all rDTIs. The concentrations for significant inhibition in the factor Xa generation test and 2 fold prolongation of the standard PT test were similar (2,5 – 5 µM) suggesting that this effect drives the PT inhibition. Results in the APTT-variant showed inhibition of factor Xa generation and of contact factors at lower concentrations (0,5 – 1,5 µM) than the PT variant. This was in agreement with the lower levels to achieve 2 fold prolongation in the standard APTT test relative to the PT. It is suggested that the contact activation inhibition of the rDTIs drives the APTT effects. Results with contact activated thrombin generation tests are pending, but this variant is expected to reveal best the main other effects of rDTIs rather than the tissue factor driven variant. It is concluded that rDTIs, by virtue of the use of high dosages exert multiple effects in coagulation of which the effect on the contact system happens already within the therapeutic range.

Monitoring rDTIs can focus on different aspects (antithrombin = lag phase TGT and ecarin clotting time), factor Xa effects (PT) and contact activation effects (APTT).

Submitted by E. Gray