

## **Working Group on Vascular Biology**

**July 13th, 2003**

**08:00 to 12:00**

**Hall 10**

**The International Convention Center, Birmingham**

Chair: Peter J. Newman (USA)

Co-chairs: Michael C. Berndt (Australia), John Griffin (USA),

Irène Juhan-Vague (France), Klaus T. Preissner (Germany)

The SSC Workshop in Vascular Biology met for the first time on Sunday, July 13th, 2003, in Birmingham, England. Though there were a rich variety of topics relevant to the field of vascular biology that might have been addressed, and may be addressed at future workshops, the Organizing Committee chose two topics that they felt would be timely and relevant to the mission of the SSC and of the ISTH: (1) Detection, measurement, function, and clinical significance of membrane microparticles derived from blood and vascular cells, and (2) Real-time measurements of blood cell interactions with the vessel wall – applications of intravital microscopy and other flow systems. Six internationally-recognized experts, three in each of these two fields, were invited to speak. The audience was estimated at 200-300, with standing room only at some points during the morning.

### **SESSION I – Detection, measurement, function, and clinical significance of membrane microparticles derived from blood and vascular cells**

**Alan D. Michelson** (Center for Platelet Function Studies, University of Massachusetts Medical School, USA) spoke on "*Detection, standardization, and clinical correlates of platelet-derived microparticles*". Dr. Michelson provided a brief historical overview of the field of platelet-derived microparticles, and went on to describe how they are formed, the cells from which they are derived, and some of the functions that have been attributed to them. Their clinical relevance, as well as human disorders associated with the generation of circulating microparticles was also discussed.

**Jean Marie Freyssinet** (Institut d'Hématologie et d'Immunologie Faculté de Médecine - Université Louis Pasteur, Strasbourg, France) spoke on "*Detection of cellular membrane microparticles in the vascular compartment*". Dr. Freyssinet brought forward a variety of timely issues that could be discussed in greater detail in future meetings, including how microparticles are quantitated, appropriate markers that could be used to identify their cellular sources, and the development of standards by which microparticles should be prepared and defined. Clinical studies and new applications were also discussed.

**Bernd Engelmann** (Institute for Clinical Chemistry - Ludwigs-Maximilians University, Munich, Germany) spoke on "*The role of microparticles in the initiation of blood coagulation*". Dr. Engelmann focused on tissue-factor-containing microparticles and their potential to promote blood clotting – a topic that received much discussion both here and during the ISTH meeting proper. A number of unresolved issues were addressed and discussed, including identification of the source of tissue factor, and its biologic activity in microparticles.

This session ended with a brief panel discussion of topics that could be discussed in future vascular biology workshop meetings. There was good consensus that the issue of blood cell-derived microparticles had much more to offer in future meetings of the SSC.

## **SESSION II – Real-time measurements of blood cell interactions with the vessel wall – applications of intravital microscopy and other flow systems**

**Barry Coller** (Rockefeller University, New York, USA, with co-authors Marketa Jirouskova, Heikki Vaananen, and Jay Degen) spoke on *"Real-Time Imaging of Carotid Artery Thrombosis in the Mouse: Studies on the Role of Fibrinogen and the Fibrinogen Gamma Chain"*. Dr. Coller brought forward a number of important issues related to the standardization of models currently in use to examine thrombosis and hemostasis in real time, including the types of anesthetics used, how blood vessels should be damaged, the positive and negative attributes of using different vascular beds for analysis, and how to best capture and quantify the images obtained.

**Steffen Massberg** (German Heart Center - Technical University, Munich, Germany) spoke on *"Platelet-vessel wall interactions in vivo -implications for thrombosis and atherosclerosis"*. There is a growing awareness of potential relationships between inflammation, thrombosis, and the development of atherosclerotic lesions, and Dr. Massberg has been at the forefront of this field. He provided a stimulating discussion of the role of platelet interactions with the vessel wall in athero-progression and in arterial thrombosis. The role of leukocytes with and without bound platelets, and the role of platelet monolayers in leukocyte recruitment was also discussed.

**Jerry Ware** (Scripps Research Institute, USA) spoke on *"Relative contributions of GPVI and the GPIIb complex to platelet adhesion, activation, and thrombus formation under flow - different models/different insights"*. Dr. Ware described the strengths and weaknesses of the most commonly-used methods for determining platelet function in vitro and in vivo, including the use of tail bleeding times, standard aggregometry, flow chamber studies, and the ferric chloride model of carotid artery denudation. The impact of variability in the reagents (e.g. types of collagens) used, the impact of shear, and the size of mouse versus human platelets was discussed. Recently developed GPVI-null mice were used as an example of how subjecting animals/platelets to different models can lead to different insights into vascular physiology and blood cell function.

The session ended with much discussion from the audience, an offer by several members of the audience to participate more actively in the development of next year's program, and a suggestion that the organizing committee entertain the notion of including additional topics in vascular biology (i.e. angiogenesis) in future workshops.

**Working Group on Vascular Biology**

**June 18, 2004**  
**14:15 to 18:15**  
**Barbantini Room**  
**Fondazione Giorgio Cini**

Chairman: P. J. Newman, USA

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

**Detection and characterization of (circulating) microparticles**

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

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

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

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

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

**Dr. Françoise Dignat-George** summarized the replies to the questionnaire, emphasizing heterogeneity of the approaches (flow cytometry, solid-phase capture assay enabling assessment

of the associated procoagulant potential, functional assays), at either pre-analytical (delay, whole blood, platelet-free plasma, anticoagulant, centrifugation conditions) or analytical stages (numeration, procoagulant activities, immunological and functional determinations). It clearly appeared that these phases need to be standardized in order to make sure investigators are considering the same membrane fragments as MPs.

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

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

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

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

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

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

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

At the end, a general consensus was reached that MPs are true pathogenic markers of prime interest and of course worth standardizing. The pre-analytical and analytical phases were discussed in depth, with particular emphasis on the definition of shed MPs as submicron fragments different from other membraneous cell-derived entities, e.g. exosomes, released organelles, or larger apoptotic bodies. Instrumentation (flow cytometer capacities and limitations) and reagents (antibody specificity and enzyme and cofactor quality for functional assays) were also considered as primary sources of inter-laboratory variability.

Information gained during this lively session was indeed useful to define new and important issues to be integrated in an extended version of the first questionnaire, which will be prepared by Françoise Dignat-George and Jean-Marie Freyssinet and circulated via the ISTH or accessible from the website. Such a detailed survey should prove of prime interest to enter the second phase of standardization with specific tasks to be discussed in Sydney, in order the WG-VB can fulfill its objective.

*Respectfully submitted by Jean-Marie Freyssinet*

## Working Group on Vascular Biology

Saturday, 6 August 2005

11:00 to 14:30

Ballroom 1

Sydney Convention and Exhibition Centre

Chairman : Peter J. Newman, ( USA )

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

The program was divided into two parts: (I) Detection and characterization of circulating endothelial cells and their progenitors, and (II) Determination and characterization of (circulating) microparticles.

Approximately 160 people attended the session.

**Session I :** Detection and characterization of circulating endothelial cells and their progenitors (Chairs: K.T. Preissner & P.J. Newman)

**Andrew Blann** ( UK): “*Circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs): Two sides of the same coin or two different coins*”? That endothelial cells detectable in blood are not a homogeneous population, but rather represent more than one species of endothelial cells was discussed. CECs are thought to arise from the vessel wall, whereas EPCs are mobilized from the bone marrow. Thus, although originally defined according to different criteria, there are also some common characteristics. The lack of consensus regarding definition and methodologies remains an important area of future work for this Working Group. The use of CD146-coated magnetic beads to identify and purify CECs was discussed, as well as the extent to which the presence of CECs reflects endothelial cell damage. The origin of CD34+ EPCs remains controversial as they are identifiable in peripheral blood and capable of forming *in vitro* colonies. Improving the array of surface markers allowing to discriminate CECs from EPCs remains an important problem for further study.

**Alexander Woywodt** ( Germany ): “*Detection of circulating endothelial cells by immunomagnetic separation (IMS) assays.*” That circulating endothelial cells are a novel marker of microvascular damage was reinforced. CD146 driven-immunomagnetic isolation appears to be the technique of choice to isolate and enumerate these cells. However, several variables influence isolation of CECs by IMS (both at the pre-analytical and analytical levels). In this respect, a standardized methodology represents an important step towards consensus regarding CECs. Moreover, given the variable phenotype of these rare cells in peripheral blood, this technique still has several pitfalls, and precautions taken to avoid them were discussed. The second part of the talk focused on the clinical utility of detecting circulating endothelial cells as a marker of ANCA-associated small-vessel vasculitis.

**Françoise Dignat-George** ( France ): “*Detection of circulating endothelial cells in the vascular compartment*”. A historical perspective of the development of CD146 mAbs as selective markers

for detecting CEC was provided, followed by a description of clinical disorders that have been associated with increased circulating CECs, including infection, malignancies, transplantation, and immune disorders like TTP. A working definition of CECs was proposed, and the clinical utility of CECs as biomarkers of endothelial damage was discussed. It was concluded that a consensual definition of the most appropriate technique is a key issue to be addressed in order to validate CECs in large cohorts of patients. The future potential for proteomic analysis to provide selective markers allowing to discriminate CECs (from damaged vessels) versus EPCs (bone marrow) was also discussed.

## **Session II : Determination and characterization of (circulating) microparticles (Chairs: J Griffin & J-M Freyssinet)**

**Françoise Dignat-George and Jean-Marie Freyssinet** (France): “*Questionnaire on microparticle detection and characterization : a retrospective analysis*”. Since MP are increasingly being viewed as markers for various pathophysiological processes, and may in addition have therapeutic applications, Drs. Dignat-George and Freyssinet have undertaken the large, important, and challenging task of surveying how investigators in this growing field prepare and characterize MP. The results of their ISTH-sponsored questionnaire were presented, and the results divided into pre-analytical and analytical procedures. Relative consensus was reached that blood should be anticoagulated with citrate, and that minimal manipulation should be involved to avoid cellular activation and inadvertent production of MP. Outstanding issues remaining on the pre-analytical side include whether whole blood or plasma is used to prepare MP, and if/how MP should be stored prior to analysis. On the analytical side, standardization of methods of quantitation, surface markers, and functional properties of MP remains an important goal. Current technologies used in this regard include flow cytometry, ELISA, determination of pro-coagulant and anti-coagulant activities, and the beginning of adoption of proteomic technologies.

**Rienk Nieuwland** ( Amsterdam , The Netherlands): “*Detection and characterization of microparticles by flow cytometry*”. Circulating cell-derived microparticles most often have procoagulant properties due to exposure of negatively charged phospholipids. Most studies on microparticles, however, have been performed on microparticles after *in vitro* manipulations, e.g. pelleting/resuspending or freezing/thawing, thereby possibly influencing microparticle structure. This group investigated whether non-manipulated microparticles expose phosphatidylserine (PS), whether this exposure is affected by *in vitro* manipulations, and if so, whether this changes their procoagulant properties. Surprisingly, in their hands only very few non-manipulated microparticles from venous blood of healthy individuals, or from pericardial blood of patients undergoing cardiac surgery exposed PS, as evaluated by annexin V binding. Upon pelleting/resuspending, freezing/thawing, or both, however, the fraction of PS-exposing microparticles increased, which was accompanied by fragmentation, change in the size of MPs, and and/or loss of particular microparticle populations. Interestingly, the extent of PS exposure did not affect the procoagulant activity or the mechanism of coagulation activation, allowing them to conclude that even low exposure of PS is sufficient to support coagulation. They cautioned that microparticles in fresh samples should not be quantified based on PS exposure. In the discussion, however, it was emphasized that the conditions for using annexin V have to be better defined with respect to the biochemistry of this PS probe.

**Johan W. M. Heemskerk** ( Maastricht , The Netherlands): “*Phosphatidylserine-dependent procoagulant potential of microparticles*”. Tissue factor-induced thrombin generation with PRP and with platelet-derived MP similarly relies on phosphatidylserine exposure. Thrombin generation in PRP is enhanced by integrin  $\alpha\text{IIb}\beta\text{3}$ -mediated shedding of MP. Platelets shed phosphatidylserine-exposing MP in the absence of activation (coagulation). This shedding is thought to be (1) secondary to F actin degradation, (2) mediated by integrins, and (3) negatively regulated by PKA (cAMP). It was concluded that integrin  $\alpha\text{IIb}\beta\text{3}$  signaling accomplishes destabilization of the membrane cytoskeleton, negatively controlled by PKA, and resulting in MP shedding from the plasma membrane.

**Yasushi Ozeki** ( Japan ): Described a new ELISA method for detecting platelet-derived microparticles that utilized an anti-GPIX mAb for capture, and an anti-GPIb mAb to detect. This commercially-available assay might be detecting very small MP that flow cytometric analysis misses.

**Nigel S. Key** ( Minneapolis , USA ): “*Tissue factor-dependent procoagulant potential of microparticles*”. Evidence for TF-dependent procoagulant activity on MP derived from platelets and monocytes was presented, and the concept of encrypted versus de-encrypted TF exposure and its associated pro-coagulant activity was discussed, as were assay variables of TF procoagulant assays and the need for standardization and normalization.

**Thomas Exner** ( Sydney , Australia ): Presented the so-called XACT assay aimed at detecting procoagulant phospholipids in plasma, based on factor Xa-activated clotting time. One advantage of this assay is that it can be performed with whole plasma samples and is insensitive to the presence of most lupus anticoagulants. Hence, it can be anticipated that procoagulant MP are also detected.

**Cheng Hock Toh** ( Liverpool , UK ): “*Anticoagulant potential of microparticles*”. Dr Toh highlighted the fact that microparticles, depending on their cellular source, are able to display not only procoagulant properties, but also/instead express EPCR-bound activated protein C, which often functions to initiate anticoagulant pathways. The quantitation and functional analysis of these particular microparticles were described.

**Bruce Furie** ( Harvard , USA ): “*Impedance-based flow cytometry for measuring microparticles: New instrument, new answers*”. TF-bearing, PSGL-1-bearing MP, likely derived from monocytes, bind to laser-damaged vessels in a P-selectin-dependent manner, and deliver TF in such a way as to promote fibrin deposition and thrombus growth. This model is currently thought to reflect inflammatory injury. Quantitation and detection of at least a sub-population of very small MP is unfortunately complicated by the fact that the particle size is on the same order of magnitude as the wavelength of light (488 nm) used for their detection. To overcome this limitation, an impedance-based instrument, which measures electronic volume and has a 10X signal:noise ratio has been developed, and found to be able to detect up to  $1.6 \times 10^6/\text{ml}$  TF-bearing MP in the blood of individuals with certain forms of cancer, including pancreatic, colon, breast, and ovarian. It is postulated that delivering TF in this way might contribute to the incidence of thromboembolism prevalent in the later stages of many cancers.

**Eric F. Grabowski** ( Boston , USA ): *Microparticles in flowing blood*". The hemolytic uremic syndrome (HUS) results from Shiga-toxin-producing strains of E. coli, and causes acute renal failure in children. Though Shiga-toxin is able to increase TF activity 2-3 fold on the surface of activated endothelium, it does *not* appear to be able to similarly activate TF on EC-derived MP, perhaps due to downregulation of MP TF activity by TFPI.

**Pudur Jagadeeswaran** ( San Antonio , USA ): *"Zebrafish microparticles from thrombocytes and their role in hemostasis"*. Thrombin and collagen were found to induce the formation of annexin V-positive MP from zebrafish thrombocytes, and these MP were capable of accumulating at sites of laser-induced arterial injury.

**Jean-Marie Freyssinet** (Paris & Strasbourg, France) - *"Round table discussion of an action plan for the standardization of the determination of microparticles"*. There was broad agreement that microparticles can generally be defined as 0.1-1  $\mu$ m cell-derived vesicular structures that lack a nucleus or synthetic capability. They can, and often do, however, contain a membrane skeleton. Microparticles have their origins in a variety of blood and vascular cell types, and mAb and proteomic analysis is likely to shed important clues as to their varied origins – much work remains to be done in this regard. MP contain varying amounts of surface-exposed PS, depending on their origins and mode of preparation/shedding. Both the phenotype and function of MP vary according to cellular origins and inducers of vesiculation. Hence, MP can be pro-coagulant or anti-coagulant, but when the balance is disrupted in favor of the former population, this reflects an increased thrombotic risk. Preferential approaches to their preparation and analysis was felt to be premature, but remains a worthy goal of future VB Workshop activities.

## Vascular Biology

Chair: J-M. Freyssinet ( France)

Co-Chairs: M. Berndt ( Australia), F. Dignat-George ( France), J. Griffin (USA), I. Juhan-Vague (France), P. Newman (USA)

The session was divided into three parts, addressing key issues in vascular biology and related disorders. None of the three topics does specifically belong to the scope of ISTH, which emphasizes the efforts to be made to maintain a leadership in the field.

Regarding microparticles (MPs), the new feature emerging from several presentations was the incidence of MPs in several types of cancers (B. Furie, N. Mackman, A. Weltermann), and more particularly with respect to the presence of tissue factor (TF). In a context where the elevation of MPs has been widely documented in cardiovascular diseases (R. Nieuwland, Y. Ahn), this considerably reinforces their pathophysiologic significance and therefore highlights the need of methodologies for better determination and characterization. A first step was precisely proposed as the “MP challenge” that would consist in the distribution of appropriate materials for the standardization of flow cytometry analysis (B. Furie, F. Dignat-George). Calibrated microbeads should enable to standardize instrument settings, then biological samples could be distributed provided shipment problems are solved with respect to specific national security regulations. The search for correlation with the procoagulant potential(s) of MPs could also be included. If the samples to be tested could be prepared by a single laboratory, pre-analytical conditions would not require particular attention at this stage. For feasibility purpose, suggestions and input from investigators interested in participating in this challenge are welcome, please contact Françoise Dignat-George at: [Francoise.Dignat-George@mail.ap-hm.fr](mailto:Francoise.Dignat-George@mail.ap-hm.fr). Although it is generally agreed that MPs stem from the plasma membrane of stimulated or apoptotic cells, it has however to be kept in mind that MPs have still to be better defined, e.g. on standardized bases since their physical properties, and probably a proportion of associated biological effects, mainly depend on the method used for their isolation. This is the other main goal of this subcommittee because it is essential to avoid confusion of functions with respect to other membraneous vehicles such as exosomes.

Receptor shedding and shed receptors as plasma biomarkers of vascular injury was the second topic. The fate of three platelet receptors was reported, GPV (J. Clemetson), GPVI (B. Nieswandt, P. Smethurst) and semaphorin-4D (L. Brass). This underscores the significance of the platelet “sheddome” in pathology. Not only the shed receptors have to be considered by themselves but also the pathways associated with, or accounting for, the shedding process. For instance, GPVI can be released as a true soluble truncated form or as a probable full-length membrane protein in MPs, depending on the conditions of platelet activation. In case studies in patients show the value of these, and perhaps other, shed receptors as biomarkers, standardized ELISA methods based on the availability of appropriate antibodies could be proposed for diagnosis and/or for the assessment of treatment efficiency.

Circulating endothelial cells (CEC) are believed to reflect endothelium damage or degeneration whereas endothelial progenitor cells (EPC) are viewed with regenerative potential, at least in cardiovascular disorders knowing they can also be mobilized in tumor development. Regarding

CEC, definition and markers appear consensual. There is a general agreement on CD146-dependent immunomagnetic separation as a reference method, and owing to cell scarcity, there is a trend to combine a first step of enrichment and flow cytometry (F. Sabatier, P. Goon). The situation is somewhat more confusing for EPC as there is no consensus on the definition itself and no appropriate methodologies taking function and phenotype into account (P. Gaussem, J. George, N. Saunders). Hence, definition of EPC constitutes an essential next goal for this subcommittee.

In summary, in its first year of existence, the Scientific Subcommittee on Vascular Biology has identified three main topics to be further investigated at methodological and standardization levels, the interest being demonstrated by the importance of the attendance with up to ~300-350 participants.

## Vascular Biology

5 July 2008  
Vienna, Austria

Chair: *Jean-Marie Freyssinet (France)*

Co-Chairs: *Michael Berndt (Australia), Françoise Dignat-George (France), John Griffin (USA), Peter Newman (USA)*

### DRAFT

The session was divided into three parts, addressing key issues in vascular biology and related disorders. None of the three topics does specifically belong to the scope of ISTH, which emphasizes the efforts to be made to maintain a leadership in the field and to attract leading speakers.

Receptor shedding and shed receptors as clinical biomarkers of vascular disease was the first topic. The fate of some platelet receptors, and cognate ligand in one case, was reported.

Compromised ITAM-based platelet receptor function ITAM was evidenced in one patient with immune thrombocytopenic purpura (ITP). In ITP patients, platelet autoantibodies can impair platelet function, and ITAM receptor dysfunction may account for bleeding tendency (M. Berndt).

Soluble P-selectin has been found a novel independent clinical biomarker for venous thromboembolism in cancer patients. Main tumor origins were breast, lung, gastrointestinal tract, pancreas, kidney, prostate, brain, haematologic malignancies, and others (I. Pabinger).

This underscores the significance of the platelet “sheddom” in pathology. Not only the shed receptors have to be considered by themselves but also the pathways associated with, or accounting for, the shedding process. For instance, P-selectin glycoprotein ligand-1 (PSGL-1) has been reported to regulate P-selectin-mediated endothelial activation and shedding of P-selectin from activated platelets. Amazingly, *psgl-1* knockout in mice leads to increased aggressivity, probably as a neurological consequence of stroke occurring in the absence of proper regulation of soluble P-selectin (D. Wagner). Receptor shedding and shed receptors as clinical biomarkers is considered an appropriate topic to be proposed by this Subcommittee for SSC Educational Sessions planned for the Boston meeting.

Circulating endothelial cells (CEC) are believed to reflect endothelium damage or degeneration whereas endothelial progenitor cells (EPC) are viewed with regenerative potential, at least in cardiovascular disorders, knowing they can also be mobilized in tumor development. Hence, the second session was dedicated to the analysis of circulating cells of endothelial origin detectable in peripheral blood. Technical challenges of standardization relies both on their scarcity and on the overlapping phenotype between EPC and mature CEC.

M. Strijbos addressed the specificity of a single platform flow cytometry (FCM) method that identified CEC in whole blood as CD34+/ CD45-/ CD31+/CD146+ events. Using DNA analysis and cell sorting, he demonstrated that these cells are in fact “giant platelets” that unspecifically bind antibody to CD146. Such interference may in fact participate in the huge discrepancy between CEC counts reported in the literature using standard FCM (few CEC per  $\mu$ l) or assays based on cell enrichment of CD146+ cells (few CEC per ml).

L. Terstappen (USA) presented an automated instrumentation for CEC analysis that combines isolation of CD146+ cells using paramagnetic nano-particles and image analysis. Criteria for CEC identification included size, morphological properties, presence of nucleus, expression of endothelial markers such as CD105 and negativity for CD45. After control of vene-puncture artefacts, normal values of a few CEC per ml, comparable to those reported in the consensus protocol (Woywodt, *J Thromb Haemost*, 2006) were observed while elevated levels were found in cancer patients.

Moving on to EPC, G. Uzan stressed the interest of immuno-depleting lineage positive cells previous to multicolor FCM. He showed that the numbers of Lin- / CD133+ / CD34+ / KDR+ / 7AAD- cells correlate with those of late EPC outgrowth colonies in normal subjects and in patients with leukaemia or systemic sclerosis.

L. Heymans (Belgium) critically compared 6 different FCM protocols for EPC analysis based on co-expression of CD34 and KDR. Besides recalling technical recommendations applied to rare cell analysis, she showed that, despite a higher potential loss of cells, previous mononuclear cell enrichment by density gradient centrifugation significantly improved counting data reproducibility as compared to whole blood techniques.

M. Pirro (Italy) added to the same technical considerations the interest of measuring both EPC and EPC-derived microparticles (MPs). The presence of KDR<sup>+</sup> MP in the circulation was proposed as a marker of apoptosis-related reduction in EPC and was shown to correlate with Framingham score in patients with vascular disorders. Therefore combinations of EPC and EMP levels could be useful to improve the assessment of cardiovascular risk.

This topic has to be developed if ISTH wants to maintain a certain leadership in the field of vascular biology, actively investigated in other specialities, not only cardiovascular biology but cancer biology. The benevolent participation of established investigators, not serving the ISTH, is certainly an encouragement in this sense.

Regarding microparticles (MPs), several years ago, B. Furie emphasized by that owing to the physical laws of light scattering, flow cytometry may not be fully appropriate for enumeration of the sole forward scatter basis. For instance, impedance flow cytometry used to detect MPs in cancer patients does not yield the same figures than flow cytometry, in patients and control subjects. The characteristics of new instruments were reviewed. However the size detection threshold remains ~ 0.4 - 0.5  $\mu\text{m}$  (B. Furie).

Hence, it is clear that MPs enumerated by flow cytometry may just represent a tiny part with respect to the pool of circulating MPs. P. Harrison presented evidence that dynamic light scattering (DLS) can yield valuable information on the size distribution of MPs, showing that only a small percentage of the MP "iceberg" can be seen by conventional flow cytometry since the main part of MPs has a size  $\leq 0.4 \mu\text{m}$ . Furthermore, the size of MPs could vary according the stimulus at the origin of MPs, the smaller ones seem to be associated with pathological conditions (J.-M. Freyssinet). Although a sophisticated technique, DLS is operational in platelet-free plasma and revealed that aggregated lipid particles can be confounded with MPs, which may well occur in hyperlipemic patients. Another point was the discrepancy between the protein or lipid content of MPs and their numbers reported in the literature. In order to better define internal standards, it would therefore be advisable to express MP concentrations in protein and/or lipid equivalents, especially in experiments aimed at exploring MP associated functions.

Thrombin generation can be a valuable tool for assessing the procoagulant potential of MPs (B. Binder). Red blood cell-derived MPs were reported having a higher thrombogenic potential probably owing to their phospholipid composition determined by lipidomics. Again, the recurrent problem is that of a reliable internal standard.

Among MPs circulating in the vascular compartment, those of endothelial origin (EMPs) have to be better defined, which has led to the re-evaluation of the criteria (R. Nieuwland). MPs harbouring the CD62E/CD144/CD146 phenotypes are the more likely to be of endothelial nature and can therefore be considered true EMPs.

The confusing situation with respect to the enumeration of MPs by flow cytometry has led Françoise Dignat-George and Nigel Key to propose a workshop on standardization by using calibrant microbeads that will be distributed free of charge by the manufacturer, in agreement with the conflict-of-interest guidelines provided by the ISTH Executive Director and SSC Chair. Calibrated microbeads should enable to standardize instrument settings, then biological samples could be distributed by the core lab (F. Dignat-George) provided shipment issues are solved with respect to specific national security regulations. Regarding the latter point, Elaine Gray who is used to biological sample exchanges at an international level, has kindly offered help. This action has been presented in the DIC and Malignancy Subcommittee sessions and has raised a real interest as 15 to 20 groups have declared being willing to participate in the workshop. The slides detailing the main aims, features

and milestones of this workshop, as well as the form of intent to participate will be available soon for download from the ISTH website.

In summary, in its 2<sup>nd</sup> year of existence, the Scientific Subcommittee on Vascular Biology has identified three main topics. Two of them, “CEC & EPC” and “Microparticles”, have to be further investigated at methodological and standardization levels while “Shed Receptors” will be developed in future SSC educational sessions.

*Submitted by J-M Freyssinet*