European Science Foundation

Standing Committee for the European Medical Research Councils (EMRC)

ESF EMRC EXPLORATORY WORKSHOP

APPLYING NEW TECHNOLOGIES TO THE STUDY OF INHERITED DISORDERS OF MEGAKARYOCYTES AND PLATELETS

Scientific Report

Naples, Italy
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Convened by: Alan T Nurden[1],
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1. Executive summary

Blood platelets mediate the initial steps leading to the arrest of blood loss following vessel injury. Through the use of the same molecular pathways, platelet accumulation within diseased coronary arteries may result in arterial thrombosis and contribute to diseases of high socio-economic importance such as myocardial infarction and stroke. A proven way of identifying proteins with vital roles in platelet function is from their causal involvement in inherited diseases affecting distinct aspects of platelet biology. A heterogeneous group of familial platelet disorders have as their cause quantitative or qualitative defects of megakaryocyte (MK) differentiation and platelet production processes. Some involve loss of adhesion and aggregation mechanisms of direct relevance for thrombosis. A feature of these rare diseases is that advanced studies and even diagnosis often requires highly specialized laboratories and is well beyond the scope of hospital platforms and individual research units. Another problem is incorrect diagnosis and improper therapies (an example is spleen removal in giant platelet syndromes or familial thrombocytopenias mistakenly diagnosed as autoimmune disease). Although genetic advances have permitted important insights into the molecular causes of some of the major rare diseases of platelets, genotyping remains slow. Nevertheless, in spite of progress the complex processes involved in platelet production and function often remain poorly understood and the abundance of patients with bleeding syndromes who do not fit into established categories suggests that a wide range of rare genetic disorders affecting the megakaryocyte lineage await definition.

Our meeting aimed at establishing European collaborative activities to:

1) establish protocols using advanced proteomics and genomics to extend our knowledge of the molecular causes responsible for defects in the signaling pathways of platelets, in secretory processes and also in platelet production;
2) design microarray assays for screening known mutations and to involve the human genome sequencing centers in genotyping of more abundant and well-characterized disorders such as Glanzmann thrombasthenia;
3) develop diagnostic flow-charts and simple diagnostic methods for patients with suspected inherited platelet disorders;
4) evaluate the necessity and the possibility to create a clinical consultancy based on a European hotline;
5) plan a post-genomic platform for the implementation of innovative approaches to develop new diagnostic and therapeutic tools;
6) structure potential projects to be funded by European organizations.

To discuss these aspects, 21 European researchers and clinicians from 10 countries were invited to the ESF Exploratory Workshop. Arturo Brunetti, a member of the Standing Committee for the European Medical Research Councils, was also present and made a valuable contribution. A highlight was an invited lecture from Dr. David Wilcox (Milwaukee, USA) who discussed the latest advances in gene therapy for diseases affecting the haemostasis and coagulation pathways. Our Workshop underlined that inherited platelet defects are rare bleeding disorders with an extensive clinical and genetic heterogeneity. Communications clearly showed how the application of new technologies to their study would offer a unique chance to define additional key components of pathways involved in regulating cell differentiation and cell surface interactions. Furthermore, the use of improved diagnostic procedures would identify specific cohorts

of patients and lead to advances in patient management and treatment. It should be emphasized that only by defining the molecular pathways of platelet function can all avenues of drug design to combat arterial thrombosis, where platelets are the major contributor, be explored. Thus the rare diseases in question provide a unique link to drug design. In this context, experts in cell biology, genetics, nanotechnology and medical care met to coordinate their activities, define their priorities and establish new collaborative projects.

Among specific discussion were:

a) the need for identifying specialized centers within Europe for performing specific tasks such as megakaryocyte culture, gene sequencing, proteomic analyses, genomics and gene profiling, site-directed mutagenesis. Two situations were identified where specific European projects could be developed either as Eurocore proposals or as part of the 6th PCRDT. The first concerns the identification of molecular causes responsible for defects in signalling and secretory pathways in platelets. The second concerned the evaluation of gene therapy as way of treating some of the more well-characterized diseases.
b) setting up joint European projects to assure the genotyping of patients with the more abundant platelet disorders such as Glanzmann thrombasthenia. This disorder of the alphaIIbbeta3 integrin is already well studied but an approximate estimate is that only one patient in five has been genotyped. The relationship between genotype and phenotype is poorly understood. Also unclear is the relationship between the molecular defect and the tendency of patients to produce isoantibodies to deficient or abnormal glycoproteins after transfusion. Genotyping is required prior to prenatal diagnosis and would be obligatory in case gene therapy come into use. If this project was managed on a European scale, then true patterns may emerge as several hundred patients could be screened. Also, a range of plasmatic and vascular risk factors could be measured in an effort to understand why some patients bleed more than others.
c) setting up task forces to produce algorithms or diagnostic flow charts to help improve the diagnosis of platelet disorders, to facilitate genotyping and to prevent improper therapies (e.g. spleen removal in giant platelet syndromes). This particularly applies to the giant platelet syndromes and will also apply to the signalling disorders as our knowledge of them increases.

The unique charm of Naples helped make this a very successful three days in which the scientific discussions were of a very high level with considerable interchange between the participants.

2. Scientific content of the event

Our meeting was subdivided into four sessions in which specialist speakers were given 30 minutes to describe recent aspects of their work and to respond to questions. Each session was followed by a 1-hour discussion open to all participants entitled *"Planning for Europe"*. These discussions were extremely productive.

Session I dealt with "Defects of megakaryocytopoiesis and platelet production". Platelets are cell fragments that are produced in large numbers from their parent cells, megakarocytes, which mature in the bone marrow. Only recently has the molecular basis of what may turn out to be a large family of related disorders affecting platelet production

started to be defined. Recently reported defects in transcription factors specific for the megakaryocyte lineage were discussed. A genetic approach to the study of disorders where insufficient circulating platelet numbers (thrombocytopenias) are associated with the presence of giant platelet forms was described. Finally in this session, it was reported how congenital amegakaryocytic thrombocytopenia may be due to the absence of receptor activity for thrombopoietin, a defect that was shown to extend to hematopoietic stem cells. The study of congenital thrombocytopenias is an emerging field still in its infancy and much remains to be done. Little is known of how genes are regulated during megakaryocyte maturation while the production of thousands of platelets by individual mature megakaryocytes is a unique event.

Session II dealt more specifically with platelet receptor defects and alterations in signalling pathways. It began with a review of recent mutations giving rise to an altered function of the GPIb-IX-complex, a major platelet adhesion receptor that recognizes subendothelial-bound von Willebrand factor. The Bernard-Soulier syndrome is an already well-characterized rare disease in which an absence or dysfunctioning of an adhesion receptor is accompanied by the production of giant platelet forms. The functioning of the GPIbbeta cytoplasmic domain as well as a potential role for platelets in tumour growth were specifically discussed. The key mediator of platelet aggregation is the alphaIIbbeta3 integrin and we were told how the affinity of the extracellular domains for adhesive protein ligands is controlled by intracellular interactions. A major physiologic agonist of platelets is ADP and disorders of the recently cloned ADP receptor, $P2Y_{12}$, were reviewed. We were reminded that ADP is a key stimulus of platelets not only for primary haemostasis, but also for arterial thrombosis. We then learned about an unusual case under study where a platelet aggregation defect and shortened platelet lifespan were associated with an abnormal functioning of a previously little studied phosphodiesterase. This case highlighted how platelets from patients with a previously unrecognized phenotype require specialized molecular characterization. An animal model with a defective alpha-actinin phosphorylation was briefly reported before the session closed with a presentation on a possible regulating role for insulin receptors on the platelet aggregation response. This long session was most stimulating because it is generally agreed that many disorders affecting signalling pathways of platelets remain to be characterized.

Session III was more clinical in emphasis and began with a specifically designated young speaker, Raffaele Badolato, who talked about the genetics and molecular diversity within the Wiskott-Aldrich syndrome. This was followed by a presentation on the diagnosis of inherited thrombocytopenias and discussion of a working algorithm for identifying individual cohorts of patients either with a related genotype or with different genotypes. It was agreed that this approach needs to be applied to other categories of platelet disorder. The relationship between genotype and phenotype in giant platelet disorders with mutations in the MHY9 gene was then discussed in terms of the relevance to patient care. Finally, we were told how diagnostic advances have permitted the recognition of a new cohort of patients with the Gray Platelet Syndrome, where a lack of secretable alpha-granule proteins is associated with a molecular deficiency of the GPVI collagen receptor.

Session IV was devoted to discussing post-genomic strategies for platelets and megakaryocytes. The session began with an introduction to transcriptional profiling of cells of the megakaryocytic lineage and microarray analysis. This technology shows how the absence or malfunctioning of a specific protein can influence gene transcription and a cell's response to its environment. This was illustrated with reference to essential thrombocythemia where gene expression profiling may lead to the recognition of cohorts

of patients either in terms of the clinical evolution of the disease or in terms of treatment. We were then told of the efforts of a collaborative project within the University of Oxford to define the platelet proteome and on how proteomics can be used to study platelet signalling pathways. Over 3000 proteins have been reported to be present in platelets. Finally, the session concluded with an evaluation on how the human genome project can be used to accelerate research into platelet biology and platelet-based disease. This led to a debate on how new technological advances can be applied to the study of rare platelet disorders. While the frequency and severity of bleeding can vary a lot between patients even with the same mutation, the quality of life in certain cases is severely impaired. A response to the question of what governs bleeding risk will be essential for optimal patient care.

Finally, the question of **gene therapy** for these diseases was discussed in a plenary lecture by David Wilcox who came from Milwaukee (USA) especially for our meeting. Using a megakaryocytic/platelet-specific alphaIIb promoter inserted into a lentivirus vector, Dr. Wilcox has been able to target deficient genes into megakaryocyte precursers and show transgene expression in cultured megakaryocytes. Early data from animal models is very promising. So, protocols for gene therapy in disorders such as Glanzmann thrombasthenia (alphaIIbbeta3 deficiency) and Bernard-Soulier syndrome (GPIb-IX-V deficiency) are being established.

3. Assessment of the results, contribution to the future direction of the field, outcome

Our workshop was the first of its kind designed to promote European research into the study of *Inherited disorders of Megakaryocytes and Platelets*. In this respect, to assemble together over 3 days many of the leading European experts in this field was in itself extremely useful. Our meeting led to intensive discussion on how to proceed. Discussions were frank and extremely positive. Up to now, Italy and France have been leading the way in that national networks have already been established. Other European countries were urged to follow suite. This is essential, because a chain of events is needed so that a patient seen by a clinician in a hospital consultancy can be referred to specialized centers where first the platelet functional abnormality can be fully characterized and then the molecular defect determined. The wide variety of the rare diseases under study means that no single specialized center can cope. The task would be much more efficient if managed as part of a coordinated European network with designated “Centres of Excellence” for genomics, proteomics, genetics, cell signaling pathways etc.

In order to promote the integration of the participants in a future effective Network, as well as to contribute to the integration of other groups, the participants agreed to prepare a written proposition for a Network. Alan Nurden and Anna Savoia will coordinate this and attempt to obtain a consensus. The decision of the ESF to suspend the funding of Networks means that this will need to be done under a different European format. It is important nonetheless to obtain a formal recognition of the group. It should be noted that aid for the diagnosis of rare diseases has been proposed by a recently appointed ethical committee as a priority for the European Commission

To reach the aims proposed at the workshop, it was thought necessary to start immediately by encouraging exchanges within Europe. As well as scientific, these should cover legal and ethical aspects linked to studies on patients and sample exchange. Specific proposals for projects involving proteomics and genomics will be prepared for

either research grants or financial supports to encourage the network. Possibilities were identified where specific European projects could be developed either as Eurocore proposals or as part of the 6th PCRDT. The proposed document will represent the basis to implement programs and define the specific area for future applications. These should be coordinated as best as possible with parallel projects on cardiovascular disease. Finally, as the Network develops, contacts and priority should be given to the development of similar networks in third world countries.

4. Final programme

Thursday 22 April 2004

13.00 - 14.30	*Registration*
14.30 – 14.45	Welcome **Alan Nurden (**France)
14.45 - 15.00	**Arturo Brunetti** (Italy, Standing Committee for the European Medical Research Councils): Presentation of the European Science Foundation (ESF)

Session I: Defects of megakaryocytopoiesis and platelet production
Chairpersons: **William Vainchenker** and **Anna Savoia**

15.00 - 15.30	**William Vainchenker** (France): Fli-1 and the Paris Trousseau syndrome
15.30 - 16.00	**Christel Van Geet** (Belgium): Transcriptional regulation of megakaryocyte maturation: role of GATA1/FOG1.
	Tea Break 15 min
16.15 - 16.45	**Anna Savoia** (Italy): Genetic approach to the study of hereditary thrombocytopenias
16.45 - 17.15	**Matthias Ballmaier** (Germany): Congenital amegakaryocytic thrombocytopenia. A hematopoietic stem cell defect.
17.15 - 18.15	Planning for Europe

Friday 23 April 2004

Session II: Platelet functional defects: signaling pathways, aggregation and secretion
Chairpersons: **Christian Gachet** and **Stephen Watson**

08.30 - 09.00	**Jeanine Clemetson** (Switzerland): Bernard-Soulier and platelet-type von Willebrand disease syndromes.
09.00 - 09.30	**Dermot Kenny** (Ireland): A novel role for the cytoplasmic domain of GPIbalpha
09.30 - 10.00	**Marco Cattaneo** (Italy): Defects of the P2Y12 platelet ADP receptor
10.00 - 10.30	**Christian Gachet** (France): Platelet aggregation defect in response to all agonists in a patient with mild thrombocytopenia and a shortened platelet lifespan: a critical role for PDE3.
	Coffee Break 15 min
10.45 - 11.15	**Nelly Kieffer** (Luxembourg): Talin and □IIb□3 activation
11.15 - 11.45	**Jan-Willem Akkerman** (Holland): A bleeding disorder in a Landseer dog associated with a defect in □-actinin phosphorylation
11.45 - 12.30	Planning for Europe
12.30 - 14.00	*Lunch*
14.00 - 15.00	Plenary Lecture: **David Wilcox** (USA): Gene therapy for Inherited Platelet Disorders

Session III: Patient care: clinical, diagnostic and therapeutic aspects
Chairpersons: **Carlo Balduini** and **Andreas Greinacher**

15.00 - 15.30	**Raffaele Badolato** (Italy): Some aspects of the Wiskott-Aldrich syndrome
15.30 - 16.00	**Carlo Balduini** (Italy): Proposal for diagnostic algorithm of inherited thrombocytopenias
	Tea Break 15 min
16.15 - 16.45	**Andreas Greinacher** (Germany): Clinical presentation and management of patients with MHY-9 mutation giant platelet syndromes
16.45 - 17.00	**Paquita Nurden** (France): Observations on the Gray platelet syndrome
17.00 - 18.00	Planning for Europe

Saturday 24 April 2004

Session IV: Post-genomic strategies for platelets and megakaryocytes
Chairpersons: **Willem Ouewenhand** and **Dermot Kenny**

08.30 - 09.00	**Diana Tronik-Le Roux** (France): Transcriptional profiling of the megakaryocytic lineage
09.00 - 09.30	**Stephen Watson** (UK): Studying platelet function using proteonomics
09.30 - 10.00	**Sergio Ferrari** (Italy): Gene expression profiling of CD34-derived megakaryocytic cells from normal donors and patients with and essential thrombocythemia
10.00 - 10.30	**Willem Ouwehand** (UK): How to use the genome information in platelet biology?
10.30 - 11.15	Planning for Europe
	Coffee Break: 15 min
11.30 - 13.00	**Forum Conclusions and Planning for the Future**

5. Final List of Participants

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6. Statistical information on participants (age structure, countries of origin, etc)

Age structure
Group 1 (>50 years): 7 participants
Group 2 (>40 and <50 years): 13 participants
Group 3 (<40 years): 2 participants

Contries of origin
Belgium: 1
France: 5
Germany: 2
Great Britain: 2
Holland: 1
Ireland: 1
Italy: 6
Luxembourg: 1
Spain: 1
Switzerland: 1
USA: 1

Male and female participants
Male:16
Female: 6