

Summary of the BVAC/ABCA program hosted by the general annual meeting of the BHS in Ghent, Friday January 25th 2013

A. Kornreich, A. Gothot

For the first time, during the course of its general annual meeting, the BHS hosted a satellite meeting organized by the Belgian Society for Analytical Cytology (BVAC/ABCA) to bring together clinicians with laboratory scientists and staff. The BVAC/ABCA program included state-of-the-art lectures, followed by a session focused on advances in genetic testing of haematological malignancies and finally a satellite symposium sponsored by Alexion on the deficiencies of complement regulators leading to thrombotic micro-angiopathies (TMA). All these presentations are available on the BVAC/ABCA website: <http://www.cytometry.be/Gent2013.html>

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State-of-the-art lectures

1. Immune monitoring in intensive care patients (Guillaume Monneret, PharmD, PhD, Cellular Immunology Unit, E. Herriot Hospital, Lyon, France)

Sepsis is still a leading cause of death in intensive care units and its incidence is higher than most common diseases. Recent data suggest that after the initial proinflammatory phase of immune activation, a protracted phase of immune suppression is ultimately responsible for viral reactivation, nosocomial infections and mortality. The objective of monitoring ICU immunodepression is to identify patients who could most benefit from immune stimulation. The best tools to achieve immune monitoring are based on flow cytometry.

Septic patients have a marked reduction in absolute T-, B- and NK-cell counts at diagnosis. Lymphocyte

apoptosis leading to defective restoration of T-cell counts after one week is predictive of mortality. In addition, an increased proportion of circulating regulatory T-cells (Treg; CD4+CD25+CD127-) is observed in sepsis, and contributes to lymphocyte anergy.

Currently, the best characterized marker of immune suppression is the decreased expression of HLA-DR on monocytes (mHLA-DR), which is a surrogate marker of their function. In septic shock, low mHLA-DR expression is independently associated with nosocomial infections and its persistent low expression predicts mortality. Additionally, it predicts unfavourable outcome and nosocomial infections in other ICU conditions (major trauma, acute pancreatitis, severe burn injury, acute stroke, decompensated liver cirrhosis), in paediatric lung transplant recipients and is correlated with the

Authors: A. Kornreich, MD¹, A. Gothot, MD, PhD².

¹President of the BVAC/ABCA, Belgium. ²Laboratory of Haematology, CHU Sart Tilman, Liège, Belgium.

Please send all correspondence to: A. Kornreich, MD, Onco-Hematology Consultant, Rue de Sotriamont 92, 1400 Nivelles, Belgium, tel: +32 487 32 71 10, email: ankornre@ulb.ac.be.

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rate of progression and overall survival in non-Hodgkin lymphoma.

In order to conduct multicentric studies, flow cytometric measurement of mHLA-DR expression has to be standardized. This can be done by using stabilized blood and calibrated beads in order to express results as the number of HLA-DR epitopes per monocyte. Inter-laboratory assessment of mHLA-DR has shown high reproducibility.

Monitoring the immune status of ICU patients with mHLA-DR expression opens new therapeutic possibilities such as blocking inhibitory receptors on lymphocytes with PD1 and CTLA4, stimulating lymphocytes with IL-7 or stimulating innate immunity with IFN-gamma or GM-CSF. A small phase-II clinical trial using GM-CSF has shown promising results in terms of side-effects, reduced ventilation time, reduced length of ICU stay and reduced length of intra-hospital stay.

Conclusion

ICU-acquired immunosuppression may be monitored with a biomarker that can predict the risk of mortality and nosocomial infection, thus allowing individualized patient care by different strategies of immuno-stimulation that still have to be assessed in future clinical trials.

2. Advances in immunophenotyping of myelodysplastic syndromes (MDS)

Matteo Della Porta, MD, Department of Haematology, San Matteo Hospital Foundation, University of Pavia Medical School, Italy

Bone marrow dysplasia is not specific of MDS and may be difficult to evaluate in some patients. Yet, bone marrow dysplasia has a prognostic value in MDS and WHO classification of MDS is correlated with post-transplantation outcome. It has already been shown that flow cytometric evaluation of bone marrow dysplasia is feasible but is based on pattern recognition analysis which requires an expert operator. Evaluation of erythroid dysplasia is particularly difficult because of the limited availability of specific markers. Until now, no single immunophenotypic marker is able to accurately differentiate MDS from normal bone marrow.

Flow cytometric scoring systems (FCSS) were developed over the years combining panels of markers and pattern recognition analysis. Recently, a simpli-

fied score was evaluated for its reproducibility by the European LeukemiaNET in a multicentric study in low-risk MDS. The score includes 4 cardinal parameters such as: % CD34+ myeloblasts, % CD34+ B-cell progenitors, lymphocyte/myeloblast CD45 expression ratio and granulocyte/lymphocyte SSC ratio. A diagnosis of MDS is suggested in the presence of a FCM-score value ≥ 2 . The score has a sensitivity of 70% for low grade MDS and a specificity of 93%. To improve sensitivity, another prospective multicentric study (ANITA) including 492 unselected subjects with peripheral blood cytopenias is under way. A diagnosis of MDS is suggested in the presence of an 8 parameter-score value ≥ 3 . Preliminary results indicate that a sensitivity of 90% and a specificity of 92% for low grade MDS can be achieved.

Conclusion

Flow cytometry may be used to evaluate marrow dysplasia in patients presenting with peripheral blood cytopenias. However, this requires a standardization of sample processing, antibody panels and data analysis. The 4-parameter score evaluated by the European LeukemiaNet have little inter-operator variability and is useful to differentiate MDS from non-clonal cytopenias. The ANITA score appears to be suitable to diagnose MDS especially when morphology and cytogenetics are indeterminate. Finally, flow cytometric evaluation of marrow dysplasia may identify subsets of patients with poor prognosis within the low and intermediate-1 IPSS risk categories.

3. Flow cytometric analysis of T-cell lymphomas

Marie-Christine Jacob, MD, Laboratory of Immunology, CHU Grenoble, France

Haematological malignancies are characterized by the malignant transformation of a cell, differentiation arrest and clonal expansion. Thus, a malignant cell subset may express aberrant markers, is clonal and demonstrates features of homogeneity. Unfortunately, flow cytometric analysis of T-cell lymphoproliferative disorders (LPD) can not rely on true aberrant markers if high sensitivity is required, and no clear-cut clonality marker is available. However, there are many indicators of homogeneity.

One challenge is to define the best markers for high sensitivity and specificity. Another is that homogeneous T-cell expansions also occur during immune

reactions, thus reactive and malignant expansions must be distinguished. In all cases, the demonstration of clonality, usually by molecular biology, is mandatory and the interpretation must always take into account clinical and other biological information in a multidisciplinary analysis.

M.-C. Jacob reviewed the different subsets of T-cells, i.e., CD4+, CD8+, CD4+CD8+, CD4-CD8-, and among the latter, those expressing TCR $\alpha\beta$ and TCR $\gamma\delta$. The prevalence of malignant clones, the reference ranges and the differential diagnosis with reactive expansions were discussed in each case. Diagnostic significance of decreased expression of pan-T antigens, such as CD7, CD26, CD5, CD2 and CD3, was also reviewed. Expansions of very minor T-cell subsets such as CD10+ T-cells mainly in angioimmunoblastic T-cell lymphoma and CD4+CD158k+ in Sezary Syndrome, were presented. Finally, the interest and limitations of TCR V β repertoire analysis by flow cytometry were discussed.

Overall, expansions of T-cells with phenotypes such as CD10+, CD4-CD8- TCR $\alpha\beta$ +, CD4+CD158k+, CD4+CD3- and CD3+CD2- are highly suspicious of malignancy. Other phenotypes are moderately specific and sensitive such as CD3+CD7-, CD3+CD5-, highly increased CD4+ T-cell counts and skewed TCR V β repertoire. Conversely, expan-

sions of CD8+, CD4+CD8+ or CD4-CD8- TCR $\gamma\delta$ + T-cells have poor diagnostic power.

Conclusion

Flow cytometry is extremely useful for T-cell LPD diagnosis providing the interpretation takes into account the bio clinical context.

B. Advances in genetic testing of haematological malignancies

1. Molecular Haematology: Overview on activity

Christian Demanet, MD, PhD, Laboratory of Haematology, UZ Brussel, Brussels, Belgium

The Royal Decree for the Centres for Molecular Diagnosis (CMDs) was published in 1998. On August 1st 2007, the article 33bis of the RIZIV/INAMI nomenclature became effective and imposed BELAC accreditation for test reimbursement. So far, 16 laboratories have been accredited for tests included in art. 33bis. Medico-economic data on molecular diagnosis are available from Econodat (Besco) from 1/8/2007 to 2011. There are no data available for Jak-2, which is reimbursed since 01/08/2010 only. In haematology, there are 6 tests available for diagnosis and 2 tests for follow-up, whereas in oncology, there are 3 diagnostic tests and 1 follow-up test. The numbers of analyses have considerably increased (45%!) in the past years (Figure 1).

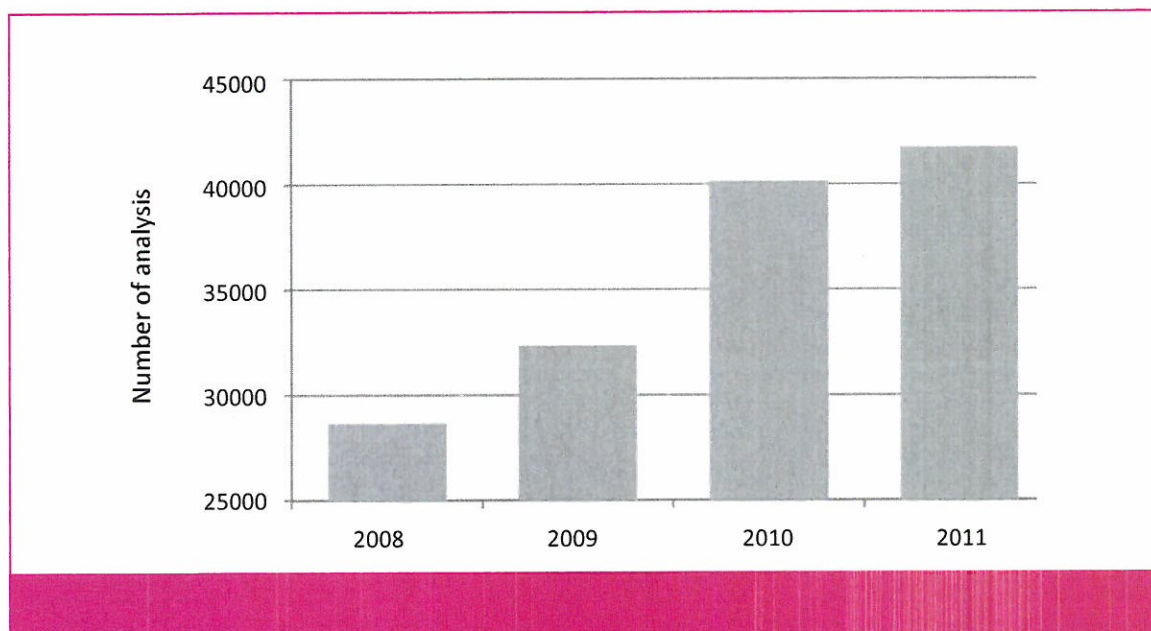


Figure 1. Total number of analysis performed during 2008-2011.

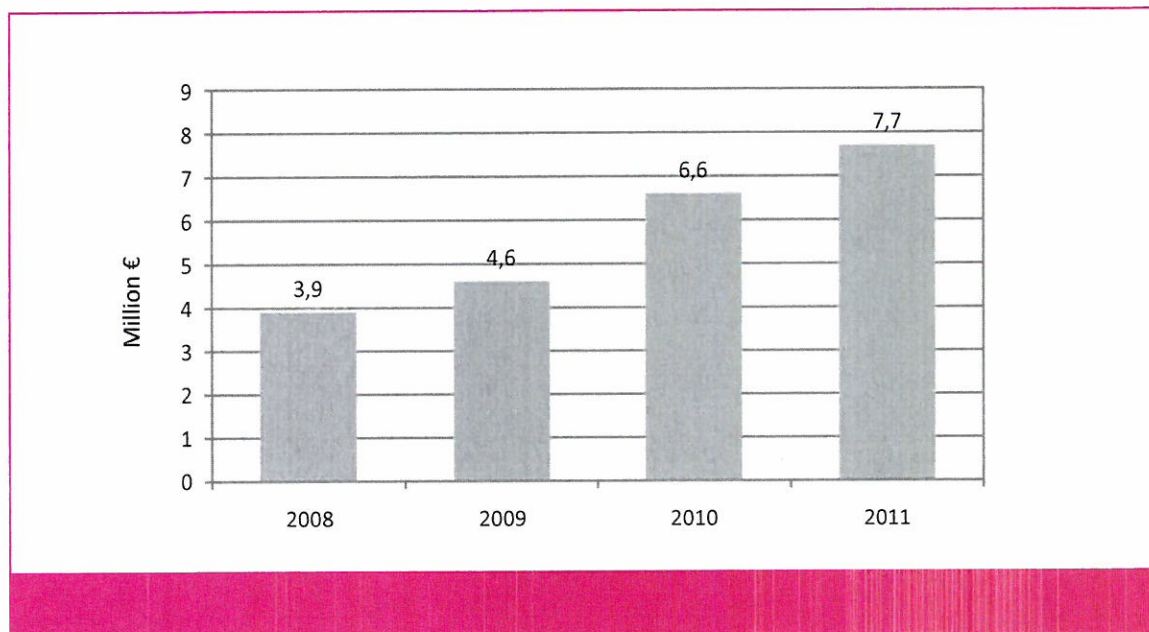


Figure 2. Expenses article 33bis during 2008-2011.

There are a number of new nomenclature proposals submitted to the commission of Clinical Biology: introduction of T-cell chimerism, introduction of a nomenclature code for chronic myeloproliferative disorders (including testing of BCR/ABL and Jak-2), CGH microarray for CLL and MM, VH-Hypermutation status in CLL.

The original budget allocated for the article 33bis in 2007 was $3,3 \cdot 10^6$ €/year. In 2011, the expenses increased to $7,7 \cdot 10^6$ € (Figure 2).

Conclusion

The use of molecular tests in haematology and oncology within article 33 bis increased significantly. This increase was questioned in the Senate in 2012 and might stimulate restrictive measures such as a clinical biology 'forfait' regulation.

2. Overview of genome wide arrays in haematological malignancies

Pierre Heimann, MD, PhD, Laboratory of Medical Genetics, CUB Hôpital Erasme-Institut Bordet ULB, Brussels, Belgium

The genetic complexities of cancer cells require a sensitive genome-wide analysis, enabling the detection of small genomic changes in a mixed cell population, as well as regions of homozygosity. Conventional karyotyping provides a genome-wide assessment but its resolution is too coarse to reliably detect small

deletions and FISH can only assess the regions of the genome targeted by the panel of probes. Conversely, array-based karyotyping allows the identification of very small copy-number alterations (CNAs) with high accuracy. Though all CGH (comparative genomic hybridization) arrays offer an excellent resolution, are performed on interphase cells and allow for the precise detection of CNAs and LOH (loss of heterozygosity i.e. deletion), SNP (single nucleotide polymorphism) arrays can also detect copy neutral LOH (cnLOH) or acquired uniparental disomy (aUPD), a chromosomal region in which both copies are acquired from the same parent. This distinction is important as cnLOH is reported to underlie 20-80% of the LOH seen in both solid and haematological cancers. However, SNP arrays also have certain limitations; they are unable to detect balanced chromosomal translocations, the sample analysed has to contain at least 20% of tumoural cells and they can not assess regions of the genome not represented on the arrays.

Nevertheless, they have promising applications in several haematological cancers:

- In Chronic Lymphoid Leukemia (CLL), copy neutral LOH have been identified at clinically relevant loci that cannot be detected by conventional karyotyping and FISH. Because arrays can display the genome at high resolution, it is becoming apparent that individual molecular lesions identified earlier by

FISH are actually heterogeneous in both genomic length and copy number, though with undetermined clinical significance.

- In Acute Lymphoblastic Leukaemia (ALL), because of poor banding and metaphase quality in a great proportion of ALL samples, interpretation of conventional karyotyping may be difficult and chromosomal aberrations may be missed or misinterpreted. The success rate of arrays is 93% in detecting CNAs compared to 65% in karyotyping. Arrays also provide additional genetic information, e.g. IKZF1 gene deletion detected in more than 10% of childhood B-cell precursor-ALL cases are highly predictive for the occurrence of relapse. As such, determination of the Ikaros deletion status is expected to become important in routine ALL diagnosis. However, FISH tests remain necessary for clinically relevant balanced aberrations.
- In Multiple Myeloma (MM), karyotyping has a low success rate (30-40% of cases) due to low proliferative rate of plasma cells. Compared to interphase FISH on enriched plasma cells, which is the gold standard and remains necessary for clinically relevant balanced translocation detection, microarray-based genomic profile was performed on enriched plasma cells yielding a 92% concordance for the identification of CNAs. Arrays allow discrimination between tetraploid and hyper diploid karyotypes and identify many novel lesions including regions exhibiting cnLOH, though their clinical relevance remains to be determined.

Conclusion

Arrays are becoming applicable in routine diagnosis for haematological neoplasms, according to their specific profiles, but they do not replace metaphase karyotyping (still required for determining the IPSS in MDS), as they don't detect balanced abnormalities.

C. Satellite symposium: Deficiencies of complement regulators lead to lifelong risk of systemic complement-mediated TMA [Sponsored by Alexion]

1. PNH: Disease evidence and clinical experience

Timothy Devos, MD, PhD, Department of Haematology, UZ Leuven, Leuven, Belgium

PNH or Paroxysmal Nocturnal Haemoglobinuria is a misnomer. Indeed, it is neither paroxysmal, nor

nocturnal and 75% of the patients present without haemoglobinuria. The 3 main characteristics of this rare acquired disease are intravascular haemolytic anaemia, bone marrow failure (with cytopenias) and thrombosis. It is due to an acquired somatic mutation in hematopoietic stem cells. The incidence is 2-6/10⁶ and the median survival is 10 years. The median age of occurrence is 42 years old. In PNH, the natural inhibitors of the complement at the surface of the RBC (CD55 and CD59) are absent due to a defect in the Glycophosphatidylinositol (GPI) anchor. This leads to uncontrolled and chronic complement activation, in turn leading to thrombosis, renal failure, pulmonary hypertension, abdominal pain, dyspnoea, dysphagia, fatigue, haemoglobinuria and erectile dysfunction. It should be noted that clone size does not correlate to symptom severity. The main reasons that warrant PNH screening are bone marrow hypo-/aplasia, MDS, Coombs-negative haemolytic anaemia, thrombosis, unexplained non-haemolytic anaemia or pancytopenia.

The leading cause of death is thrombosis (40-67% of PNH patients) followed by renal failure (8-18%). The only treatment presently available for PNH is Eculizumab, which is a humanized anti-C5 antibody. It has been shown to reduce the risk of clinical thrombo-embolism and provides long-term patient survival similar to that of the normal population. The only subgroup for which HSC transplantation is still justified is "PNH in the setting of another specified bone marrow disorder" when MDS-RA (refractory anaemia) or AA (aplastic anaemia) becomes predominant in a rather fit patient.

Conclusion

PNH may be more common than we think and the availability of an effective treatment warrants testing in higher risk patient population.

2. Identifying patients at high risk for PNH: recommendations for screening/testing

Jan Philippé, MD, PhD, Laboratory of Haematology, UZ Gent, Ghent, Belgium; Bernard Husson, Laboratory of Haematology, Jolimont Hospital, La Louvière, Belgium
A number of symptoms and signs of PNH are highly frequent but not specific, thus delaying the diagnosis for 1 to more than 10 years. In most instances, PNH screening will be ordered by the clinician,

but as 93% of patients with PNH have peripheral blood abnormalities, the laboratory might suggest the test in some cases. The gold standard for PNH testing is flow cytometry with FLAER (Fluorescently Labelled Aerolysin) which specifically binds to the GPI anchor that is missing on monocytes and granulocytes in PNH. Lineage specific markers are also used: CD15 for granulocytes and CD33 for monocytes. However CD33 is also expressed on dendritic cells (DC) and on immature granulocytes which can dimly express the GPI anchor and can be found in the blood. Therefore, it is argued that CD64 is better suited as it is strongly expressed on monocytes but not on DCs and early myeloid precursors. It is recommended to combine two GPI-specific reagents per lineage: CD24 for granulocytes and CD14 for monocytes are usually assessed together with FLAER. A detection threshold of 1% will be used in most cases but in aplastic anaemia, that will only yield a 20% sensitivity. Lowering the threshold to 0.01% will allow the detection of up to 70% of positive patients. For red blood cells (RBC), glycophorin A (CD235a) and CD59 are used for gating and GPI evaluation, respectively. Only RBC testing allows the distinction between type I, II and III cells, but the technical procedure must be optimized: the clone used for CD59 detection must be carefully validated, titration of the antibody is required to avoid RBC agglutination and "racking" the test tubes immediately prior to the analysis is mandatory. Quantitative results should be reported.

Conclusion

Effective treatment and reliable standardized testing procedures are compelling reasons to implement PNH screening by flow cytometry in haematology laboratories.

3. New insights in thrombotic microangiopathies (TMA): TTP (thrombotic thrombocytopenic purpura) and aHUS (atypical haemolytic uremic syndrome)

Catherine Lambert, MD, Department of Haematology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

The last few years have been marked by a better understanding of the underlying mechanisms of TMA, better management and the development of targeted therapies. The fibrin micro thrombi that occur in TMA will lead to a variable degree of organ dysfunction. Renal impairment is predominant in aHUS whereas neurological abnormalities will be foremost in TTP but there are overlaps between these entities. The cornerstone of therapy for TMA patients remains plasma exchange (PE) with fresh frozen plasma (FFP) restitution. However, there are suboptimal responses in 20% of the cases, relapses in 20-40% of TTP, irreversible organ damages and even though haematological remission is obtained in 70-80% of the cases with PE, a 10-20% mortality rate remains. Therefore, new strategies are used to manage suboptimal responses such as early Rituximab that will decrease the production of anti-ADAMTS 13 antibodies by depleting the pool of B-cells and Eculizumab that will inhibit the complement pathway through its anti-C5 activity.

Other therapies are being tested: inhibitors of von Willebrand Factor (vWF)-platelet GP1b binding to prevent platelet aggregation and the apparition of micro thrombi; recombinant ADAMTS 13 has been tested in mice, by overcoming anti-ADAMTS 13 inhibitors; gain-of-function ADAMTS 13 variants with a modified exosite in the spacer domain that will reduce antibody binding and preserve or enhance cleaving activity; N-acetylcysteine that will inhibit vWF polymerisation by reducing the disulfide bonds; TT30, a selective inhibitor of the complement alternative pathway with the ability to provide durable local tissue binding and protection from haemolysis with only minimal transient systemic inhibition.

Conclusion

Early recognition of TMA is crucial, prompt PE remains the first line of treatment but the new therapeutic developments are very promising.