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Dear ESCCA members and Conference Attendees,

Thank you for joining us at ESCCA 2019 up North - we are looking forward to meeting you for 3,5 intensive days of discussions and presentations.

Some changes have been introduced to this years’ programme based on repeated requests from former conference participants:

All lectures are open to all participants – you may switch between the educational tracks Clinical Cytometry, Advanced hematology-Oncology and Immunology.

The Clinical Cytometry track has been designed to suit newcomers to the field: it starts by introducing basic concepts followed by presentations of a selection of recommended approaches and consensus guidelines in the field.

To provide scientists with an opportunity to present their data and gain exposure, we’ve reserved ample time for oral presentations and poster flash presentations. Poster flash presentations in particular are meant as a “taster” to attract the audience to the physical poster for details. Posters will be at display in the Exhibition area – pass by during the conference for more detailed information.

I encourage you to go and listen to your peers!

Those of you attending ESCCA for the first time will be welcomed separately to ease the entry into our society.

I hope you’ll find useful information at the courses, lectures and booths and I am looking forward to a lively and informative conference – do participate, interact and give your feedback.

Sincerely,

Paula Fernandez
ESCCA president
Mass Cytometry in Haematology
Bjørn T. Gjertsen
University of Bergen / Haukeland University Hospital

Mass cytometry is a single cell analysis technique that uses time-of-flight mass spectrometry of stable metal isotopes to detect multiple parameter in each cell. Mass cytometry is still in its technological childhood but has demonstrated the power of multiparameter single cell analysis. The surplus of measuring points per cell has made it simpler to include both the standard extracellular markers with newer intracellular markers, as well as opening the pristine avenue of nucleotide analysis in single cells. This widen the typical repertoire of phenotypical and functional markers of cells dramatically. The level of multiparameter data has required development of bioinformatics with new algorithms and machine learning to mine the data for biological information. Current limitations of mass cytometry compared to conventional flow cytometry include fewer validated and premade antibodies, limited hardware options, slow data collection and loss of material during preparation and analysis. Haematology as a medical field include benign and malignant diseases, including or bordering into autoimmune conditions. It is obvious that better access to multiparameter single cell analysis has a huge potential in hematological diagnostics of lymphoproliferative diseases and myeloid neoplasia. The multiparametrics of mass cytometry may translate into faster and more precise diagnostics, and it allow more resolution in the phenotyping of the disease. With novel therapeutics for malignant blood diseases, an increased demand for precise diagnostics and response evaluation has emerged. There are several examples that functional analyses at the single cell level is available for disease evaluation related to disease phenotype. Some examples of functional diagnostics will be discussed, and how this may transform the way we treat our patients with blood cancer. Finally, comparing leukemia and autoimmune diseases, we will speculate if the multiparametric analysis of single cell could transform our understanding of immunological diseases and how some of the most active therapeutics actually works to successfully treat our patients.

Improving cytometry based diagnostics using Artificial Intelligence?
Yvan Saeys
VIB - Ghent University, Gent, Belgium

Introduction
During the last decade, many new technological advances have shaped the field of cytometry. These include among others multi-parameter flow and mass cytometry that are able to measure tens of parameters at single cell level, and imaging flow cytometry, enabling high-throughput imaging at single-cell resolution. The classical analysis using manual gating on biaxial plots has severe limitations when analyzing these high-dimensional data sets. Manual gating is often subjective and biased, difficult to reproduce and not scalable to a high-dimensional panels.

Results and discussion
In this presentation I will discuss the potential of techniques from the field of Artificial Intelligence (AI) to perform analysis of high-dimensional cytometry data. AI is a field that encompasses many different technologies for automated knowledge representation and reasoning, and recently many medical challenges have been addressed using AI based techniques. Also in the field of cytometry, AI based techniques have been adopted by the community.
I will start by giving a broad introduction to AI based techniques, showcasing how they can advance analysis of cytometry data. These include novel methods for automated gating of cytometry data, visual analytics, biomarker discovery and automated patient diagnosis. In addition, I will also show some examples of recently developed methods to get more insight into the underlying dynamics of cellular processes, such as modeling cell developmental trajectories.
In the second part of the talk I will describe a clinical example, introducing a novel automated cytometry based workflow for diagnosis of myelodysplastic syndromes. This AI-based workflow outperforms the conventional flow cytometry analysis in terms of accuracy, objectivity, material usage and time investment.
PLENARY SESSION 2

Keynote Lecture: The Flow Cytometry Laboratory: reality and perspectives
Katherina Psarra
EVANGELISMOS HOSPITAL, Athens, Greece

Flow Cytometry has evolved into an important diagnostic and research tool in several areas of medicine and science. The flow cytometry laboratory therefore is a part of the Hematology or Immunology departments within the structures of hospitals and a core lab in a research institute. We are envisioning clinical labs with new preanalytical machines (most of them of big volume), small modern compact cytometers of 18+ (?) colours, a small number of medical technicians preparing samples and acquiring events, modern 'R' software uploaded on computers for the analysis. The tubes - dry reagents are kept in room temperature cupboards and the rest of the monoclonals in the preanalytical machines. The colors are not the 'old friends' well known organic dyes. New not spilling bright dyes are replacing them. The samples, peripheral blood, bone marrow and lots of biological fluids, cerebrospinal fluid, ascites, pleural effusions, are coming from hematological or patients receiving new 'precision individualized immunological drugs' in large quantities. The results are 'flowing' quickly to the GDPR checked recipients and the files of the patients are packed with valuable information, while new software produces exact diagnosis from the patient's data.

Perhaps smaller labs away from the big centers with compact flow cytometers designed to automatically answer certain questions are covering urgent or simple local needs.

Beautiful modern core labs in big research centers feature 20 colors flow cytometers next to image and mass cytometers and spectral cytometers and combinations of them, sorters next to NGS machines and the whole big data information is analyzed in huge (the repetition of the word is purposeful) computers loaded with dedicated software.

Where is the cytometrist standing in this new world of 'accurate' machines? Does he understand the machines function? Does he understand the color combinations? Does he understand biology, chemistry, or medicine? Or is he a computer scientist? Is he mesmerized, is he fascinated, is he charmed by this unbelievable technology? Does he take it for granted? Is he needed in a future flow cytometry lab? Is he/she helpful and part of the science?

PLENARY SESSION 3

New therapeutic approaches and their challenges for flow monitoring
Concetta Quintarelli, Francesca Del Bufalo, Biagio De Angelis, Matilde Sinibaldi, Luciana Vinti, Pietro Merli, Mattia Algeri, Annalisa Ruggeri, Federica Galaverna, Valentina Ciriolo, Maria Giuseppina Cefalo, Giuseppina Li Pira, Giovanna Leone, Valentina Bertaina, Franca Fassio, Monica Gunetti, Stefano Iacovelli, Franco Locatelli
Bambino Gesù Hospital, ROME, Italy

Introduction: Adoptive cellular immunotherapy with chimeric antigen receptor (CAR) T cell has changed the treatment landscape of B-cell Acute Lymphoblastic leukemia as well as non-Hodgkin's lymphoma (NHL), especially for aggressive B-cell lymphomas. Single-center and multicenter clinical trials with anti-CD19 CAR T-cell therapy have shown great activity and long-term remissions in poor-risk leukemia and diffuse large B-cell lymphoma (DLBCL) when no other effective treatment options are available. Two CAR T-cell products have obtained approval for the treatment of refractory leukemia or DLBCL. With the increase of the clinical use of CAR T cells, their related toxicity has already been very well defined. Moreover, despite the huge successes, there are still a number of hurdles to overcome before CAR-T cells can be effective in mainstream oncology beyond hematological malignancies. Bambino Gesù Hospital in Rome, has started two academic Phase I-IIa clinical trials based on CAR T cells for the treatment of both resistant/relapsed Acute Lymphoblastic Leukemia patients and resistant/relapsed neuroblastoma patients.

Methods: The 'Cell and Gene Therapy Lab' of the hospital had developed two novel CAR constructs in frame with the suicide gene inducible caspase 9. Moreover, all the GMP manufacturing processes for CAR T as well as GMP Quality Control drug release are performed in the internal GMP Facility, namely 'OPBG Officina Farmaceutica'. After the infusion, patient immunomonitoring is performed at specific time points by applying in parallel flow-cytometry, ELISA and molecular biology. In particular CAR T cells PK is constantly monitored by flow-cytometry thanks to the
evaluation of CD34+ CD3+ cells (OPBG CAR vector has been designed with the incorporation in the transgene of a human CD34 epitope recognized by a commercial available mAb).

Results: 17 children were enrolled into the trial and received the iC9-CD19-CAR T cells. With the validated flow-cytometric assay, we were able to detect CAR+ T cells in all the infused patients. In particular, iC9-CD19-CAR T cells expanded in vivo and were detectable by both flow-cytometry and molecular biology in the blood, bone marrow and cerebrospinal fluid of the responders. One CD19-negative relapse 3 months after infusion was recorded, while 3 additional patients relapsed with CD19+ leukemia blasts.

Conclusions: Our data indicate that PK evaluated by flow-cytometry highly correlates the molecular evaluation of vector DNA in both peripheral blood and BM. The observed outcome achieved in the treated patients have encouraged the clinician and the research to go on the Phase II portion of the study.

PLENARY SESSION 5

The stem-cell niche in myeloproliferative neoplasms

Simón Méndez-Ferrer, PhD
Welcome-Trust Medical Research Council Cambridge Stem Cell Institute and Department of Haematology, University of Cambridge, and National Health Service Blood and Transplant, Cambridge Biomedical Campus, Cambridge, UK

Abstract will follow
ORAL PRESENTATIONS

OP-01

A proposed novel monocyte subpopulations redefinition, adding CD300e to CD14 and CD16. Evaluation in a wide variety of clinical samples

Nikolaos J. Tsagarakis1, Elpiniki Kritikou-Griva2, Stefanos I. Papadhimitriou2, Eleni Goumakou2, Georgios Oudatzis3, Paraskevi Vasileiou4, Georgios Paterakis1

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Background: Monocyte subpopulations defined by CD14 and CD16 have been classical (M1, CD14+/CD16-), intermediate (M2, CD14+/CD16+) and nonclassical (M3, CD14-/CD16+). In this study, CD300e (IREM2), a well-known antigen of monocytic maturation, was combined and evaluated in clinical samples, leading to a novel monocyte subpopulations definition.

Methods: A specific FC gating strategy was applied in a variety of clinical samples (n=65): 20 peripheral blood specimens, 39 bone marrow (BM) aspirates and 6 body fluids. Samples were obtained from 49 patients (24M/25F, median age 62, range 4-92), and were classified in the following categories: group 1 (21/65) was derived from patients with acute myeloid leukemia (AML) (in several time-points of induction therapy) (AML-MRD), group 2 (8/65) was from patients with AML in diagnosis (AML-Dx), group 3 (16/65) was from patients diagnosed with myelodysplastic/myeloproliferative neoplasm (MDS/MPN), group 4 (2/65) was from patients with chronic myeloid leukemia in diagnosis (CML), group 5 (16/65) consisted of reactive samples (heterogeneous group with infection or monocytosis - Reactive) and group 6 (2/65) included control BM. FC gating strategy was based on the combination of CD14, CD16, CD33, CD34, CD45, CD64, CD66 and CD300e. Results were expressed as (phenotype, median % of total WBC, range of %). Statistical analysis was performed with SPSS 17.0.

Results: The newly identified subpopulations were: A1 (CD14+/IREM2-/CD16-, 1.62, 0.00-35.76), A2 (CD14+/IREM2-/CD16+, 0.03, 0.00-0.83), B2 (CD14-/CD45weak/CD16-/IREM2weak, 0.05, 0.00-6.32), B3 (CD14-/CD45+/CD16-/IREM2+, 0.09, 0.00-21.24) and B4 (CD14-/CD45+/CD16weak/IREM2+, 0.01, 0.00-0.45). The A3 (CD14+/IREM2+/CD16-, 0.02, 0.00-16.49) and B5 (CD14-/CD45+/CD16strong/IREM2+, 0.12, 0.00-11.52) corresponded to the classical M1, M2 and M3, respectively. A1, A2, B2 and B3 were considered intermediate monocyte precursors significantly increased in regenerating AML marrows. A1, A2 corresponded to classical A3 (M1) and intermediate C1 (M2). B2, B3 and B4 were considered monocyte precursors of non-classical B5 (M3) monocytes. B1 subpopulation (CD14-/CD45weak/CD16-/IREM2-, 1.23, 0.00-92.42) was correlated with immature precursors (monoblasts and promonocytes). Finally, C2 (CD14+/IREM2+/CD16+/CD66+, 0.26, 0.01-60.11) was significantly increased in fluids and correlated with macrophages.

Conclusions: The application of IREM2 contributed to the identification of novel monocyte subsets, which were differentially expressed in various clinical samples. The most significant finding was the identification of B2 and B3, IREM2+, double negative for CD14 and CD16 monocyte precursors, in regenerating marrows. A possible false positive MRD interference was noted in AML. CD14-/IREM2+ populations were considered precursors of normal non-classical monopoiesis, contrary to CD14+/IREM2- populations, which were considered precursors of classical and intermediate monocytes.
Evaluating clinical utility of new immunophenotypic markers in determination of minimal residual disease (MRD) in childhood acute myeloid leukemia (AML)

Surbhi Lahoti, Prashant Tembhare, Sumeet Gujral, P G Subramanian, Shirpad Banavali, Gaurav Narula, Nikhil Patkar, Sitaram Ghogale, Y Badrinath, Nilesh Deshpande, Gaurav Chatterjee, Dilshad Dhaliwal, Shefali Verma, Jagruti Patil

Tata Memorial Hospital, Mumbai, India

Introduction: Recent studies have demonstrated that the flow cytometric minimal residual disease (FC-MRD) status in AML is a highly relevant risk factor in the prediction of clinical outcome and is better than traditionally defined morphological complete-remission status. However, currently available standard FC-MRD detection is limited to 80% of patients possibly due to the usage of limited markers. Hence, there is a need to explore new markers which will help to increase the sensitivity and applicability of the AML FC-MRD assay. We evaluated the utility of new markers in FC-MRD determination in childhood AML.

Methods: We prospectively studied flow-cytometric expression-pattern of CD39, CD69, CD73, CD86, CD96, CD166, CD184 and CD244 in non-APML childhood (age ≤ 15 years) AML patients. The flow-cytometric study was performed with 10-12 color antibody panel using Navios and Cytoflex flow cytometers (Beckman Coulter) and Kaluza software. Expression-patterns of new markers were studied in normal myeloid blasts (NMB) from control samples and leukemic blasts from AML patients. Differential expressions of these markers in leukemic blasts were studied and their utility in the detection of FC-MRD was evaluated.

Results: We studied eight markers in 55 cases of pediatric AML at diagnosis and at follow up (MRD) as well as in NMB from 18 control- bone marrow (BM) samples. New markers were studied in 109 BM samples from 55 AML cases (53 diagnostic and 56 MRD samples). Of these, CD69, CD73, CD39 and CD96 demonstrated overexpression while CD166 and CD244 demonstrated underexpression in AML blasts (p-value<0.05). CD86 and CD184 were also found to be overexpressed but failed to demonstrate statistical significance (p-value>0.05). 56/66 MRD samples were studied for new markers, of which 23/56 (41%) were positive for MRD. Median and range of MRD levels were 0.93% and 0.03 - 62.32%. CD166, CD244, and CD96 were found most useful in discriminating MRD from NMB and in detecting lower levels of MRD (<0.1%). Among MRD positive samples, three samples were positive by new markers, but undetectable using standard markers leading to false negativity.

Conclusion: Amongst the eight new markers, CD166 followed by CD244 and CD96 were most useful in discriminating MRD from NMB and in detecting lower-level MRD. Addition of these new markers to the existing standard panel can improve the sensitivity and applicability of AML-MRD assay.
Results: Of the 31 patients diagnosed with biphenotypic acute leukemia (BAL) using the EGIL classification 12 did not fulfill the WHO criteria for MPAL. Only 2 B-Myeloid cases according to the WHO classification did not fulfill EGIL criteria. We reclassified all cases following the WHO criteria and resulted in 32 MPAL cases (2.8% of the cohort; 24/32 B-Myeloid, 7/32 T-Myeloid, 1/32 B-T-MPAL).

Aberrant T-cell marker expression was seen in 6 patients with B-Myeloid MPAL, CD7 expression being the most frequent. In T-Myeloid MPAL, the cytoplasmic CD79a B-cell marker was aberrantly expressed in 4/7 cases (57%). HLA-DR was expressed in all MPAL cases. TdT was positive in all Ph+ MPAL, compared to 26% in non-Ph+ MPAL cases. Seventy-three percent of the cases were classified as ALL by morphology. Cytogenetic data available for 29/32 patients were as follows; normal karyotype= 5 (17.2%), t(9;22) translocation=7 (24.1%), complex karyotype (CK)=14 (48.2%), monosomy 7=5 cases (17.2%). Response to treatment and outcome were available for 24 and 29 patients, respectively; ALL-type treatment induced a response in 72% of the patients, whereas AML-type in 33.3%; All refractory patients (7/24) had a complex karyotype. Median overall survival (OS) was 18.7 months, 12 and 27 months for patients with CK and t(9;22) respectively, while the 5-year survival rate was 12.5%. In contrast, BAL patients classified according to EGIL had median OS 23.3 months.

Conclusions: In our study, reclassification of BAL (EGIL) patients as per WHO, resulted in a more narrow characterization of acute leukemias with mixed phenotypic features. Our study confirms that MPAL/BAL displays a uniformly poor outcome especially in patients with CK. The addition of TKI in the treatment of Ph+ patients probably ameliorates MPAL poor prognosis.

OP-04

A standardised, reproducible flow-cytometric panel and scoring-system to discriminate patients with aplastic anemia from MDS and reactive causes of cytopenia

Jan Dirks, Beatrice Drexler, Julia Engels, Dimitrios Tsakiris
University Hospital Basel, Basel, Switzerland

Introduction: In the last 15 years a huge variety of flow-cytometry based scoring systems for myelodysplastic syndrome (MDS) has been published, each differing substantially in aspects such as antibody panels, sample preparation protocols, gating strategies and properties included into the final score. The lack of consensus and standardisation is arguably the biggest obstacle for flow-cytometry based MDS diagnosis, preventing inter-centre comparability and reliability of results. Furthermore one of the main differential diagnosis in patients with unexplained, persistent cytopenia – aplastic anemia – is not included in current scoring systems.

The aims of this study were:
● Improve the reproducibility of flow cytometry-based MDS diagnostics by applying the best multi-centric validated scoring system to the best standardised antibody-panel.
● Establish an algorithm, which helps to discriminate patients with aplastic anemia from patients with MDS and patients with reactive causes of cytopenia, using a common antibody-panel.

Methods: We measured bone marrow samples of a total of 102 individuals with MDS (n=38), aplastic anemia (n=14), reactive causes of cytopenia (n=28) and healthy bone marrow donors (n=22) with the AML-MDS tubes 1-4 according to the Euroflow protocol (van Dongen et al, Leukemia 2012) in our center. Flow-cytometric data was subsequently analysed according to the MDS scoring system published by the Groupe Francophone des Myélodysplasies (GFM; Bardet et al, Haematologica 2015). Diagnosis of MDS and aplastic anemia was done integratively with morphology and genetics.

Results: The MDS scoring system by the GFM could be applied to the flow-cytometric-data acquired using the Euroflow AML-MDS panel leading to highly reproducible results (inter-operator congruence between: two experienced flow cytometrists = 100%; an experienced flow cytometrist and a trainee = 84%).

Using the same panel we identified a distinct pattern with low myeloblasts (CD45dimCD34+CD117+ myeloblasts <0.08% in representative bone marrow aspirate containing ≥ 0.002% CD117+ mast cells) in all 14 patients with aplastic anaemia, discriminating this group from patients with MDS (specificity=97%), cytopenias of reactive cause (specificity=100%) and healthy bone marrow donors (specificity=100%) all having higher blast counts.

Conclusions: The MDS scoring system by the GFM can be applied to the AML-MDS panel from Euroflow and enables highly reproducible results.

Patients with aplastic anemia can be reliably discriminated from patients with MDS and reactive cause of cytopenia. As both analysis for MDS and aplastic anemia can be performed in parallel with the same standardised panel, the value and indication for performing flow-cytometry in patients with unexplained cytopenias should be reconsidered.
OP-05

Normal CD19-negative early B-cell precursors in bone marrow impair flow cytometric MRD-monitoring in childhood B-cell ALL after CD19-targeting treatment

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Introduction. CD19 is the most widespread pan-B-cell antigen, expressed during all stages of B-lineage maturation. Strongly expressed in nearly all cases of B-cell precursor acute lymphoblastic leukemia (BCP-ALL), this marker is often lost under the selective pressure of CD19-directed immunotherapy. As the minimal residual disease (MRD) persistence is the strongest indicator of targeted treatment failure, detection of CD19-negative leukemic blasts expressing other B-lineage antigens (CD22, CD10, CD24, iCD79a, etc) is crucial for correct evaluation of the response to immunotherapy. Nevertheless, particular therapeutic approaches could lead to very specific regeneration patterns, not completely matching B-cell development in normal bone marrow. Thus CD19-negative early BCP, normally undetectable, can appear and lead to false-positive MRD-detection results after CD19-directed immunotherapy. The aim of our study was to assess the possibility of normal early CD19-negative precursor appearance in bone marrow after CD19-targeting treatment.

Methods. Since December 2015 till June 2019 62 children and adolescence with relapsed/refractory BCP-ALL completed at least one course of blinatumomab, while 25 patients were treated with CD19-CAR-T-cells. MRD-monitoring was performed by 10‑12-color flow cytometry. CD22 and CD24 were added to conventional antibodies combinations to avoid negative influence of potential CD19 loss on cytometric data interpretation.

Results. In 23 out of studied 87 cases (26.4%) during MRD monitoring after CD19-directed therapy, we observed CD19-negative BCP (median 0.077% of all nucleated bone marrow cells) with strong expression of CD22, weak expression of CD10 and CD45 also being positive for CD34, CD38 and negative for CD20 and all leukemia-associated markers studied (CD58, NG2, myeloid). In all cases described cells represented exactly the same antigen profile completely matching cytometric patterns of early BCP, except lack of CD19. In 3 samples all other stages of B-cell maturation were presented, completing solid pattern with detected CD19-negative BCP. Moreover, in other 4 cases CD19-negative leukemic blasts (CD10-positive and CD10 negative in 2 cases each) were also detectable on the background of CD19-negative BCP. As 14 of these patients underwent allo-HSCT prior to CD19-targeted immunotherapy, CD19-negative BCP were sorted for chimerism studies. In all three studied cases sorted cells were confirmed to be of donor origin and thus non-leukemic.

Conclusion. Our data show that normal CD19-negative very early BCP can be detectable in bone marrow of substantial proportion of children with BCP-ALL after CD19-targeting treatment. These cells mimicking residual leukemia could appear both after blinatumomab and CAR-T treatment and require special attention and investigation to confirm non-malignant nature.

OP-06

Evaluation of CRLF2 over expression in B cell Acute Lymphoblastic Leukemia and its association with post induction Minimal residual disease

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Introduction: Ph-like B-cell acute lymphoblastic leukemia (Ph-like BALL), is a recently defined high-risk subtype of BALL. Cytokine receptor–like factor 2 (CRLF2), located on chromosomeXp22.3 and Yp11.3,4 has been found overexpressed in 50-60% of the Ph-like BALLs. CRLF2 gene is a part of the thymic stromal lymphopoietin receptor (TSLPR) and its over expression is studied using antibody against TSLPR. It has been shown that frequency of Ph-like BALL varies with age and ethnicity. Incidence of CRLF2+ Ph-like BALL has been shown to 78% in BALL patients with Hispanic ethnicity. Till date, there is no data on Ph-like and CRLF2+ BALL in Indian patients. We present a study of CRLF2 overexpression-pattern and frequency in BALL patients diagnosed in Tata Memorial Centre, Mumbai, India.
Methods: CRLF2 overexpression was studied using two different clones of anti-TSLPR antibody (clone 1A6, PerCP-eFluor™ 710 from eBioscience and clone 1B4, PerCP-Cy5.5 from Biolegend) and better clone was selected. Samples were acquired on a 10-color 3-laser Navios-cytometer. CRLF2 evaluation method that including controls (for every sample) was standardized and a specific template was developed for data-analysis using Kaluza-V2.1. BALL blasts with ≥ 10% CRLF2+positivity was defined as CRLF2 overexpression. Post-induction MRD-analysis was performed using 10-color antibody panel.

Results: CRLF2 expression was prospectively studied in diagnostic samples from 545 BALL patients (358 children and 177 adult BALL). Of 545, CRLF2 overexpression was found in 39 (7.15%) samples (25/362, 6.9% in children and 14/183, 7.65% in adult BALL). In 3/39 cases CRLF2 positive expression was detected at the time of relapse. These results match with previously published data. Median and range of CRLF2+ blasts in all samples was 0.75% and 0.03%-99.1%. MRD was available in 484 patients, of them 258 (53.3%) were MRD-positive (MRD median, 0.029% & range, 0.0001-77%). MRD was available in 26 CRLF2+ patients, of which 16/26 (61.5%) were positive for post-induction MRD (0.002-35%) against only 52.8% MRD positivity in CRLF2 negative BALL. Thus, CRLF2+ cases showed higher percentage of MRD positivity.

Conclusions: CRLF2 expression assay can be easily standardized using flow cytometry for the diagnosis of Ph-like BALL. The frequency of CRLF2 expression in Indian pediatric and adult BALL patients closely matches with published data from western countries and possibly indicates similar incidence of Ph-like BALL in Indian patients. The post induction MRD-positivity rate was found to be relatively high in CRLF2+ BALL.

OP-08

Altered immunophenotypes on leukemic and/or monocytic cells from acute myeloid leukemia highly predict for nucleophosmin gene mutation


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Introduction: Nucleophosmin gene mutation (NPM1mut) occurs in around 30% of acute myeloid leukemia (AML) patients, frequently with favourable prognosis. Due to the acquired knowledge on the clinical impact of NPM1mut and its associated molecular background (e.g. FLT3-ITDmut), more expeditious diagnostic approaches would contribute to early diagnosis and prognostic stratification of these patients. Herein, we investigated the association of immunophenotypic features of leukemic and monocytic cells with the presence of NPM1mut in AML.

Methods: Bone marrow (BM) leukemic and monocytic cells from 399 AML patients were studied by 8-color flow cytometry using the EuroFlow AML panel. NPM1mut was present in 220 patients, whereas remaining 179 cases showed wild type gene (NPM1wt). Presence of FLT3-ITDmut was found in 74 of 371 cases studied (20%), while concomitant NPM1/FLT3mut was observed in 51 cases (14%). Leukemic cells with monocytic differentiation were detected among 60% vs. 38% of AML with NPM1mut vs. NPM1wt cases, respectively. In those AML cases lacking blast cell monocytic differentiation (myeloid AML), remaining monocytic cells were also studied.
Results: Abnormal monocytic patterns were more recurrent among NPM1mut vs. NPM1wt cases, both on leukemic (98% vs. 34% of monocytic AML cases) and remaining monocytic cells (76% vs. 23% of myeloid AML cases) (p<0.001). Most frequent phenotypic alterations among NPM1mut monocytic leukemic cells were asynchronous CD300e prior CD14 (78% vs. 5% NPM1/FLT3-ITDwt cases, respectively; p<0.001) and/or CD35 prior CD14 (83% vs. 30%) patterns independently of FLT3-ITD. Similarly, remaining monocytic cells from myeloid AML also showed a higher frequency of CD14/CD300e asynchrony on NPM1mut (64% vs. 7% of NPM1wt cases; p<0.001) and of CD35 vs. CD14 (58% vs. 20% of NPM1wt cases, respectively; p<0.001). Noteworthy, coexistence of both asynchronous monocytic patterns was specific for NPM1mut (64% vs. 0% NPM1wt patients; p<0.001). Moreover, CD9 aberrant leukemic cell expression was present in a high proportion of all AML cases (56%), although being more frequent in AML with NPM1mut (73% vs. 46% vs. NPM1wt) but not in AML with FLT3-ITDmut. However, aberrant CD25 was otherwise linked to FLT3-ITDmut (73% vs. 18% of FLT3-ITDwt cases; p<0.001), as well as the presence of a higher leukemic cell immaturity, as reflected by a significantly higher median CD34+ leukemic cells among NPM1mut/FLT3-ITDmut vs. NPM1mut/FLT3-ITDwt (65.5% vs. 0.3%; p<0.001).

Conclusions: Detection of asynchronous monocytic patterns among leukemic or monocytic cells from AML patients is highly predictive for NPM1mut and may contribute to early diagnosis and upfront therapy selection of these patients.

OP-09
Discrete cell subsets self-generated by unsupervised FlowSOM analysis of normal bone marrow with classical flow cytometry panels
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Introduction: Multiparameter flow cytometry (MFC) has developed as a sensitive tool to analyze complex immunophenotypic patterns and thus identify cell subsets not accessible to morphological examination. Yet, classical MFC relies on gating strategies where each step depends on the operator’s appreciation of relevant patterns. With the development of mass cytometry and its generation of huge amounts of information, new tools had to be devised, allowing for a comprehensive appreciation of MFC, taking into account multiple parameters in dimensional spaces outside of the reach of the human brain. Among those tools, FlowSOM was developed as a user-friendly and rapid solution, clustering cell subsets with enough similarity in distinct nodes.

Methods: FlowSOM was used by our group to explore the complexity of normal bone marrow, using merged samples from different individuals in order to obtain a representative “matrix” of normal hematopoiesis. Four different ten-color panels, devised respectively for the exploration of the myeloid and lymphoid lineages in the context of acute leukemias, were used to obtain four reference minimal spanning trees (MST). The combination of FlowSOM R-packages/scripts and the Kaluza® software of MCF analysis allowed for an in-depth examination of the “nodes” identified by FlowSOM in an unsupervised fashion.

Results: Here we will report on how FlowSOM segregated subsets difficult to isolate with classical methods. Within the lymphoid lineage, FlowSOM was able to separate CD4+, CD8+ and NK-cells while only using CD7, CD4 and CD56 as lineage-specific markers. Among CD19+ B-cells, the various stages of maturation (hematogones) were properly segregated as individual nodes, based on the expression or not of CD38 and CD16. Within the monocytic lineage, the combined presence of CD14 and CD16 isolated non-classical monocytes as a single node. Immature forms of monocytic maturation were also clearly separated. Even in the absence of CD10, maturation stages between immature and mature granulocytes could be readily divided. Some even more immature forms clustered as single nodes or clusters of granulocytic progenitors or metamyelocytes. CD34+ progenitors were neatly subdivided according to the level of concomitant expression of CD38 or not. Even more interestingly, depending on the panel, FlowSOM was able to isolate dendritic cells, basophils, eosinophils and plasma-cells.

Conclusions: All in all, this new comprehensive approach of unsupervised immunophenotypic exploration of normal human bone marrow allows for an in-depth definition of hematopoietic cell subsets, independent of gating strategies liable to minimize or overestimate such discrete populations.
Detection of minimal residual disease by flow cytometry after anti-CD19 blinatumomab treatment

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Introduction: Minimal residual disease (MRD) is one of the most important prognostic markers in B-acute lymphoblastic leukemia (B-ALL), as patients with detectable MRD need more intensive therapy. MRD detection by eight color multiparametric flow cytometry (MFC) is highly sensitive and used worldwide. CD19 is a specific marker of normal and neoplastic B cells and MFC classically identify B cells through CD19 expression. It's also a therapeutic target and the use of anti-CD19 is increasing. Blinatumomab, a bispecific chimeric antibody that binds B cell CD19 to T cell CD3, directs T effector cell response against B neoplastic cells. In such cases, CD19 expression may be altered, leading to false negative - absence of B cells - in MFC studies. Therefore, new labeling and gating strategies in MFC must be developed to correct MRD detection.

Methods: We analyzed 72 samples of 9 consecutive multi-refractory B-ALL patients receiving blinatumomab therapy (5 male, median age=9 years, range=4-18, 3-27 samples/patient). MRD was performed in eight color MFC, through standard lab panel, containing CD19, CD10, CD20, CD34, CD38, CD45, CD58, CD81, CD44 and CD66c (10^6 events). To detect eventual CD19 negative B cells a new tube was created: TdT, CD79a, CD10, CD19, CD38, CD22 and CD45. To evaluate if CD19 suppression was clone-specific, we tested CD19 clones HIB19, SI25C1, HD37 and J3119.

Results: One case of 9 was refractory and presented CD19+ disease. Of the 8 responsive cases, standard approach (i.e. gating within CD19+ events) detected 1 case as MRD+ during follow up. The new approach detected other 4 cases with MRD+, through analyzes of CD79a, CD10 and TdT (0,2%-44% of neoplastic cells). Even in the MRD+ by standard approach, the abnormal population was larger, due to CD19- events, raising from 2% to 15%. The 4 “new” MRD+CD19- cases received salvage treatment protocols and two relapsed as CD19- (3 and 18 months of follow up) and other as a partial CD19+. Of the 3 patients initially MRD- by both approaches, 2 had late relapses as CD19+ cases. No differences among antibodies clones were noticed. Interestingly, normal regenerative B cells were always CD19+.

Conclusion: The new approach was very effective at MRD detection, as it identifies CD19- neoplastic cells. CD19 loss does not seem to be simply due to a competitive obstruction of CD19 labeling, as CD19- relapses occurred even 18 months after treatment, but a real, though in some cases transient, loss of the antigen.

Flow cytometric standardization of PAX5 expression and its utility in B-cell lineage assignment in acute leukemias in a clinical setting

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Introduction: PAX5 is a nuclear protein in the paired-box (PAX) family of transcription factors. It is a pan-B-cell marker expressed from pro-B-cell stage to the early plasma cells. Expression of PAX5 by immunohistochemistry is widely used as a lineage-specific marker for B-cells. However, its expression by multicolor flow-cytometry (MFC) has not been studied in the clinical setting. On the other hand, the assignment of B-lineage on MFC can be complicated in challenging cases as the WHO criteria require multiple additional markers along with CD19. We standardized PAX5 expression by MFC and studied its utility as a B-lineage marker in the lineage-assignment of acute leukemia.

Methods: Anti-PAX5 (PE, clone-REA140) antibody staining was standardized and studied in the blasts from acute leukemia samples. The bone marrow samples were processed by the lyse-wash method followed by surface staining. We evaluated four permeabilization reagents: FACS Lyse (BD), Fix-&-Perm (Invitrogen), Foxp3-fixation-kit (eBiosciences) and True-nuclear transcription-factor staining buffer-set (Biolegend). MFC was performed on Cytoflex flow cytometer (Beckman-Coulter) and data was analyzed using Kaluza-v2.1-software. Expression-levels of PAX5 in the blasts was determined as a normalized mean-fluorescent-intensity (nMFI) as described by Sędek et al. (doi:10.1016/j.jim.2018.03.005). The pattern of expression (homogenous/heterogeneous) was determined as coefficient-of-variation of immunofluorescence (CV-IF).
Results: Of the four permeabilization reagents, Foxp3-fixation-kit (eBiosciences) had shown the best results for the anti-PAX5-antibody expression with titration volume of five-microlitre. PAX5 expression was studied in 21 AML, 25 B-ALL patients and 2 mixed phenotypic acute leukemias (MPAL, B/myeloid). T-cells and normal B-cells were taken as negative and positive controls with a median (range) MFI of PAX5 1.84 (0.18-1.4) and 5.7 (3.19-17.3), respectively. Median (range) of nMFI of PAX5 in B-ALL and AML patients were 14.7 (3.18-33.1) and -0.62 ((-2.4)-10.8). All the AML patients expressed negative PAX5 (nMFI<3) except one t(8;21) positive AML, which also showed aberrant CD19 expression. Strong expression of PAX5 (nMFI>7) was expressed exclusively in B-ALL blasts (17/23 samples, p<0.001) except the t(8;21) positive AML. Median (SD) CV-IF of PAX5 in B-ALL and AML cases were 58.9 (41.7) and 101.5(131.8) respectively, indicating homogenous expression in B-ALLs (p=0.048). PAX5 was also expressed in two CD10 negative pro-a ALLs and two B/Myeloid MPAL patients.

Conclusion: We first-time standardized flow cytometric evaluation of PAX5 expression in the clinical setting. Foxp3-fixation-kit (eBioscience) found to be the best permeabilization reagent. Flow cytometric PAX5 expression was highly specific for B-lineage ALLs and is a useful additional marker in determining B-cell lineage in acute leukemias.

OP-12

Comparison of Kaluza® and Infinicyt® softwares for the detection of MRD in the follow-up of B-cell progenitor acute lymphoblastic leukemia

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Introduction: Lately, the landscape of flow cytometry was reconfigured with the launching of the Infinicyt® software. This software makes it possible to compare a population of selected cells against a reference database, facilitating the standardization of flow cytometry analysis and the detection of abnormalities in the maturation of a cell lineage. The purpose of this work was to compare the sensitivity of detection of minimal residual disease (MRD) in B Cell Progenitor Acute Lymphoblastic Leukemia (BCP-ALL) between Infinicyt® and the multi-parameter flow cytometry (MFC) software Kaluza®.

Methods: First, 41 samples of normal bone marrows, containing at least 1000 events in the B precursors gate, were selected using a “10 colors B tube” (Kappa, Lambda, CD34, CD10, CD19, CD20, CD22, CD38, CD45, CD5), the Navios® cytometer and Kaluza®. Second, a gating strategy for the B cell lineage was developed on Infinicyt®. Then, the Automated Population Separator (APS) was selected in order to draw a maturation pathway and create a database. Afterwards, 5 bone marrow samples with BCP-ALL and the follow up of the MRD for these samples were analysed using variable tools on both softwares : on Infinicyt® the reference image, the supervised APS and the reference maturation pathway; on Kaluza®, the comparison of marker’s expression between the BCP-ALL blasts and the hematogones using the 10 markers of the B tube and other markers such as CD58, CD81, CD123, aberrant T/myeloid with MFC analysis.

Results: Firstly, as expected the CD19 and the CD22 markers appeared to be essential markers for the selection of the B cell population. Moreover, the CD34 and the CD10 were essential to separate on one hand the lymphoblasts from the mature B lymphocytes and on the other hand two important stages of the hematogone maturation. We were able to draw a maturation pathway in 7 stages and the data obtained was consistent with the literature data. Finally, the comparison of pathological bone marrow samples to the reference database allowed the detection of maturation abnormalities in the 5 BCP-ALL at the diagnosis. The range of sensitivity for the MRD analysis for these samples was comparable between the MFC analysis on Kaluza® and the one on Infinicyt®.

Conclusion: Both methods of analysis gave comparable results for the search of MRD in the follow-up of BCP-ALL without evidence of superiority of the Infinicyt® analysis over the MFC analysis on Kaluza®.
A single tube to screen for both plasma cell dyscrasias and leukemia/lymphoma in the presence of monoclonal gammapathy

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Introduction: The finding of a monoclonal heavy and light chain restricted antibody also referred to as a paraprotein or M protein, both in routine diagnostic testing or following relevant clinical symptoms is discerned is usually followed by the study of the possible entities associated with this condition. Plasma cell dyscrasias including multiple myeloma (MM) and monoclonal gammapathy of undetermined significance (MGUS) are the most frequent causes associated with this condition but neoplastic lymphocyte proliferations must also be ruled out. As such an in house tube was developed in order to identify the affected B or plasma cells, which will subsequently be fully characterized using a specific panel.

Methods: A single 8-color tube including combined detection of surface and intra-cellular antigens was designed: CD45 PB/CD138 PO/Clambda FITC/CD56 PE/CD5 PerCP-CY 5.5/CD19 PC7/cKappa APC/CD38 APC-H7, and applied in 962 samples from October 2013 until May 2019. Light chain restriction, either kappa or lambda can be identified in the plasma cell pool when cells are gated on CD38 and CD138 or in the B cells when gated on the CD19+/low side scatter cells. The presence of CD5 in the tube is also useful as both an internal negative control (surrogate T-cell marker) or in the case of CD19 co-expression (B-Cell Chronic Lymphoproliferative Disorders).

Results: From the 962 processed and analysed samples (98.7% of which were bone marrow aspirates), we identified 335 MM, 6 Plasma Cell Leukemias, 2 Solitary Plasmacytomas, 1 Lymphoplasmacylastic Lymphoma, 313 MGUS (12 concomitant B-CLPD), and 73 B-CLPD. The use of subsequent panels led to the diagnosis of 3 B-CLL, and 70 immunophenotypes compatible with 68 Lymphoplasticmyctic Lymphoma/Waldenström Macroglobulinemia/Marginal Zone Lymphoma, 1 Mantle Cell Lymphoma, and 1 Diffuse Large B-Cell Lymphoma. 214 samples were considered normal/reactive.

Conclusions: The screening of monoclonal gammapathy-associated disorders using this single tube proved to be very useful and efficient in the diagnosis and exclusion of single disorders, and also to enhance the diagnosis of concomitant diseases. It is time and cost-effective and particularly relevant in the context of today’s increasing average life expectancy.

Single platform quantitative analysis of circulating plasma cells and lymphocytes in patients affected by Multiple Myeloma, MGUS and Smouldering Myeloma

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Introduction: Multiple Myeloma (MM) is characterized by bone marrow (BM) infiltration and proliferation of clonal plasma cells. Recent findings have suggested that malignant plasma cells can be isolated in peripheral blood (PB) of patients affected by MM (circulating plasma cells CPCs) and that their identification correlates with a worse outcome, pointing towards their role as possible biomarkers.

Methods: We analyzed 108 samples of patients (pts) affected by MM (27 pts), Smouldering Multiple Myeloma (SMM)(27 pts) and MGUS (54 pts) at diagnosis and 20 healthy controls. The immunophenotype evaluation was performed on EDTA whole blood samples, using a lyse-no-wash technique and a panel of directly conjugated antibodies (CD3FITC-CD56PE-CD4ECD-CD138PC5.5-CD27PC7-CD20APC-CD19A700-CD38A750-CD8PB-CD45KO, Beckman-Coulter). The analysis were performed using a Navios cytometer (Beckman Coulter) and Navios software. The single platform method was used to determine absolute counts, employing fluorospheres (Flow-Count™ Beckman-Coulter). The analysis of lymphocyte subpopulations was performed on lymphocyte population gate, using quadrant dot plot statistics. The absolute plasma cell count per microliter was measured by gating for CD38+CD138+ on all leucocytes. The maximum number of total events to be collected was 500000 or an acquisition time of 600 seconds. A qualitative comparison of surface markers was performed between CPCs and BM plasma cells (BMPCs) for pts with MM and SMM. The clinical data were collected for all patients. Statistical analysis was performed using Graph Pad Prism Version 8.
Results: CPCs were identified in MGUS, SMM and MM of new diagnosis but not in healthy controls. The absolute number of CPCs in MM pts was higher compared with those of MGUS and SMM pts (P value: 0.0168)(mean: 17.57, 0.46 and 0.59 cells/µL, respectively). All CPCs were CD45+, while BMCPs in the same patients were mostly CD45-.

In the MM group, there was a significant correlation between the concentration of CPCs and the level of beta-2 microglobulin, percentage of BMPCs in both biopsy and aspirate, percentage of CD45- BMCPs, and the R-ISS prognostic score (P value:0.0033, P:0.0047, P<0.0001, P:0.0024, P:0.03 respectively), while there was no correlation with the prognostic scores in MGUS and SMM patients. MGUS patients showed a significant higher concentration of NK cells compared to SMM, MM and controls (P value: 0.0139).

Conclusions: Flow cytometry analysis using a single platform-quantitative method is a useful tool for the identification and absolute quantification of CPCs and lymphocyte subpopulations. This technique opens up new perspectives for the biological and prognostic investigations of plasma cell dyscrasias.

OP-15

Plasma cell multiparametric flowcytometric identification in Lymphoplasmacytic Lymphoma, demonstrates characteristic antigen expression profile contributes in diagnosis evaluation and patients' monitoring

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Introduction: Flowcytometric Immunophenotype (FCI) of lymphocytes in Lymphoplasmacytic Lymphoma/Waldenstrom’s Macroglobulinemia (LPL/WM) is considered as the major diagnostic tool for the initial disease diagnosis. However, the exact role of clonal plasma cells (PCs) in diagnosis and outcome is still under investigation.

Methods: B cells and PCs from bone marrow (BM) samples were analyzed. Two samples from patients with IgM-secreting disease (Multiple Myeloma and Marginal Lymphoma) were used as controls; FCI was performed using 6-8 color antibody panels on the SYSMEX CUBE8 or NAVIOS flow-cytometer and data analysis was performed using FCS express V4 or KALUZA Software V2.1.

Results: Bone marrow samples from 13 patients with LPL/WM (M/F:6/8, median age: 71, range 53-85) were analyzed; 10/11 evaluated patients were positive for MYD88 L265P mutation; 9/14 patients were treated with rituximab-based combinations; 7 achieved partial response and 2 stable disease; Median B lymphocytes were 13,5% (1,2-75%) and median PCs were 0,4% (0.04-18%). Clonal B cells were positive for B cell markers i.e. CD19, CD20, CD45, CD22 and surface light chain (LC) and negative for CD5, CD10, CD11c, CD103, FMC7 and CD43; CD38 and CD25 ranged from negative-positive as previously described. In addition, CD24 was positive (moderate to bright) and CD27 was negative to moderate; CD20 was negative to moderate; PCs expressed the same surface LC as neoplastic B cells, albeit at lower level compared with intracellular detection. Surface LC expression was dimmer on PCs compared with B cells; CD38, CD138, CD19, CD45 and CD27 expression pattern on PCs was close to that of normal bone marrow PCs; however, surface CD20 displayed dim expression and CD56 was negative; BM samples from 6 treated patients were evaluated for minimal measurable detection after therapy. All responding patients reduced both B-cell and PCs compartment. Of note, non-responders increased BM clonal PCs even though B cells were dramatically reduced.

Conclusions: Our results confirmed the presence of a typical immunophenotype of clonal B cells of LPL/WM; a possible role of an extended immunophenotype including CD20, CD27 and CD24 needs further investigation; PCs’ immunophenotype of LPL/WM patients revealed a specific profile, different from normal PCs and MM PCs, as they all bared surface LC clonality; this could help in the differential diagnosis between LPL/WM and MM. Interestingly, patients who did not respond to therapy increased PCs compartment suggesting that PCs could be used for response evaluation and disease monitoring, however this warrants further examination in a larger number of patients.
Study of the expression pattern of CD147 (EMMPRIN) in multiple myeloma using multicolor flow cytometry

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Introduction: CD147 (extracellular matrix metalloprotease inducer, EMMPRIN), a transmembrane glycoprotein, is expressed by many different cell types. Its over-expression is associated with increased angiogenesis, tumor growth, lower progression-free survival and poor clinical outcome in non-hematological cancers. Recently, an interaction of extracellular-CyPA secreted by bone marrow endothelial cells and CD147-protein complex has been shown to play a role in multiple myeloma (MM) plasma cell (PC) colonization and proliferation. The abnormally elevated levels of soluble-CD147 have also been suggested to be associated with poor outcome in MM. However, the expression-pattern of CD147 in MM-PCs and normal-PCs has not been studied. We investigated the expression-pattern of CD147 in MM and normal-PCs using multicolor flow cytometry (MFC).

Methods: We analyzed expression-pattern of CD319 (BV605, clone-HIM6) in clonal-PCs from newly-diagnosed MM and normal-PCs from control samples (uninvolved staging BM). MFC characterization was performed on Cytoflex (Beckman-Coulter) flow-cytometer and data-analysis was performed using Kaluza-v2.1-software. Expression-levels of CD147 in PCs was determined as a normalized mean fluorescent intensity (MFI) and it was determined as the ratio of MFI in PCs to CD147-negative lymphocytes (denoted as nMFI). The pattern of expression (homogenous/heterogeneous) was determined as coefficient-of-variation of immunofluorescence (CV-IF).

Results: Expression-pattern of CD147 was studied in 37 MM and eight control samples. Median (standard deviation, SD) of total PCs/viable cells (TPCs) in MM and control samples were 6.6% (11.5%) and 0.58% (0.57%). Median (range) of nMFI of CD147 in clonal-PCs and normal-PCs was 8.1 (2.8-18.5) and 5.7 (3.4-7.5). clonal-PCs had significant overexpression in comparison with normal-PCs (p<0.05). CD147 showed overexpression (nMFI above upper-level of normal-PCs) in 23/37 (62.16%) cases. Median and SD CV-IF of CD147 in PCs was 59.49 and 12.02, indicating its homogenous expression in PCs. Median (SD) of CD147-positive plasma cells (out of total-PCs gated with CD38vCD138vCD45) was 98.9% (6.17%). Median (range) of CD147-nMFI in rest of non-PCs hematopoietic cells (HCs) was 2.8 (1.4-5) and the expression-level of CD147 in PCs was revealed to be brighter than HCs (p<0.001).

Conclusion: In this study, we first time demonstrated the expression-pattern of CD147 in clonal-PCs from MM and normal-PCs using MFC. CD147 showed the bright and homogenous expression in PCs as compared to rest of hematopoietic cells and the expression levels of CD147 was higher in clonal-PCs than normal-PCs. It was found overexpressed in 62% of MM cases.

OP-17

The flow cytometric approach seems not to discriminate double hit lymphomas from Burkitt or high-grade B-cell lymphomas with MYC translocations

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Introduction: Although a consensus has not yet been reached to provide immunophenotypic guidelines supportive of a double hit (DH) lymphoma diagnosis, which would warrant further cytogenetic investigation, flow cytometric (FC) studies have suggested certain immunophenotypic features like CD20 and CD38 intensities in expression, to be associated with a DH lymphoma diagnosis.

Methods: We retrospectively analyzed the FC findings of all patients diagnosed with de novo DH lymphoma, during the last 10-year period, in our hospital. At the same time, all MYC+ Burkitt lymphomas and MYC+ Diffuse Large B-Cell Lymphomas (DLBCL) were retrieved and immunophenotypically compared with the DH group. The size of lymphoma cells was assessed by morphology and by forward scatter (FS) and side scatter (SS) properties compared to normal T-lymphocytes (FS B/T, SS B/T). The antigen expression pattern (% of expression and normalized median fluorescence intensity, MFI) was analyzed and compared between the groups, for CD19, CD20, CD5, CD10, CD38, CD43, CD27, FMC-7, CD79b, CD23, CD22, surface immunoglobulins IgM, IgD, IgG, IgA, Ki-67, bcl-2 and CD45. Statistical analysis was performed with SPSS Statistics 17.0.

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Results: Our patient cohort consisted of 35 patients (26M/9F, median age 67, range 21-88): 13 patients diagnosed with DH (4 MYC+/BCL2+, 4 MYC+/BCL6+, 2 MYC+/CCND1+, 2 CCND1+/ampfMYC, 1 ampfBCL2/MYC+), 14 patients diagnosed with MYC+ Burkitt lymphoma (BL group) and 8 patients diagnosed with MYC+ DLBCL (non-Burkitt MYC+, NB group). The most significant findings were: higher CD38% in BL vs DH (p<0.05), higher CD10% in BL vs NB (P<0.05), higher normalized CD10 MFI in BL vs DH and BL vs NB (both, p<0.05), higher Ki-67% in BL vs DH and BL vs NB (both, p<0.05) and higher FMC-7% in BL vs NB (p<0.05). The differences in CD20, CD38 and CD45 intensity were not statistically significant. Also, lower (SS B/T) and (FS/BT) were observed in DH, although differences were non-significant.

Conclusions: Despite an obvious overlap of FC features between DH, BL and MYC+ DLBCL, CD10, Ki-67 and SS B/T may prove of usefulness in the differential approach. On the contrary, neither CD38 nor CD20 suggested by other authors proved discriminative in our study.

OP-18

The advantage of using multidimensional dot-plots in the diagnosis of acute promyelocytic leukemia and B-cell chronic lymphoproliferative disorders

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Introduction: Flow cytometric analysis is positioned at the beginning of diagnostic algorithms of hematologic malignancies because this method can orientate the diagnostic workout in these heterogeneous diseases. Due to the fast development of flow cytometric examination, more and more information can be obtained on the malignant cells. Efficient data handling is a real challenge and can be achieved by using multi-dimensional dot-plots. Our aim was to establish analysis protocols based on multi-dimensional dot-plots to improve the specificity and sensitivity of flow cytometric examination in acute myeloid leukemia (AML) and B-cell chronic lymphoproliferative disorders (B-CLPD).

Methods: Of the two protocols we developed, the one for identification of acute promyelocytic leukemia (APL) cases included only four multidimensional dot-plots and was created by reanalysis of APL (n=8) and non-APL (n=12) AML cases which mimicked the immunophenotype of APL. In the subsequent 18 months the efficacy of this protocol was tested. Another protocol was established that facilitates the differentiation between various types of B-CLPD, which were categorized to four patient groups: (variant) hairy cell leukemia (vHCL, HCL, n=9), marginal zone lymphoma (MZL, n=5), mantle cell lymphoma (MCL, n=5), chronic lymphoid leukemia (CLL, n=5). Reanalysing the results of these cases, the positions of the different types of pathological cells could be predicted. The protocol was tested in CLL with 12 trisomy cases (n=8) and MCL suspected patients (n=7).

Results: The percentage of pathological cells was well above the cut-off value (90%) in the gates corresponding to their types and never reached the cut-off value in gates different from the investigated cell type in any of the multidimensional dot-plots. Even when this value exceeded 90% in a single tube, the pattern was markedly different from that observed in typical cases. The sensitivity and specificity of the AML protocol proved to be 100% in the testing period. The B-CLPD protocol was able to identify atypical CLL and MCL cases.

Conclusion: Pathological cells are located in specific positions in multidimensional dot-plots, therefore the protocols based on such dot-plots can improve the sensitivity and specificity of the flow cytometric evaluation. The analysis of atypical cases with multidimensional dot-plots provides a more accurate measurement of the percentage of pathological cells.
15 parameter 13-color flow cytometric assay for detailed immune cell profiling in post allogenic bone marrow transplant immune reconstitution studies

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Introduction: Immune cell profiling has become essential to study outcomes in allogenic hematopoietic stem cell transplant (HSCT). Detailed analysis of the immune cell profile has a tremendous potential to distinguish patients at risk for specific infections or immune-mediated diseases and inform vaccination strategies. However, the total white blood cell (WBC) counts usually vary after HSCT and detailed immune cell profiling gets challenging in samples with low WBC counts using standard 4 to 8-color immunophenotyping. Hence, there is a need to standardize immune cell profiling assay using higher parameters for routine clinical practice. In this study, we standardized a two-tube 13-color flow cytometric assay that allows an evaluation of 66 immune cell subsets in peripheral blood (PB).


Results: Immune cell profiling was performed in PB from 20 healthy controls and 37 post-HSCT samples at different time-points. The results (median and range) of major immune cell subsets in 37 post-HSCT samples were as follows. In total WBCs: CD3+ T cell%-16.56(0.01-51.75), B-cell%-0.78(0.004-58.19), NK-cell%-2.44(0.14-33.64), classical monocyte%-7.02(0.01-24.44), nonclassical monocyte%-1.15(0.01-17.09), basophil%-0.38(0.001-9.15), plasma cell%-1.68(0.00-71.50), plasmacytoid dendritic cell%-0.09(0.00-13.59), myeloid dendritic cell%-0.23(0.00-2.95)). In total B-cells: naïve B cell%-71.04(0.01-97.26) and memory B cell%-6.71(0.00-93.91). In total CD4+T-cells: TH1%-1.96(0.00-57.00), TH2%-19.3(0.34-87.24), TH17%-2.73(0.00-25.20), T-reg%-2.47(0.07-38.15). In total CD8+T: TC1%-0.79(0.006-41.37), TC2%-23.17(0.86-84.04), TC17%-2.48(0.00-71.92). Gamma/Delta-T%-5.06(0.10-51.20), LGL%-8.84(1.33-98.81) were in total CD3+T-cells. Time period for restoration of individual components of immune system was measured. Our results demonstrated that innate-immunity recovers much earlier compared to adaptive-immunity. Complete immune recover may take several months to up to 2 years post transplant. Current study provides the deep insight into immune reconstitution pattern and discuss the limitations and strategies to enhance immune monitoring.

Conclusion: We standardized the two-tube 13-color flow cytometric assay that allows quantitation of 66 immune cell subsets in human peripheral blood. We determined its utility in the evaluation of post-HSCT immune-reconstitution in routine clinical practice.

OP-20

Simple adaptation of the Basophil Activation Test to the Euroflow staining and instrument settings improves identification of basophils.

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Introduction: Basophil activation is a promising tool in detection of hypersensitivity type I (allergy) reactions with high sensitivity and specificity. However, proper identification of basophils is impaired in current protocols due to red cell debris formation during the preparation of the sample for flowcytometry (FC) and mostly low numbers of basophils are identified (200-300). In routine FC analysis of blood samples using Euroflow protocols basophils are easily identified. Therefore, we hypothesized that if staining and FC measurement was performed according to the Euroflow protocol basophils would be better defined and basophil activation could be more sensitively determined.

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Methods: A volume of 100µl sodium-heparinized blood was subjected to basophil negative (stimulation buffer) or positive (anti-IgE, fMLP or IL-3) activation controls and stopped with washing buffer (PBS/0,05%NaAz/0,2%BSA) containing 10mM EDTA at room temperature. Washed cells were stained according to the Euroflow AML protocol with CD45-V500 and HLA-DR-V450, IgE-FITC, CD63-PE and CD203c-APC. Cells were further treated according to the Euroflow protocol and measured on the BD FACSCantoII (8 color) using Euroflow instrument settings. We determined basophils in samples as SSC<sup>hi</sup>/CD45<sup>mm</sup>/HLA-DR<sup>neg</sup>/IgE<sup>pos</sup> and basophil activation as CD63 and/or CD203c expression. The test was validated with 27 healthy volunteers using birch pollen.

Results: Although all buffers and centrifugation steps after activation were kept at roomtemperature, CD203c and CD63 expression changes with the different activators were as expected from literature while red cell debris was reduced. An average number of 1268 basophils (0,7 % of the leukocytes) was identified in the 27 validation samples using half the available test volume. A subpopulation of basophils showed an increase in Forward Scatter after activation, indicating a shape change, and this correlated with bright CD63 expression. Preliminary results after storage of the blood for 24h before activation showed that CD203c was not suited to identify activation anymore since it was upregulated in the negative control, while activation was still detected if based on CD63 upregulation. Sensitivity and specificity for birch pollen was 86% (6/7) and 95% (19/20) respectively.

Discussion: To detect basophil activation Euroflow buffers and protocols for centrifugation are well suited if supplied with EDTA. This simple adapted method for basophil detection allows easy implementation in routine laboratory diagnostics, especially if Euroflow protocols are already in place. Since basophils are distinctly recognized and CD203c and CD63 upregulation can be clearly separated, this method may be used to discriminate different types of basophil activation in different conditions.

OP-21

LOU064 - a highly selective, potent covalent oral BTK inhibitor with a promising pharmacodynamic efficacy in healthy volunteer studies

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LOU064 is a low molecular weight molecule, and a highly selective irreversible covalent inhibitor of Bruton’s Tyrosine Kinase (BTK). BTK is a cytoplasmic tyrosine kinase selectively expressed in cells of the immune system such as B cells, macrophages, mast cells, basophils and thrombocytes. It is hypothesized that inhibition of BTK is an attractive therapeutic concept to treat various autoimmune and chronic inflammatory diseases mediated through the Fc epsilon receptor 1 (FceR1 for Immunoglobulin (IgE) E) or the B cell antigen receptor (BCR). We report BTK downstream pharmacodynamic (PD) effect on relevant pathways in peripheral blood basophils and B cells. Two fit-for-purpose flow cytometry assays have been developed and validated at the Biomarker Department of Novartis Institutes for Biomedical Research, and transferred to Contract Research Organization laboratory. Briefly, whole blood was stimulated ex-vivo either with an anti-IgM/IL-4 to activate signal transduction via the BCR or with an anti-IgE to trigger FceR1 pathway. Activation of basophils was investigated by analyzing CD63 and CD203c markers expression, while B cells activation was assessed for expression of CD69 before and after LOU064 dosing. To assure high quality of clinical biomarker data we have performed on-site trainings at involved clinical sites and we set up proper logistics for the shipment of the samples. Emax modeling has been applied to characterize the relationship between LOU064 and BTK target engagement.

The safety, pharmacokinetics, and PD of LOU064 were investigated after single and multiple doses. The first-in-human studies with healthy volunteers included asymptomatic atopic subjects. A dose- and time-dependent inhibition of ex-vivo activated whole blood basophils and B cells was observed following single and multiple-dose administration of LOU064. Blood basophil activation (measured by CD63 expression) reached close to 90% inhibition at single dose of 60 mg at 24 h post dose. In the multiple doses of ≥ 50 mg LOU064 led to near complete inhibition of blood basophil degranulation. The B cell activation marker CD69 was inhibited by ≥ 50% at doses of ≥ 30 mg. Consistent with its covalent mode of action LOU064 provided prolonged PD effect in targeted cells.
The flow cytometry-based assays were key to monitor BTK pathway engagement in clinically relevant cells, and to support dose selection for future trials. These data make LOU064 a strong candidate for the treatment of chronic diseases driven by B cell autoimmunity such as Sjögren’s Syndrome and basophil/mast-cell driven skin diseases such as chronic spontaneous urticaria.

OP-22

Causes and diagnosis of CD3+CD4-CD8- double-negative (DN) T-cell lymphocytosis in adults and children
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Introduction: Small numbers of CD3+CD4-CD8- DN T-cells (<2.5% of T-cells) are found in the peripheral blood (PB) of normal subjects. Autoimmune and inflammatory processes are often associated with mildly elevated DN cells. However, major expansions of DN are rare and challenging to diagnose. We aimed to define the causes and immunophenotypic characteristics of DN lymphocytosis, taking >10% as a diagnostic cutoff.

Methods: We collected clinical and laboratory data on 40 patients (males/females=22/18) with DN lymphocytosis from January 2010 to January 2019, including 31 adults (median age, 66.5 [22-84]) and 9 children (median age, 11 [3-16]). Specific indications for immunophenotyping included presence of lymphocytosis, PB abnormal/atypical lymphocytes, neutropenia, pancytopenia, autoimmune and inflammatory processes are often associated with mildly elevated DN cells. However, major expansions of DN are rare and challenging to diagnose. We aimed to define the causes and immunophenotypic characteristics of DN lymphocytosis, taking >10% as a diagnostic cutoff.

Results: 6 children had autoimmune lymphoproliferative syndrome (ALPS). These DN cells were TCRβ+/CD2-CD5-CD7+, with median percentage 11.15% (10-18.4) and count 215.5/μL (151-597). 3 children had reactive TCRγδ+ lymphocytosis with median DN percentage 28.2% (1155/μL), which was higher than ALPS (P<0.05). Among adults, 1 had ALPS (mother of a child with ALPS). 11/31 (35%) had T-LGL leukemia (7 Tγδ-LGL, 2 Taβ-LGL, 2 biclonal Taβ/γδ-LGL). Diagnosis of T-LGL was based on prominent/coarse granules and/or expression of ≥1 NK marker. In 5 Tγδ-LGL cases we noted loss/weak expression of CD7 and/or CD5. Excluding 1 rapidly aggressive case, Tγδ-LGL ran an indolent course. 5/31 (16%) had hepatosplenic lymphoma (HSPL): 4 TCRγδ+, 1 TCRβ+/TCRδ+. Diagnostically HSPL from Tγδ-LGL was difficult: loss/weak CD7 was seen in 57% Tγδ-LGL vs 60% HSPL; CD57 was expressed in 67% Tγδ-LGL vs 20% HSPL. Prominent/coarse granules were not characteristic of HSPL, however, 1/5 had “dust-like” granules. Median DN were 45.1% (1571/μL [90-10973]) in Tγδ-LGL vs 75% (2973/μL [432-84000]) in HSPL (P=0.36). 2/31 patients (6%) had eosinophilia>5000/μL, an unusual phenotype (TCRβ+/CD7-CD5+CD2+CD16-CD56-CD57), and clonal TCRβ, consistent with hypereosinophilic syndrome (L-HES). 7/31 (22.6%) had reactive DN lymphocytosis (6 TCRγδ+, 1 TCRβ+/TCRδ+); 4 had an underlying condition (CVID, Hodgkin lymphoma, fever, chronic idiopathic neutropenia). Reactive TCRγδ+ lymphocytosis was CD2+CD5+CD7+V62+, with median DN 16.6% (447.5/μL [299-1757]). The remaining patients had TCRβ+/CD56 T-cell lymphoma (2), relapsed cutaneous γδT-NHL (1), and 1 long-standing, asymptomatic, TCRβ+/CD3-CD4-CD8-CD16-CD56+ non-LGL lymphocytosis.

Conclusions: Most common disorders underlying DN lymphocytosis are ALPS or reactive in children and Tγδ-LGL/HSPL or reactive in adults. Features that indicate reactive rather than Tγδ-LGL/HSPL are DN <1800/μL (ROC=0.79) and CD5/CD7/Vδ2 expression. To differentiate between Tγδ-LGL/HSPL, morphology is required.
Normal peripheral blood leukocyte-counts in TCRab-CD8+ LGLL according to the STAT3 mutational status and the presence of bi- vs. monoclonality

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Introduction: TCRab CD8+T-cell large granular lymphocytic leukemia/lymphocytosis (CD8T-LGLL) is frequently associated with other clonal hematological disorders, i.e. B-cell lymphoid disorders and bone-marrow failure syndromes. However, coexistence of synchronic LGL disorders have not been systematically explored, as well as its relationship with the presence of somatic STAT3 mutations and the immune system status. Our aim was to relate the frequency of STAT3 mutations with the presence of mono- or bi(multi)clonality, the distribution of peripheral blood (PB) leukocytes and its clinical impact, in CD8T-LGLL patients.

Methods: PB samples from 31 cases diagnosed with CD8T-LGLL were immunophenotyped by flowcytometry, using EuroFlow panels (LST and T-cell CLPD) (http://www.euroflow.org) to identify abnormal/normal immune cells; 10 age-matched healthy donors were included (controls). Both sequencing of STAT3 (SH2 20/21 exons) and confirmation of TCR clonality were performed on FACS-sorted LGLs by conventional molecular techniques.

Results: 13/31 cases (42%) had ≥2 different PB clonal LGL populations. In turn, the frequency of STAT3 mutation in the whole clonal LGL populations (n=45) was 34%, without differences (p>0.05) in the frequency of bi(multi)clonality between STAT3 mutated (MUT, 5/13 -38%) and wild type (WT) cases (4/14 -29%). Noteworthy, the number of circulating clonal LGLs was not significantly different between either MUT vs WT or mono vs bi(multi)clonal cases. However, PB counts of virtually all normal leukocyte subsets were reduced in MUT (but not in WT) cases (vs. controls), differences reaching statistical significance (ps<0.05) for neutrophils, eosinophils, dendritic cells and total lymphocytes, which were similarly reduced in both monoclonal and bi(multi)clonal MUT cases, while NK-cells and TCRgd-cells were decreased (vs. controls) only in the former group. In contrast, WT cases did not show such a general reduction of PB leukocyte-subset counts. Accordingly, monoclonal WT cases only presented reduced (ps<0.05 vs controls) counts of neutrophils and NK-cells; in turn, bi(multi)clonal WT cases did not show decreased numbers of leukocyte subsets, and even had increased counts (ps<0.05 vs controls) of basophils, classical monocytes and TCRabCD4+CD8-cells. No clinical differences were observed between mono vs. bi(multi)clonal cases, the major impact depended on the presence of STAT3 mutations (higher incidence of neutropenia and other autoimmune diseases, and treatment requirement in MUT vs WT; ps<0.05).

Conclusions: In contrast to WT cases, presence of generalized reduced numbers of leukocyte subsets in CD8T-LGLL carrying STAT3 mutations (independently of having one or ≥2 clones) might be reflecting their association with a more profound immunodeficiency, potentially related with the pathogenesis of the disease.

OP-24

Co-expression of strong CD38 and homogenous CD10 is a highly useful feature to identify Burkitt lymphoma using multicolor flow cytometry

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Introduction: Burkitt lymphoma (BL) is an aggressive B-NHL which requires rapid diagnosis and distinct treatment strategies. The characteristic morphological features, immunohistochemical expression of BCL2(-), BCL6(+), CD10(+) and 100% MIB-1(+) aid in the diagnosis of BL. However, these features may overlap with other CD10+ B-NHL like diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma (FL) transforming to DLBCL making the final diagnosis challenging. Molecular genetic studies for c-MYC, BCL2, and/or BCL6 rearrangements play a crucial role in further categorization. Unfortunately, these tests are time-consuming, relatively expensive and routinely unavailable at many clinical laboratories (especially in resource-limited settings), requiring submission to a reference laboratory, which can delay accurate diagnosis.
Flow cytometric immunophenotyping (FCI) is a widely available, fast and inexpensive technique that routinely uses CD10 and CD38 for the diagnosis and monitoring of hematological neoplasms.

**Methods:** We investigated the utility of CD10(APC, clone-ALB1) and CD38(BV605, clone-HB7) in the rapid diagnosis of BL by analyzing the expression-pattern of these markers in CD10+ B-NHL. The final diagnosis was performed on histopathological and immunohistochemical evaluation. FCI was performed on Cytoflex (Beckman Coulter) and data-analysis was performed using Kaluza-v2.1-software. Expression-levels of CD10 and CD38 were determined as a normalized mean fluorescent intensity (nMFI) as described by https://www.ncbi.nlm.nih.gov/pubmed/?term=S%C4%99dek%20%C5%81%AAuthor%5D&cauthor=true&cauthor=false&tag=29530508 et al. (doi:10.1016/j.jim.2018.03.005).

**Results:** We studied 36 cases of CD10+ B-cell NHL (16 BL; age, 4-67 years and 20 non-Burkitt NHL; age, 31-78 years). Non-Burkitt NHL included 15 FL and 5 GCB-DLBCLs. Median (range) of nMFI of CD10 and CD38 in BL, FL and GCB-DLBCL were 8.3 (0.05-75.9) and 8.71 (0.03-33.4), 9.95 (0.3-33.6) and 0.35 (0.008-6.8), 9.73 (2.1-19.3) and 0.6 (0.2-2.6) respectively. Strong expression was defined as nMFI>7. CD38 was strongly expressed in BL (13/16) versus non-Burkitt NHL (0/20) (median 8.71 vs 0.35, p<0.001). CD10 expression-level didn’t reveal a significant difference, but all 13/16 BL showed homogenous expression of CD10 versus 10/20 in non-BL. Co-expression of strong CD38 and homogenous CD10 was found in 12/16 BL but 0/20 non-BL NHL. Sensitivity and specificity of co-expression of strong CD38 and homogenous CD10 for the diagnosis BL was 100% and 83.3% (p<0.001) respectively. Cytogenetics was available in six BL cases and showed typical MYC:IGH translocation.

**Conclusion:** The co-expression of strong CD38 and homogenous CD10 is highly sensitive and specific in the differential diagnosis of BL from other CD10+ non-BL NHL.

**OP-25**

**Flow cytometry application in Hemostasis: Flow Cytometric Analysis of Platelet Activation and Immune-mediated Platelet Disorders. Basic and Clinical Application**

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**Introduction:** Flow cytometry (FCM) is a useful tool for the study of platelet activation process, *ex vivo* platelet responsiveness to stimulants and inhibitors, ongoing, real-time prothrombotic activity, and immune-mediated platelet disorders.

**Methods:** Analysis of *ex vivo* platelet activation state and microbead assays for antibody-mediated disorders.

**Results:** During coronary angioplasty, blood samples demonstrated activation of platelet GPIIb/IIIa and P-selectin, correlated with vascular re-occlusion.

Women with pregnancy-related hypertension (n-68) demonstrated high expression of p-selectin. During preeclampsia, platelet procoagulant activity increased with highly-activated platelet microparticles (30%) with high-binding (100-fold) of annexin A5. The FCM findings corroborated by elevated plasma markers of thrombin generation and correlated with pregnancy outcome.

Sickle cell disease patients (n-33) showed increased platelet activation which was markedly increased during ischemic painful-episodes, with highly-activated procoagulant platelet microparticles (30%), corroborated by increased plasma markers of thrombin generation and fibrinolytic activity.

Autoimmune thrombocytopenia patients (ITP) (n-62) studied using FCM microbead assay, demonstrated circulating autoantibodies against platelet-specific receptors including CD41a (GPIIb/IIIa), CD42b (GPIb), CD61 (GPIIa), CD41b (GPIIb), CD42a (GPIX), and CD51 (aV). The overall performance of the FCM (ROC plot analysis) showed an area under the curve of 0.96 (p<0.001). At sensitivity of 86%, specificity approached 100%. Neonatal-alloimmune thrombocytopenia tested similarly, demonstrated circulating Anti-platelet HPA-1a specific autoantibodies.

Anti-phospholipid syndrome (APS) studies using annexin A5 competition assay, showed 62 (94%) of 66 APS and 20 (51%) of 39 SLE patients are positive for anti-platelet-phospholipids autoantibodies. Sensitivity was a combination of anti-cardiolipin and lupus-anticoagulant results, and was significantly high in patients with arterial (97.0%) and gestational vascular complications (100%), with overall assay sensitivity of 95% and specificity of 97%.

Using FCM, a rapid, sensitive and specific functional assay was developed for the diagnosis of pathogenic heparin-induced thrombocytopenia (HIT) antibodies, capable of *in vitro* activation of platelets in the presence of heparin. Of 650 samples, 99 (15.3%) were positive by the PaGIA-Heparin/PF4immunoassay and 31 (4.8%) by FCM correlated with the radioactive serotonin-release assay. FCM showed significantly higher correlation with the clinical presentation of HIT than PaGIA (ROC-plot analysis, AUC 0.93 vs. 0.63, p<0.001). At 92% sensitivity, specificity was 96%.
**Conclusion:** FCM is a useful tool for the studies of ex vivo platelet activation and responsiveness, real-time procoagulant activity and the determination of antibody-mediated platelet disorders.

**OP-26**

Detection of circulating tumor cells using highly-sensitive flow cytometry can replace the painful bone-marrow biopsy in the clinical-staging of neuroblastoma

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**Background:** Neuroblastoma with bone marrow (BM) involvement is classified as a stage-4 disease and is associated with poor clinical-outcome. Currently, BM-aspirate/biopsy (BMA/Bx) evaluation is a gold standard for BM metastasis. However, BMA/Bx is an invasive, painful and costly procedure that needs to be performed under anesthesia in children. Hence, there is a need for a non-invasive, safe, reliable and cost-effective method for the assessment of systemic metastasis. Circulating tumor cells (CTC) are the dissociated tumor cells circulating into the bloodstream that provides the source for systemic metastasis. Hence, the detection of CTC indicates systemic metastasis. In this study, we determined the utility of CTC detection using highly sensitive (high-event) single-tube multicolor flow-cytometry (MFC) in the prediction of BM metastasis in Neuroblastoma.

**Methods:** We studied CTC in peripheral blood (PB) and bilateral BMA/Bx for metastasis in pediatric Neuroblastoma. Immunohistochemistry was performed in histopathological suspicious BM biopsies. CTC and bilateral BM aspirate were studied with ten-color highly-sensitive MFC using an antibody-panel that included GD2, CD3+CD19+CD36, CD9, CD13, CD45, CD56, CD81, CD90, CD99, and CD271.

**Results:** We studied 79 patients of neuroblastoma with median age- 3 years (range, 0.6-13 years) and M:F ratio of 2.7. Median (range) events acquired for MFC of BM and CTC analysis were 1,595,890 (3,53,017-11,594,763) and 2,744,713 (214,223-15,459,159). On BMA/Bx examination, 32/79 (40.5%) showed BM-metastasis (bilaterally, 20/79 (26.6%); unilaterally, 13/79 (16.4%)). MFI-analysis revealed BM-involvement in 42/79 (53.2%) with median tumor-cells 1.5% (range, 0.003-49.14%). In 10/79 (12.7%) patients, MFC-BM detected minimal-metastatic-disease (MMD) which was found negative on BMA/Bx examination. In these patients, median-level of MMD was 0.016 (range, 0.003-0.65). CTC were detected in 34/79 (43%) with median tumor-level of 0.0085% (range, 0.0003 to 11.5%). CTC were found in all 32 patients with BMA/Bx metastasis and also in two patients with BM-Bx negative but unilateral MFC-BM involvement. CTC-positivity showed high correlation with MFC-BM-positivity (r=0.82) with 100% specificity and 81% sensitivity for MFC-BM involvement. These findings suggested that CTCs may not be detected in patients with very low-level MFC-BM involvement (<0.05%); however, are definitely detected in all patients with BMA/Bx involvement.

**Conclusion:** Detection of CTC using highly-sensitive MFC can predict BMA/Bx involvement in all patients and is highly specific and sensitive in the prediction of systemic metastasis in neuroblastoma. Hence, CTC detection using highly-sensitive MFC can replace the bilateral bone-marrow biopsy in the clinical staging avoiding painful, invasive, costly and time-consuming BMA/Bx examination for risk-stratification in neuroblastoma.

**OP-27**

Differential alkaline phosphatase activity in CD34+ hematopoietic stem cells as a potential predictor of engraftment

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**Introduction:** Alkaline phosphatase (ALP) is an enzyme highly expressed in primitive stem cells. Hematopoietic stem cell transplantation (HSCT) involves the intravenous infusion of autologous or allogeneic CD34+, cells to reestablish hematopoietic function. HSCT can involve peripheral blood leukapheresis to collect CD34+ cells from autologous or allogeneic donors.
This procedure may be the only curative option in many hematological malignant and nonmalignant conditions, and it is generally safe and well-tolerated. We previously adapted a fluorescent microscopy-based assay for quantifying ALP<sup>high</sup> and ALP<sup>low</sup> cells by flow cytometry in combination with immunophenotyping. The aim of the present study was to use this rapid quantitative assay for detection of ALP activity in CD34<sup>+</sup> hematopoietic cells after and before stem cell collection, as a potential biomarker for short and long term engraftment.

**Methods:** Mobilized peripheral blood and apheresis products (n=21 healthy donors and n=21 multiple myeloma patients) were used in this study. ALP staining was combined with CD34 immunophenotyping and no-lyse no-wash methods. Vybrant DyeCycle™ Violet Stain (Thermo Fisher) was used to discriminate nucleated cells from erythrocytes and debris. Alkaline Phosphatase Live Stain (Thermo Fisher) was used to detect ALP activity as described (Rico, LG. et al., 2016). CD34+/ALP<sup>high</sup> and CD34+/ALP<sup>low</sup> cell counting was directly obtained on the Attune™ NxT Flow Cytometer (Thermo Fisher). For statistical analysis, differences between absolute values in each group were compared using two-sided Wilcoxon rank sum tests. A p-value < 0.05 was considered statistically significant.

**Results:** CD34+/ALP<sup>high</sup> cells were detected in both mobilized peripheral blood and apheresis products. Healthy donors showed significantly higher CD34+/ALP<sup>high</sup> cells numbers when compared with multiple myeloma patients, for mobilized blood (p-value = 0.01, 95% CI = 3.8 – 43.6) and for apheresis products (p-value = 0.007, 95% CI = 115.4 – 1047.4).

**Conclusions:** Differential ALP activity found in CD34+ mobilized cells may suggest that committed progenitors existing in this source may be functionally different. CD34+/ALP<sup>high</sup> cells may be associated with a more primitive stem-like population essential for long term engraftment, whereas CD34+/ALP<sup>low</sup> cells may be associated with a less homogeneous and more differentiated populations with short-term repopulating activity. More studies will be needed to better understand how this promising marker can be useful for indicating, obtaining, processing, and evaluating graft quality.

**OP-28**

Flow cell sorting followed by donor chimerism evaluation is an easy-to-perform tool for MRD verification in patients after allogeneic HSCT

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**Introduction:** Minimal residual disease (MRD) monitoring is a crucial component in the selection of appropriate treatment strategy for leukemia patients after hematopoietic stem cell transplantation (HSCT). Persistence or reappearance of MRD after HSCT is an indicator of inevitable subsequent relapse. Despite the fast development of highly sensitive molecular technologies, multicolor flow cytometry (FCM) remains an indispensable tool for MRD detection. Nevertheless, an immunophenotypic profile of normal and leukemic cells after HSCT (especially with preceding targeted treatment) may be ambiguous, complicating cytometric MRD monitoring. The PCR-based chimerism testing of the preselected suspicious cell population may assist in ascertaining the presence of MRD.

The aim of the present study was to assess the ability of flow cell sorting and further donor chimerism evaluation to verify flow cytometric MRD detection results.

**Patients and methods:** Twenty-six bone marrow (BM) samples were obtained from 20 patients with various hematological malignancies (7 with T-lineage neoplasms, 12 with B-cell precursor ALL (BCP-ALL) and 1 with AML) after allogeneic HSCT. Follow-up MRD studies by 10-12 color FCM revealed doubtful testing results. In 20 cases MRD-suspected cells were sorted, while in the remaining 6 ones normal cells with slight immunophenotypic differences from the BM regeneration patterns were isolated. Sorted cells were then analyzed for donor chimerism by RQ-PCR.

**Results:** The chimerism distribution in sorted cells was successfully evaluated in the vast majority of samples (n=24, 92.3%). The minimum number of sorted cells was 1078. All cell populations being interpreted as MRD demonstrated up to 99% of recipient chimerism, while the cells considered as normal precursors demonstrated complete donor origin. In one case of BCP-ALL, sorted cells populations with an ambiguous immunophenotype were proved to be normal very early BCP of donor origin. This finding helped to disprove relapse development. In one case two tumor populations (CD19<sup>+</sup> and CD19<sup>-</sup>), as well as early BCP, were sorted after the blinatumomab treatment. The chimerism analysis showed that almost all of the CD19<sup>+</sup> cells were normal precursors of donor origin with just slight immunophenotypic deviations. This finding allowed for discontinuation of CD19-directed therapy. Nevertheless, in one case chimerism evaluation didn’t allow to confirm MRD, as the suspected CD45<sup>-</sup> blasts were to be distinguished from BM stromal elements that also could have recipient origin.
**Conclusion:** It has been shown that rather fast and cheap chimerism evaluation in sorted cells is a beneficial tool for MRD verification in almost all complicated cases after HSCT.

**OP-29**

**Characterization of microvesicles, exosomes and friends using multiple scatter combinations**

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**Introduction:** Traditional flow cytometry (FCM) has utilized the intrinsic properties of cells to scatter light as a primary characterization tool since the inception of the technology. The 488 nm laser is most commonly used for this purpose; however, 405 nm laser light is superior for resolution of microvesicles. Particle analyzers like the NanoSight select the laser based on the nature of the material to be characterized. However, only one laser can be used at a time and the parameters are limited to concentration and size distribution of the particles within the sample.

**Method:** In FCM, multiple lasers are used consecutively for measuring many parameters in a sample including the intrinsic light scattering properties from the 488 or 405 nm lasers. Side Scatter of various lasers have been collected simultaneously to various sample types using the range of laser lines on the BD FACSAriaIIIu, Beckman Coulter CytoFLEX, CytoFLEX S and CytoFLEX LX in order to investigate their utility for label-free characterization. Microvesicles from cell lines and clinical samples have been tested using this concept.

**Results:** The results demonstrate that the combination of multiple scatter signals can elucidate patterns that correlate to cell treatment or patient progression.

**Conclusions:** The combination of multiples SSC from the different lasers shows a huge potential for the identification and characterization of different types of populations of cells, microvesicles and/or particles. Since they are label free parameters, the workflow is simplified and the potential for introducing error is minimized. Manufactures and researchers have an opportunity to work side by side to explore and expand these possibilities and develop instruments which are clearly expanding the possibilities of conventional FCM.
POSTER FLASH PRESENTATIONS

PFP-01

Evaluation of myelodysplastic syndromes by flow cytometry. Experience of a center
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Introduction: Myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal hemopathies characterized by ineffective hematopoiesis and cytopenias in peripheral blood, with risk of progression to acute myeloblastic leukemia. It mainly affects elderly individuals, with an annual incidence of 4-5 / 100,000 inhabitants. Diagnosis is based mainly on morphological and cytogenetic studies. Flow Cytometry (FC) is a sensitive technique that identify immunophenotypic alterations in cellular populations. The objective of this work is to evaluate the contribution of FC when there is suspicion of MDS or clinical data of one or more cytopenias.

Methods: Bone marrow (BM) samples from patients with one or more cytopenias and/or with suspicion of MDS from the Maciel Hospital in Montevideo, from January 2015 to June 2019 were analyzed. A five-color cytometer FC500 of Beckman Coulter was used. Labeling was carried out using the lysis and washing technique. A five-tube myeloid screening panel was used (Tube 1: CD4 / CD8 / CD3 / CD45 / CD19, Tube 2: HLADR / CD11B / CD45 / CD13 / CD117, Tube 3: HLADR / CD34 / CD38 / CD45 / CD117, Tube 4: CD64 / IREM2 / CD14 / CD56 / CD45, Tube 5: CD71 / CD117 / CD19 / CD34 / CD45). The fluorescents used were FITC, PE, ECD, PECy5 and PECy7. The monocyte, myeloid, erythroid, lymphoid, and immature precursors population were evaluated. The results were compared with the morphology (myelogram or BM biopsy), which is the gold standard for the diagnosis of MDS. This work complies with the Helsinki Declaration Principles.

Results: 62 patients between 17 and 83 years (median 65 years) were studied. In 28 (45.2%) the morphological diagnosis was MDS and in 34 (54.8%) the diagnosis was not conclusive with MDS. Considering these two groups, the following tendencies were observed by FC in the group of MDS: 1) greater frequency of hypogranularity of the granular series, 2) greater frequency of maturational delay of the granular, monocytic and erythrocyte series, 3) greater frequency of atypia (CD56 expression) in the monocyte series, 4) maturational delay together with aberrant expression in the monocyte series, 5) association of monocytic and granulocytic maturational alteration and atypia in monocytes.

Conclusion: FC phenotypic alterations of the MDS studied correlated with the dysplasia found by morphology, being a useful technique that contributes to the diagnosis. The trends found should continue to be evaluated in subsequent studies.

PFP-02

Comparative evaluation of mast cell detection methods in the bone marrow of patients with myelodysplastic syndromes
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Introduction: The percentage of bone marrow mast cells (MC) might be elevated in some hematologic malignancies such as myelodysplastic syndromes (MDS). Previously we have shown that a flow cytometry scoring system including the MC percentage provided high sensitivity and specificity and an excellent discriminative power between MDS and non-MDS samples. In this study one of our objectives was to explore the optimal conditions for the measurement of MCs by flow cytometry. Furthermore, we investigated the association between percentage of MCs and bone marrow fibrosis, since MC plays a key role in fibrosis of different tissues.

Methods: Seven bone marrow samples collected in K3-EDTA and Na-heparin were labelled and analysed by flow cytometry right after aspiration and on two consecutive days. In addition, in 35 patients the MC percentage was evaluated by flow cytometry and trephine biopsy was used to detect the grade of fibrosis by silver staining and to evaluate the MC percentage by CD117 and tryptase staining.
**Results:** The percentage of MCs were significantly increased in samples collected in K3-EDTA not only at day-0 but also at day-1 or day-2 (p=0.03, p=0.016, p=0.047, respectively). Delayed sample handling did not influence significantly the percentage of MCs. The intensity of CD117 expression on MCs proved to be stable regardless of the type of anticoagulant. On the basis of silver staining, the distribution of patients regarding myelofibrosis (MF) was the following: MF-0 (n=6), MF-1 (n=20), MF-2 (n=8), and MF-3 (n=1). The percentage of MC detected by flow cytometry correlated with the percentage evaluated by tryptase or CD117 immunohistochemical staining (r=0.41, p=0.016 and r=0.38, p=0.028, respectively). Significant correlation was found between the grade of fibrosis and the percentage of MCs detected by flow cytometry (r=0.48, p=0.04).

**Conclusions:** The most suitable sample for MC detection by flow cytometry was the bone marrow aspirate collected into K3-EDTA. The difference of MC percentage depending on the type of anticoagulant was not caused by technical factor, as the gating marker (CD117) intensity did not differ significantly between samples collected in K3-EDTA or Na-heparin. An increased MC percentage was detectable, which correlated with the adverse fibrotic stage of the bone marrow. This raises the possiblity that MC might have a role in the development of fibrosis in some MDS cases.

**PFP-03**

**Eosinophilia and flow cytometry (FC) related studies: a Spanish multicentre experience**

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**Introduction:** Non-haematopoietic conditions and haematopoietic neoplasms (HN) may be associated to eosinophilia. This work describes the FC protocols applied for the study of samples requested for eosinophilia and aims to determine its diagnostic yield.

**Methods:** A group of Spanish clinical FC laboratories answered a survey to investigate their current workflow when “eosinophilia” was the reason for consulting.

**Results:** Between 2016 and 2018, the 9 centres that joined the study received a total of 60,687 immunophenotyping studies. Eosinophilia was the reason for request in 266 samples: 210 (0.34%) peripheral blood, PB; 55 (0.09%) bone marrow, BM and 1 urine. The frequency of this request per year among laboratories ranged between 0.05-1.74% in PB, and 0.07-0.7% in BM. Only 17 patients had serial evaluations. Median eosinophil count (218 cases had available information) was 1,200/mm³ (<100-33,563).

After reception, 46 samples (17.3%) were discarded for FC study because eosinophilia wasn’t confirmed. A sequential strategy for staining was used in 8/9 centres. All included CD3, CD4, CD8 and CD45 in their screening panel, 7 added CD5 and B-cell markers, and 2 centres studied CD7. To characterise abnormal T-cell populations, laboratories designed heterogeneous home-made panels, with agreement on 12/36 reagents in >50% laboratories. Definitive clinical diagnosis was available in 150/220 cases (68.2%), mostly reactive conditions (n=102/150, 68%). In the remaining 48 cases (42 patients), eosinophilia was associated with myeloid malignancies (5 myeloid neoplasms with PDGFR/A/PDGFBR rearrangement, 2 acute leukemias, 2 myelodysplastic syndromes, 1 systemic mastocytosis); lymphoid malignancies (6 T-NHL, 5 B-CLL, 2 Hodgkin’s disease, 2 clonal gammopathies, 1 NK malignancy); eosinophils disorders (14 hypereosinophilic syndrome, 5 lymphocyte-variant hypereosinophilia); and other entities (2 autoimmune thrombocytopenia, 1 hyper-IgE syndrome).
FC detected abnormal T-cell populations in 18 cases: 5 lymphocyte-variant hypereosinophilia, 1 angioimmunoblastic T-NHL, 1 large granular lymphocytic leukemia and 3 reactive conditions. Eight cases lacked conclusive diagnosis. Median percentage of abnormal T-cells as referred to total lymphocytes was 12.5% (0.02-50%).

Conclusions: A FC screening test for all lymphoid populations seems a reasonable approach to a “eosinophilia” request because most of them are finally catalogued as reactive. In this series, lymphomas were infrequent, with a similar FC detection of B-CLL and T-NHL. Further studies are necessary to determine whether eosinophilia associated with B-CLL is reactive or a manifestation of a synchronous myeloid malignancy. FC was crucial to diagnose lymphocyte-variant hypereosinophilia but follow-up and TCR molecular studies are often needed to determine the clinical significance of abnormal T-cell populations.

PFP-04

Validation of a10-color 21-monoclonal antibody screening panel for multi-parametric diagnostic Immunophenotyping
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Introduction: The rapid and sensitive multi-parameter detection, even at a single cell level, renders flow cytometry (FCM) to a powerful tool to distinguish malignant cells from normal cells. In particular, detection of MRD or characterization of very small cell populations (e.g. in cerebral spinal fluid and hypoplastic bone marrow) that can only be analysed by a one-tube determination, needs high-level multi-color analyses. Due to the limited number of PMT’s in most widely used flow cytometers for clinical purposes, a stacking of conjugates in one color is needed to enlarge the number of parameters to detect aberrant populations. Therefore we developed recently a screening panel of 10 colors containing 21 MoAbs directed against target antigens to detect malignancies in all lineages.

Method: After identification and selection of target antigens and an extensive search for suitable MoAb clones, all MoAbs were titrated to find the correct concentration. Subsequently, compensation of spectral overlap and extensive balancing of the panel were performed to establish suitable and correct combinations of MoAbs within one color and preservation of their capacity to separate and identify cell populations. This resulted in the development of a 10-color 21 MoAb screening panel. To test the feasibility of this panel a validation study was performed together with our standard 6-tube 5-color MoAb screening panel.

Results: To validate our new 10-color 21-MoAb screening panel, forty-two bone marrow and peripheral blood samples from normal individuals and patients with various hematologic malignancies were screened with both panels using a Navios 3-laser 10-color instrument. KALUZA analysis software was used to compose protocols for sample evaluation tested with both panels. The capacity to detect subpopulations of all lineages and malignant aberrancies was compared. This analysis resulted in comparable detection of malignancies with both screening panels.

Conclusion: This validation study of a one tube 10-color 21-MoAb panel showed that normal and malignant subpopulations in bone marrow and peripheral blood samples can be equally identified as with standardly in-use panels. By using one tube only, a very high resemblance in detection of subpopulations of all lineages and malignant aberrances, despite the use of multiple combinations of MoAbs within the same color, can be found. The key issue to create such a high-level powerful screening tool is the performance of a correct balancing of the panel and the use of correct analysis protocols.
Immunophenotyping features in specific genetic subgroups with FLT3-ITD and NPM1 mutations in acute myeloid leukemias

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Introduction: In acute myeloid leukemia (AML) is recommended that all patients be tested for the presence of FLT3-internal tandem duplication (FLT3-ITD) mutations. and allelic ratio (AR) (FLT3-ITD/FLT3-WT) be determined in order to establish the genetic risk category. Previously reports showed that FLT3-ITD mutations induce a specific phenotype in leukemic blasts which is characterized by high levels of CD33 & CD123, and that CD33 & CD123 levels are dependent of FLT3-ITD AR levels.

Methods: Thirty-six FLT3-ITD AML cases without APL were included in the current analyses, of which 18 (50%) were positive for NPM1 mutations. Control group - 84 FLT3-ITD negative AML cases. Gating strategy was based on selecting leukemic blasts from the CD45/SSC gate, verified by CD117, CD34 and HLA-DR backgating. The antigen panel tested was: CD4, CD7, CD9, CD13, CD14, CD33, CD34, CD56, CD64, CD71, CD117, and CD123. Quantitative antigen expression was determined as the ratio between the geometric mean fluorescence intensities (MFIs) of leukemic blasts which were normalized to the MFIs of negative lymphocyte populations for the respective markers. FLT3-ITD DNA and RNA analyses were performed, under the same conditions, by capillary electrophoresis.

Results: Of the 36 FLT3-ITD cases, 26 patients had good quality RNA samples. Compared with the control group, FLT3-ITD cohort presented significantly higher CD7, CD33 and CD123 levels (Mann-Whitney U Test). Impact of FLT3-ITD levels on antigen expression was assessed by separation of patients into 2 groups according to: (1) AR <0.5 (15 patients) & AR ≥0.5 (21 patients), according to current AML guidelines; (2) RR < 0.8 (11 patients) & RR ≥0.8 (15 patients); (3) FLT3-ITD mRNA abundance RR/AR= 1 (5 patients) & RR/AR > 1 (20 patients), 1 excluded patient presented RR/AR ratio <1. There were no statistical differences between CD7, CD33 and CD123 levels for all sets of 2 groups (Mann-Whitney U Test). The correlation between CD7, CD33, CD123 MFI ratio values and the 3 FLT3-ITD quantitative parameter values was also tested, without significant correlations (Pearson’s correlation).

Conclusion: Presence of FLT3-ITD mutations induces a specific antigen profile in AML blasts, however the profile is essentially unchanged among the FLT3-ITD cohort regardless of the FLT3-ITD expression level. Our data does not correlate with previous reports in which both CD33 & CD123 expression were influenced by the FLT3-ITD AR. Possible reasons for these results could be due to differences in the statistical tests used, the different AR cut-off, or it could represent differences between patient cohorts.

Identification of High Risk Immunophenotypic Profile Correlating with Primary Induction Failure, Shorter Overall and Disease-Free Survival in NPM1-Mutated AML

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Introduction: Although sharing a driver gene mutation, NPM1-mutated (NPM1mut) acute myeloid leukemia (AML) is a clinically heterogeneous disease with variable outcome; to date a comprehensive description of the immunophenotypic landscape in NPM1mut AML has not been reported; our aim was to investigate the association between specific immunophenotypic patterns and clinical outcome in this AML subset.

Methods: We identified 64 NPM1mut AML cases treated between 2010 and 2018 with intensive chemotherapy. Flow cytometry was performed on diagnostic bone marrow samples (BM) in order to fully characterize the immunophenotypic profiles of leukemic populations. We focused our attention on myeloid cells in the CD45dim SSClow blast area. Based on CD34 expression we identified two main cell subsets: CD34+/CD45dim versus CD34-/CD45dim. The two subgroups were analyzed by using a comprehensive antigen panel. Parameters were expressed as percentage of positive cells and/or as mean fluorescence intensity; 68M samples from healthy donors were used as controls to set normal references.
Results: Cluster analysis led to the identification of 3 immunophenotypic clusters (C1, C2, C3). Distinctive features of C1 were expression of immature antigens (CD117, HLA-DR) on both CD34+/CD45dim and CD34-/CD45dim cell compartments as well as recurrent asynchronous CD11b expression and cross-lineage CD7 and CD25 expression. In C2, CD34-/CD45dim population showed an acute promyelocytic leukemia-like phenotype (CD117-/+, HLA-DR-) albeit with low SSC and CD13dim. In C3 the leading trait was differentiation to monocytic lineage, with low frequency of aberrant antigen expression. Survival analysis by cluster revealed significant differences in terms of DFS and OS (P=0.006; P=0.05, respectively). Patients belonging to C1 had a higher relapse risk and a relatively poor outcome when compared to patients classified as C2 and C3. Since the latter categories had a substantially superimposable outcome, we compared patients from C1 to the rest of the population as a unique entity: C1 cluster correlated with significantly shorter survival. Along with FLT3-ITD and age, C1 phenotype emerged as an independent predictor for DFS and OS. While CR rates after first induction cycle were comparable between C1 and Non-C1 patients, C1 was enriched with patients not achieving CR after 2 or 3 induction cycles (36% vs 11%; P=0.016; OR 2.024). In multivariate analysis, a C1 profile was the sole predictive factor for not achieving a CR anytime.

Conclusion: Our analysis led to the identification of 3 distinctive immunophenotypic profiles significantly correlating with clinical outcome. C1 cluster enucleated patients at high risk of refractory disease and shorter DFS and OS.

PFP-07
Wide panel screening for new useful markers for MRD monitoring in pediatric BCP-ALL

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Introduction: There are several well-established antigen markers commonly used in flow cytometric (FC) diagnostics of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Apart from cell lineage confirmation, a number of other antigens is used to determine possible genetic abnormalities or facilitate subsequent monitoring of minimal residual disease (MRD). To enhance the sensitivity and specificity of MRD detection, new markers should be used, preferably in ≥10-color panel setting. The aim of our study was to select new candidate markers for MRD monitoring from 12 antigens tested at diagnosis. Here we present the preliminary results of the screening phase.

Methods: The study group consisted of 66 consecutive pediatric BCP-ALL patients treated in 14 centres of the Polish Pediatric Leukemia and Lymphoma Study Group. The bone marrow samples were processed and analysed centrally at Medical University of Silesia in Zabrze, according to sample preparation protocol recommended by EuroFlow. 12 new surface markers were screened at diagnosis: CD371, CD45RA, CD72, CD44, CD27, CD50, CD43, CD82, CD40, CD273, CD274 and CD74 in 10-color setting. The frequency of all markers at BCP-ALL diagnosis was determined, as well as their relative expression level. For this purpose, median fluorescence intensity (MFI) values of blasts were compared with the MFI of positive and negative reference populations, determined individually per marker. 4 levels of expression were distinguished: negative, dim (low), strong and overexpression.
Results: 5 of 12 antigens: CD72, CD273, CD82, CD45RA and CD43 showed the highest occurrence rate on leukemic blasts at BCP-ALL diagnosis (95%, 92%, 82%, 84% and 79% of cases, respectively). The expression levels were variable; in majority (68%) of cases expression level of CD82 was strong or higher than that observed on positive reference population. Conversely, CD72, CD273, CD45RA, CD43 showed dim expression in majority of cases. On the other hand, CD27 and CD274 were most often negative or showed dim expression in 9–26% of cases. CD371 was positive only in 3% of cases, however its expression level was strong in all cases. Finally, expression of CD40, CD50 and CD44 was heterogeneous and similar to that observed on normal lymphocytes.

Conclusion: At least 4 of the tested antigens: CD72, CD82, CD273 and CD371 are potentially useful markers for MRD monitoring in BCP-ALL, which require further investigation. Conversely, CD40, CD50 and CD44 exhibited the lowest performance as MRD markers.

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PPF-08

Candidate White Cell Counting (WCC) Reference Method by Flow Cytometry

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Introduction: The current reference method for WCC as stipulated by the International Council for Standardization in Haematology (ICSH) is a single channel semi-automated electronic counter using the aperture-impedance principle. However, analytical errors are inherent, including inaccurate displacement sample volume, recirculation of cells inside orifice tube, and coincidence events. Our aim is to derive a flow cytometric assay that is more accurate, precise and robust.

Methods: A cohort of ‘normal’ peripheral blood (PB) samples were analysed on EDTA whole blood collected <24 hours. Method 1 was based on the ICSH Reference Platelet method, 5uL of CD41 FITC, 5uL of CD61 FITC, 2uL of CD45 V500 were added to 5uL of PB and 100uL of PBS. Sample was incubated in the dark at room temperature (RT) for 15 minutes. A 1:2000 dilution was made and acquisition using fluorescence thresholding off FITC and V500. WCC was calculated by dividing the haematology analyser platelet count by ‘R’ (platelet events/white cell events). Method 2 was based on the CD34 I SHAGE strategy, 2uL of CD45 V500 was added to 100uL of PB and incubated in the dark at RT for 15 minutes. Addition of 2mL NH4Cl followed by incubation in the dark at RT for 10 minutes. 100uL of FlowCount (Beckman Coulter) beads were added before acquisition on the cytometer. Similar methodologies were employed for Perfect-Count microspheres (Cytognos) and TruCount (Becton Dickinson) beads.

Results: Precision was determined in triplicate with a low (0.8), normal (5.1) and high (250) PB. Respectively, the CV were 1.7, 2.8 and 0.7 for method one, and 1.2, 1.1, 2.1 for method two. Accuracy was determined by using Cell-DYN CD29-Plus Control (Streck). Results were within the manufacturer’s acceptance limits for low (3.04±0.4), normal (7.04±0.8) and high (15.9±3.0). Linearity was achieved by serially diluting a high WCC to 133, 66, 16, 4, 1, 0.25. Correlation of 10 patients between the Haematology Analyser (CellDyn Sapphire) WCC with method 1 yielded an R2 value of 0.98, and an R2 of 0.99 for method 2.

Conclusions: Performance specifications of the linearity and precision were excellent. Accuracy were within acceptance limits of the manufacturer’s reference ranges. Correlation was excellent between all three WCC techniques. Further patient accrual is ongoing and will be performed to determine the optimal method. Whilst a long term goal will be to utilize one of these methods in a multicenter study.
Flow cytometry challenge for measurable residual disease (MRD) monitoring in B-acute lymphoblastic leukemia (B-ALL) patients receiving targeted immunotherapy (TI)


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Introduction: TI with Blinatumomab (Bln) or Inotuzumab ozogamicin (IO) are a salvage option B-ALL patients with MRD, in which high rates of MRD negative can be achieved, allowing a bridge to transplantation and longer survival. However, some patients relapse with a phenotype lacking CD19 or CD22 expression can be found in blast cells. Those escape variants have also been described under CAR-T cells therapy and have to be considered in flow cytometry immunophenotyping (FCM) analysis when monitoring MRD. We propose to review the monitoring of MRD by FCM of patients with B-ALL treated with TI in our center and who relapsed.

Methods: FCM monitoring were performed in bone marrow aspiration (BMA) samples, using 8-colour combinations of monoclonal antibodies adapted to the TI received and diagnosis phenotype. Stained cells were measured in FACS Canto II cytometer equipped with the FACS DIVA software and analyzed with the Infinicyt software with a sensitivity (S) 10⁻⁴.

Results: Between 2015 and 2018, 2 B-ALL patients relapsed after TI, but only one showed immunophenotypic changes. The patient was a 72-year-old woman diagnosed of common B-ALL who started PETHEMA-LAL-OLD-07 protocol. BMA at the end of induction was in morphological complete response (CR) with MRD positive (0.12%). After 3rd consolidation cycle (C), MRD persisted (0.04%), so she started Bln and achieved MRD negative (S 10⁻⁴) after 1°C. However, after 3rd C, a new CD19- blast cell population was detected (0.07%), with a lower expression of CD20, while the other markers were similar to the diagnostic. In normal B cells (0.05%) the intensity of CD19 expression was preserved. Blast cells increased after 4°C (2.9%) with an associated morphological relapse (21%). She started IO and reached CR and MRD negative (S 10⁻⁴) after 1°C. Treatment was stopped after 4°C due to upper gastrointestinal bleeding, and in the BMA previous to the next C programmed, CD19- CD20dim CD34+ blast cells were detected again (0.55%). All blasts lost CD22 expression, and only 9% maintained the CD10 expression, and the rest lost it. The patient was not candidate to any other therapy, she started palliative treatment and died 2 months later.

Conclusions: From our experience, changes in immunophenotype of B-ALL blast cells are frequent after TI, due to loss of CD19 and CD22 expression. Therefore, FCM analysis in patients treated with TI demands a high degree of expertise and attention, to avoid the loss of detection of new populations that could emerge with different phenotype.

Identical clinical performance of the Fetal Cell Count™ kit version IV and III for quantification of Fetomaternal Hemorrhage

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Introduction: In Fetomaternal Hemorrhage (FMH), fetal red blood cells (fRBCs) enter the maternal circulation, which may result in severe fetal anemia or alloimmunization against fetal blood groups. Quantification of the amount of FMH is important in obstetrical management of pregnant women. The Fetal Cell Count™ (FCC) kit (IQ Products, Groningen, The Netherlands) is a flow cytometric assay for fast, sensitive, and specific quantification of FMH, using antibodies against fetal hemoglobin (HbF) and maternal carbonic anhydrase-II (CA-II). Currently, the FCC kit is improved by the substitution of a polyclonal anti-CA-II antibody (FCC-III) with a monoclonal anti-CA-II antibody (FCC-IV). The aim of this study is to evaluate the clinical performance of the FCC-IV.
**Methods:** Peripheral blood samples of 184 pregnant women suspected of FMH and spiked samples containing 0% – 5% cord blood cells were included in this study at two different medical centers. Quantification of fRBCs was performed simultaneously with both the FCC-III and FCC-IV according to the manufacturer’s instructions. Erythrocytes were gated based on logarithmic amplification of the forward scatter and side scatter properties. Next, positively and negatively stained erythrocytes were quantified by quadrant dot-plot analysis, determined by the use of fluorescence minus one controls. Within the erythrocyte population, fRBCs were identified as HbF-CA⁺, and adult RBCs as identified as HbF-CA⁻. To assess the agreement between FCC-III and FCC-IV Passing-Bablok and Bland-Altman analyses were performed.

**Results:** The results demonstrated good linearity and precision of both kits for spiked samples (correlation of 0.9921 at location A and 0.9995 at location B). Additional Bland-Altman analysis of the HbF CA⁺ and HbF CA⁻ populations in spiked samples demonstrated no significant deviation between both FCC versions. Analysis of 184 patient samples exhibited equal clinical performance of the FCC-III and FCC-IV with a non-significant bias of 0.998 (CI95%: 0.991-1.005). Furthermore, analysis of eight FMH positive patients by both versions of the FCC kit revealed an outstanding agreement and no significant differences between FCC-III and FCC-IV (bias of 1.005 (CI95%: 0.904 – 1.106)).

**Conclusions:** This clinical performance evaluation study demonstrates identical results between the FCC-IV and FCC-III. The substitution of the polyclonal anti-CAiI antibody with a monoclonal anti-CAiI antibody did not change the performance specifications of the FCC kit. Therefore, the Fetal Cell Count™ IV kit can be used for the detection and accurate quantification of fRBCs in maternal samples, providing gynecologists and obstetricians with reliable results in cases of FMH.

**PFP-11**

**Ambiguous immunophenotypic data in Burkitt lymphoma: rare cases of discrepancies in diagnostics**

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**Introduction:** Complete differential diagnostics of Burkitt lymphoma/leukemia (BL) is based on integration of flow cytometric, morphologic and cytogenetic features. The discrepancies in these laboratory findings may challenge the correct diagnostics. Flow cytometry (FCM) provides a rapid assessment of the leukemic blast immunophenotype in bone marrow or peripheral blood. The presence of surface IgM (sIgM) alone or with the light chain restriction indicates a mature blast phenotype usually observed in BL. Nevertheless sIgM expression is seldom observed in case of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). FAB L3 morphology of bone marrow blasts and MYC-gene rearrangements together with FCM data complete the classical complex of BL laboratory signs. In present study, we report 10 cases of these features discordance.

**Methods:** Between 2010 and 2016 10 pediatric BL patients (aged 1-18 years; median age 10 years; M:F 8:2) with discrepant immunophenotypic, cytomorphologic and cytogenetic features were studied. These children were diagnosed as BL because of clinical and pathological studies. FCM (6-8 color) was used for evaluation of CD10, CD19, CD20, CD22, CD24, CD34, CD45, CD58, CD38, iCD79a, NG2, CD13, CD33, CD117, CD15, immunoglobulins heavy/light chains expression by leukemic blasts in bone marrow. The conventional cytogenetic analysis included G-banded karyotyping and fluorescence in situ hybridization (FISH).

**Results:** Majority of cases (n=8) were negative for sIgM but had typical rearrangements of the MYC gene, e.g. t(8;14)[q24;q32], t(8;22)[q24;q11] or t(14;18)[q32;q21]. Two cases exhibited L2 blast morphology. Two cases were negative for MYC rearrangements or sIgM expression. Two MYC rearrangements, t(8;14)[q24;q32] and t(14;18)[q32;q21] were found simultaneously in one sIgM-negative patient. The L2 morphology was found in two patients, although MYC abnormalities typical for BL were detected. In one case, undifferentiated blasts were positive for sIgM and negative for MYC rearrangements. The presence of L2 blasts in one case contradicted with either immunophenotypic or cytogenetic data. Among patients who did not have any data on extramedullary lesions, one sIgM-negative case was diagnosed as BL according to cytogenetic and morphologic studies. Another case demonstrated discrepancies between the cytogenetic and immunophenotypic/morphologic findings.

**Conclusions:** The presented cases taken together with the published data demonstrate the importance of a thorough multidisciplinary approach in the assessment of laboratory results. Lack of sufficient set of laboratory data and leaning only on FCM results poses a risk of incorrect or delayed diagnosis and inadequate therapy.
PFP-12

**Patients with hematogones after allogeneic stem cell transplantation for acute lymphoblastic leukemia show reduced GvHD severity and improved overall survival**

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**Introduction:** Benign precursors of B lymphocytes, termed hematogones (HGs), are observed in the regenerative state of hematopoiesis following chemotherapy or allogeneic hematopoietic stem cell transplantation (allo-HSCT). Recent reports suggest that emergence of HGs is associated with better outcomes following allo-HSCT. The aim of this study was to investigate the clinical and prognostic significance of HGs in clinical outcomes of patients with acute lymphoblastic leukemia (ALL) who underwent allo-HSCT.

**Methods:** The patient cohort included 52 ALL patients, 35 males and 17 females, with a median age of 34 years (16-60 years). Disease status at the time of transplantation was complete remission 1 (CR1) in 40 patients, CR2 in 8 patients and refractory disease in 4 patients. The median number of CD34+ cells transplanted was 6.5 x 10^6/kg (range 2.24 to 13.28 x 10^6/kg). Graft source was peripheral blood stem cells (PBSC), bone marrow (BM) and umbilical cord blood (UCB) in 46, 5 and 1 patients respectively. Conditioning regimens consisted of total body irradiation/cyclophosphamide (TBI/CY) for 39 patients, busulfan (BU/CY) for 4, and fludarabine-based reduced-intensity conditioning for 8 BMT recipients, respectively. The median follow-up period for survivors after allo-HSCT was 390 days (range 35-2169). HGs were studied by flow cytometry analysis of bone marrow samples on day 30 and on day 90 in 37 and 38 patients respectively, while 30 patients had available data for both timepoints.

**Results:** HGs were confirmed in 24/37 (=64.8%) patients on day 30 and in 19/38 (=50%) on day 90. Among the HG-positive patients, the frequency of HGs did not exceed 1%. The median time of engraftment was 13 and 14 days for the neutrophils and platelets respectively. There was no significant relationship between the day of engraftment and the frequency of HGs. Due to few BM/UCB grafts and RIC conditioning regimens, no association with HGs could be made. Other factors such as recipient age, donor age and donor type or HLA-matching were not associated with HGs. Interestingly, only 1/24 patients with positive HGs on day 30 developed acute grade III-IV graft-versus-host disease (aGvHD) (p=0.02). Moreover, there was a trend for an advantage in overall survival (OS) in patients presenting HGs on day 90 sampling. (68% versus 54% 2-year OS, p=0.067)

**Conclusions:** The presence of HGs in ALL patients after allo-BMT shows a favorable impact on aGvHD severity and post-transplant overall survival. Longer follow up in larger cohorts may reveal additional correlations in graft-recipient interaction.

PFP-13

**Comparison of cell counting methods: Standard Hematology Analyzer versus Volumetric Cell Counting Flow Cytometry**

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**Introduction:** Cell counting is crucial for the hematology laboratory and many methods are available nowadays. Major neutrophils, lymphocytes and its subsets enumeration is very important for diagnosis and monitoring of a variety of conditions affecting the immune and hematopoietic system. Hematology analyzers use impedance and flow cytometry for cell number and percentage determination, as SYSMEX XE 2100 lines. New flow cytometry technologies also use beadles volumetric counting for single platform enumeration showing precision and reproducibility. In this study we demonstrated the comparison of volumetric cell counting and the standardized hematology analyzer one in peripheral blood (PB) and bone marrow (BM) samples.

**Methods:** 14 BM and 17 PB samples were processed at the SYSMEX XE 2100 analyzer (one time) and at Beckman Coulter CytoFLEX flow cytometer (three times). The samples were antibodies free, and the results were compared using Paired t-test and Pearson’s correlation.
Results: 14 BM samples had media count of 32571 (2450-108930) / uL cells in the SYSMEX XE 2100 and 32146 (2596-107008) / uL cells in the CytoFLEX ($X^2 = 1 \ p< 0.001$ / paired t-Test $p = 0.128$). The 17 PB samples had media count of 8545 (3860-23230) / uL cells in the SYSMEX XE 2100 and 8713 (3697-23509) / uL cells in the CytoFLEX ($X^2 = 0.996 \ p< 0.001$ / paired t-Test $p = 0.127$). PB neutrophil absolute counting: media count of 4178 (900-13250) / uL cells in the SYSMEX XE 2100 and 4125 (950-13150) / uL cells in the CytoFLEX ($X^2 = 0.994 \ p< 0.001$ / paired t-Test $p = 0.217$). PB Lymphocyte counting : media count of 3357 (540-6100) / uL cells in the SYSMEX XE 2100 and 2536 (529-7399) / uL cells in the CytoFLEX ($X^2 = 0.979 \ p< 0.001$ / paired t-Test $p = 0.454$). PB monocyte count : media count of 672 (113-3706) / uL cells in the SYSMEX XE 2100 and 798 (80-1620) / uL cells in the CytoFLEX ($X^2 = 0.961 \ p< 0.001$ / paired t-Test $p = 0.04$).

Conclusions: These results show a good correlation between the two methods allowing the use of beadless volumetric cell counting for hematopoietic cell determination. Based on this we are intending to perform lymphocyte subset (CD4, CD8, CD19, CD20, CD16, CD56) enumeration and compare this new methodology with the standardized beads based one, looking forward to have less costly and time saving workflow.

PFP-14

Proficiency testing of Paroxysmal Nocturnal Haemoglobinuria (PNH) using electronic data files.

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Introduction: PNH, is a rare acquired stem cell disorder, characterised by GPI-linked surface antigen loss. Flow cytometry is recognised as the method of choice to detect/quantify PNH red blood cells (RBC) and white blood cells (WBC) enabling accurate treatment and monitoring. The UK NEQAS LI proficiency testing (PT) programme has identified interlaboratory variation in detection and quantification of PNH clones. To identify the proportion of variation arising from sample testing versus that from subsequent data analysis, a series of electronic PT cases have been developed. This study looks at the analysis of anonymised data files from actual PNH cases and highlights the findings of the first electronic PNH PT distribution.

Methods: UK NEQAS LI issued 4 FCS data files to all participants (n=191) in the PNH PT programme. Patient files were obtained with differing clinical scenarios and anonymised prior to issue. Samples were stained using the 2018 ICCS/ESCCA consensus guidelines 5 colour protocol. 150,000 events were acquired on a FACSCanto II analyser for each sample giving a potential limit of detection of 0.02%. Centres were asked to analyse all samples and return results to UK NEQAS LI.

Results: 78/191 (40.8%) returned results for data file analysis. 78.6% of laboratories stated that they followed the 2018 guidelines; however, analysis found that most (62% RBC, 83% WBC analyses) were missing essential time plots. Inter-laboratory agreement with respect to clone presence/absence was 91% for RBCs, 96.8% for monocytes and 98.9% for neutrophils, comparable to levels seen when using stabilised blood samples in the existing PT programme. Comparison of PNH clone sizes from the electronic exercise to matched stabilised samples issued in the existing PT programme found average variation of clone size, as measured by inter-quartile range, was almost ten times higher in the wet samples (average IQR 1.64%) than the electronic analysis (average IQR 0.17%).

Conclusions: The results highlighted that data analysis is not the main cause of variation when performing PNH testing, but issues remain related to data analysis and guideline adherence. The lower variation in clone size measurement seen in the electronic exercises suggests that testing methodology and not analysis is the main cause of variation in PNH testing, although further exercises are required to prove this. This exercise also suggests that whilst electronic exercises are a useful PT tool, they must be used in conjunction with PT programmes using wet samples to facilitate full process monitoring.
**EuroFlow-guided comparative evaluation of technical features and performance of newly-available high-end cytometers for leukocyte immunophenotyping**

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**Introduction:** Flow cytometry (FC) is currently rapidly expanding in its (pre)clinical applications, particularly for immune monitoring. This requires novel technical features, such as analysis of more cells, less sample volume, less time, more colors, better resolution. Our aim was to critically evaluate the performance of several instruments as valid alternatives for current reference instruments.

**Methods:** Four different >20-color high-standard flow cytometers recently available on the market were tested to evaluate vendors’ claims and identify critical factors impacting on data quality; they were evaluated in parallel in two EuroFlow centers (LUMC and USAL). The aim of the study is beyond recommending equipment purchasing. Therefore technical details linking to the vendor’s identity were excluded. The following parameters were assessed (March, 2018 – June, 2019): optical configuration and actual number of fluorochromes detectable simultaneously; overtime behavior (over-time instrument quality-checks; several months-intervals); acquisition stability (45-minute long-runs); carryover; abort rates; effect on data quality of sample concentration (four concentrations, three technical replicates) and sample input rate (depending on system) using both beads and cells. For cell-based tests, normal peripheral blood samples were stained using multi-color marker combinations (16 to 25 colors) for leukocyte characterization (designed per individual cytometer).

**Results:** All tested cytometers claimed the usage of >20 fluorescent parameters (n=21-27). However, considering redundancy in detectors, signal interaction/spread and fluorochrome availability, on average only 84% (76-92%) of the fluorescent parameters (n=19-25) could be used simultaneously. All tested cytometers were stable overtime, but for 2/4 cytometers stability of signal during long-runs (Height and Area) was not optimal. In 2/4 systems a significant effect (absolute coefficient of variation – CVs of signal of dim and bright peaks >5%) of flow rate >60µL/min (not of concentration) was documented in bead-based assays. Staining indexes (SI) variability related to each marker-detector pair at different speed and concentration were calculated in cell-based assays. Variations in intra-assay %CV of SI within +/-15% were considered acceptable (i.e. no significant impact on data quality). This information, together with abort rate was used to calculate the maximum acquisition speed (4.700-38.000 events/second). The maximum speed value claimed by the companies (30.000-100.000 events/second) was confirmed in 1/4 cytometers.

**Conclusions:** Keeping up with the new requirements in clinical flow cytometry, instrument replacement becomes a challenge. The current study highlights the necessity to investigate whether the manufacturer’s technical claims of the new instruments meet the eligibility criteria and requirements, as needed to further progress towards routine clinical FC.

**Development of a dried-down, multicolor reagent solution for enhanced flow-cytometric applications**

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**Introduction:** Multicolor flow cytometry is a powerful tool to identify, analyze and enumerate multiple cell populations phenotypically. As part of its custom reagents program, BD offers dried-down reagent cocktails for a diverse range of flow cytometry applications. The dried-down cocktails provide enhanced reagent stability, simplify the assay workflow, enhance operational efficiency and enable assay standardization across instruments, operators and testing sites.

**Methods:** The emergence of BD Horizon Brilliant™ dyes has resulted in significant demand for these dyes in multicolor reagent panels. However, cocktailing multiple BD Horizon Brilliant™ dyes result in unwanted dye-dye interactions. To address this issue, BD has developed a technology that enables the delivery of multiple BD Horizon Brilliant™ dyes in a single-use, dried-down format. We designed a 7-color panel (CD3, CD4 CD45A, CD25, CD127, CD15s, CD161) for the identification of Tregs and the characterization of the different Treg subsets (naïve, effector and transitional) in a given sample. Additionally, the panel allows the assessment of the different functional subsets of Tregs, including the IL-17-producing Tregs (CD161⁺), as well as the potently immunosuppressive CD15s⁺ Tregs.
**Results:** As part of assessing the feasibility of the reagent-drying technology, we compared the performance of the 7-color Treg panel in a dried-down state and as a liquid cocktail using the BD FACSLyric™ flow cytometer. Our results show that the dried-down cocktail is free of unwanted dye-dye interactions and is equivalent to the liquid cocktail in terms of resolution of the different functional Treg subsets. Specifically, the percent-positive cells measured for the different Treg subsets in a given sample are comparable when using the dried-down cocktail or the liquid cocktail. This data demonstrates the feasibility of the reagent-drying technology and sets the stage for its application in diverse areas of flow-cytometry-based research.

**Conclusion:** The development of the dry down technology enables manufacturing of dried-down reagent cocktails containing up to five BD Horizon Brilliant™ reagents.

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**PFP-18**

**Automation of workflow in a clinical laboratory: Integration of Pre-analytical set-up using the BDFACSDuet™ and analysis on the BD FACSLyric™ cytometer**

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**Introduction:** Manual set up and processing of flow cytometry is an error prone process which utilises a significant amount of scientific staff time. In addition, manual work, including steps such as antibody selection, pipetting, and transfer of primary and secondary tubes have been identified as potential sources of error in flow cytometry. Here we investigate parallel set up of (a) manual processing and (b) using an integrated system made of a sample preparation unit and a flow cytometer.

**Methods:** An observational study was conducted at a National Health Service acute care clinical laboratory in collaboration with a lean specialist for the purposes of workflow analysis. The laboratory workflow for diagnosis of primary immunodeficiency (CD4 and TBNK) was mapped for 2 days, over 15 hours. Videotape was reviewed and analyzed to measure the two methods of laboratory time spent testing samples.

**Results:** In processing a batch of 10 samples, hands on time was reduced by 46%, with a batch of 20 samples hands on time was reduced by 69%. Individual processing steps were reduced by 71% and critical error prone steps were totally eliminated.

**Conclusion:** This observational study quantified manual work resulting in substantial cost to the laboratory in indirect costs and opportunities for error. The results suggest that, in addition to reducing the potential for error, minimizing unnecessary manual steps in the flow cytometry workflow can reduce laboratory indirect costs and improve efficiency.

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**PFP-19**

**Multiple myeloma minimal residual disease detection in stem cell apheresis product**

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**Introduction:** Multicolor flow cytometry (MFC) is well-suited to study biological samples containing plasma cells. Multiparameter cytometry with evaluation of at least 8 markers in a single tube can identify abnormal plasma cell phenotypes at MRD levels if sufficient cell numbers are evaluated. Because the aberrant phenotypes of clonal plasma cells are readily distinguishable from normal plasma cells, flow MRD in applicable in virtually every MM patient. Autologous stem cell transplantation (ASCT) is part of first line therapeutic approaches for fit patients with multiple myeloma (MM). MRD negativity after ASCT has been previously shown by a few groups to be predictive of favorable progression free survival (PFS) and overall survival (OS). Although post ASCT MRD has been well studied, MRD by MFC in stem cell apheresis product has never been described.
Our objective was to investigate whether it is possible to identify abnormal plasma cells in stem cell apheresis products. This study was approved by UNICEUB ethic board and was conducted according to the declaration of Helsinki.

**Methodology:** Up to three hours after collection, 1mL of peripheral blood stem cell apheresis product was processed according to bulky lysis previously described protocol. Two eight colors tubes were prepared using antibodies combinations including CD19 APC-H7, CD27 PerCP-Cy5.5, CD38 PE-Cy7, CD138 APC, CD45 V500, CD56 BV421, CD81 FITC, CD117 PE, KAPPA FITC and LAMBDA PE. Samples were acquired with a FACSCANTO II (BD Bioscience) and analyzed using Infinicyt (Cytognos) software.

**Results:** Six samples from different patients were studied. Number of events analyzed ranged from 6822250 to 8020156 (mean 7179334). Abnormal monoclonal plasma cells were identified in two samples: in one this population corresponded to 0.003% (205 events) and in the other to 0.01% (970 events).

**Conclusion:** The clinical significance of contaminating MM tumor cells in apheresis product is controversial. Previous studies (López-Pérez et al, 2001 and Galimberti et al, 2003) used gene scanning PCR to detect residual disease and had different findings according to its role in PFS and OS. We demonstrated that MM MRD assessment by MFC in peripheral blood stem cell apheresis product is possible. As previous studies used different techniques to detect MRD and were conducted at a different MM treatment era, we believe the role of contaminating MM tumor cells in apheresis product should be reassessed in clinical trials.

**PFP-20**

MRD detection in Mantle cell Lymphoma using multicolour Flow cytometry in a clinical Laboratory setting

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**Introduction:** Mantle cell Lymphoma (MCL) represents 3-10% of mature B cell neoplasia with an incidence in Europe of 0.45 per 100,000 persons. MCL is characterised by immunophenotyping (CD19+,CD5+, sIgM+,CD20+,CD79b+,CD23- & CD200-), over expression of Cyclin D1 and the t(11;14) translocation. Recent advances in treatment have improved CR rates however low levels of residual disease is a significant cause for disease relapse. In addition to the molecular methods currently used; PCR of clonal IgH rearrangements and detection of the t(11;14) translocation, multicolour flow cytometry methods have been shown to have similar sensitivities.

**Method:** This study validated for use in the clinical laboratory an 8 colour single tube MRD assay described by Chovancov et al using (CD19, CD5, CD20, CD23, CD62L, CD200, CD45 & CD3) with a sequential gating strategy and compared the results for treated MCL patients to those obtained by molecular techniques.

**Results:** Our results showed that this multicolour flow cytometry assay is able to reach a robust sensitivity of 0.025%. A comparison between this method and current molecular methods was carried out on 10 MCL patients post treatment, with very good correlation observed.

**Conclusion:** Our results show that this MRD assay using multicolour flow cytometry is suitable for testing treated Mantle cell Lymphoma patients and is suitable for use in the clinical laboratory setting.


**PFP-21**

Radar plots facilitate quick evaluation of the lymphoid cell compartment using 10-color 15-antibody panel

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**Introduction:** We have previously validated a 10-color 15-antibody lymphoid subpopulation screening panel in two laboratories (Toronto, Canada and Lund, Sweden) using harmonized settings (IntJLab Hematol. 2017;39;Suppl 1:76-85). We have now developed radar plots (RP) to visualize B- and T-cell subsets with the aim to quickly detect abnormal patterns.
Methods: In Toronto, list mode files of previously reported samples (n=695) were retrospectively re-analyzed. In Lund, 655 consecutive samples were re-analyzed blindly and categorized as normal or abnormal. The results were then compared with the previously issued reports. RPs were also applied to 20 additional samples with known abnormal T-cell population.

RP analysis: After removal of dead cells and debris, a "lympho/mono" gate was applied to the CD45/side scatter (SSC) plot. B cells were gated as CD19+ or CD20+ lymphocytes with exclusion of plasma cells. A six-parameter RP showed: kappa and lambda horizontally from east to west and CD20/CD10 respectively CD5/CD23 vertically north and south. In normal blood samples, a clear separation of kappa and lambda positive B-cells was seen, while in bone marrow or lymph nodes CD10+ B-cells were also present. T cells were gated as "lympho/mono" with exclusion of CD14+/SSCintermediate/high and CD19+ cells. A six-parameter RP displayed CD4 and CD8 horizontally from east to west and CD5/CD10 and CD57/ CD38 vertically north and south, respectively.

Results: In Toronto, all 458 cases with normal B-cell and T-cell populations were correctly identified using RPs. B-cell populations with a typical CLL/SLL phenotype were detected in 145/146 cases (sensitivity: 99%). Other CD5+ B-LPD were detected in 29/30 cases. CD10+ monotypic B-cell populations were detected in 4 cases. CD5-/CD10-mono B-cell populations were detected on RPs in 45/48 cases. In Lund, a concordance was found in 370 cases reported as normal. RP were difficult to evaluate in 30 cases due to a low total lymphocyte count. In 17 cases, an abnormal RP was found but cases were reported as normal after additional studies. In 173 cases, concordant results concerning an abnormal B-cell population were found. In 25 samples, a small abnormal B-cell population (<1%) was reported but it was not easily seen on RPs. In 43 cases with a reported abnormal T-cell population, it was identified on the RP in 37 samples (86%). In 6 cases, abnormal T-cells were found only in additional analyses.

Conclusions: The proposed RPs can potentially improve turn-around time as they quickly differentiate between normal and aberrant lymphoid patterns.

PFP-22

Optimization and evaluation of a 10-color antibody panel for flow cytometric immunophenotyping of B-cell lymphomas

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Introduction: Flow cytometric immunophenotyping (FCI) is widely used in cytopathology to distinguish between reactive lymphocytic proliferations (RLP) and B-cell lymphoma (BCL). The aim of this study was to evaluate and introduce a new 10-color antibody panel for the diagnosis of B-cell lymphoma in our routine laboratory work.

Methods: 48 samples (39 fine needle lymph node aspirations biopsies, 6 surgical excision lymph node biopsies and 3 samples of pleural effusion) were analyzed with an existing 4-color antibody panel (tube 1: CD3 FITC/CD19 PE/CD45 PerCp-Cy5.5/CD20 APC; tube 2: SmIg FITC/SmIgA PE/CD19 PerCp-Cy5.5/CD10 APC; tube 3: FMC7 FITC/CD23 PE/CD19 PerCp-Cy5.5/CD5 APC) and a new 10-color antibody panel (SmIgk FITC /SmIgλ PE/CD5 PerCp-Cy5.5/CD19 PE-Cy7/CD20 APC/CD3 APC-Cy7/CD23 APC-R700/FMC7 V450/CD45 V500c/CD10 BV605). The percentages of positive cells and mean fluorescence intensity (MFI) values for all markers were determined and correlated (Spearman correlation). The cytopathology diagnoses for all samples were based on morphological examination and FCI data and compared to the final diagnoses which were set by histology examination. This study was approved by The National Medical Ethics Committee of the Republic of Slovenia (0120-574/2017/3).

Results: Statistically significant correlations for the percentages of positive cells and MFI values were observed for all B-cell markers and CD3 (p<0.05). There were no differences between the immunophenotypes of malignant cells and the diagnoses determined with 4-color and 10-color antibody panels. 10 cases had a final diagnosis of RLP and 38 of BCL (7 follicular lymphomas, 6 mantle cell lymphomas, 10 chronic lymphatic leukemias, 7 diffuse large B-cell lymphomas, 6 marginal zone lymphomas and 2 composite lymphomas). In 42 cases (87.5%) the cytopathology diagnosis matched the final diagnosis. In 4 cases (8.3%) a diagnosis of BCL was established but the lymphomas were wrongly classified. In 2 cases (4.2%) a diagnosis suspicious for BCL was given.

Conclusions: Our study showed that the new 10-color antibody panel is suitable for everyday routine use for the diagnosis of BCL. As only one tube is required for the analysis, the new 10-color antibody panel will ensure a more reliable diagnosis of BCL, especially for the samples with few cells.
Comparison of anti-Kappa and Lambda light chains antibodies from Dako and Beckman Coulter
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Background: Each mature B lymphocyte expresses surface immunoglobulins bearing only one class of light chain, either Kappa or Lambda. Assessment of immunoglobulins light chains expression on B-cells by flow cytometry is the hallmark of B-cell malignancies studies. Indeed, while in healthy individuals the ratio of kappa+ to lambda+ B cells is roughly 3:2, this ratio is skewed by the expansion of clonal malignant cells. Kappa/Lambda flow cytometry assays can sometimes be challenging for laboratories, as results interpretation may be difficult. It is especially the case when non-specific staining occurs using non-optimized protocols or reagents. Dako’s rabbit polyclonal anti-kappa and lambda antibodies are widely known as the gold standard, delivering best-in-class performances. In this study we compared Dako’s antibodies to Beckman Coulter’s new goat polyclonal anti-Kappa and Lambda antibodies, conjugated to FITC and PE.

Methods: Both suppliers’ reagents were compared 15 times in various conditions. The Stain/Wash/Lyse protocol recommended by the International Clinical Cytometry Society (ICCS) was used in three variants, incorporating 1, 2 or 3 sample washes. 5 whole blood samples were processed with the three variants. Comparison between conjugated antibodies was based primarily on the assessment of the staining index, calculated as (MFI positive population – MFI negative population)/(2xSD of negative population).

Results: Performances of both supplier reagents are similar. Beckman Coulter’s antibodies provided a greater staining index when using all non-B lymphocytes as a negative population. Restricting the negative population to B-cells when calculating the staining index, Dako’s antibodies delivered a better staining index on average. Performing a single washing step of the samples did not allow to gate and discriminate positive and negative subsets. In contrast, two washes greatly facilitated the distinction and three washes offered a clear separation of Kappa+ and Lambda+ populations, no matter the supplier.

Conclusion: Each laboratory must perform their own protocol optimization and validation, as there are no real consensus protocol for Kappa and Lambda light chains assessment. It is particularly important to determine the number of washing steps required to deliver accurate and trusted results. Either Beckman Coulter or Dako anti-Kappa and Lambda antibodies could be used in this process, as performances are overall similar.

PTCL-NOS with leukemic presentation resembling Sézary syndrome: a case report
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Introduction: Peripheral T-cell lymphomas not otherwise specified (PTCL-NOS) are a heterogeneous group of diseases involving lymph nodes and extranodal sites deriving from the clonal expansion of mature T-lymphocytes. The cell of origin is an activated mature CD4+ lymphocyte. The phenotype is usually CD4+/CD8-. PTCL-NOS accounts for approximately 4-7% of all non Hodgkin Lymphomas and 30-70% of all mature T-cell lymphomas. Most patients present with peripheral lymph node involvement. Lymphoma cells can be detected in the peripheral blood smear, but leukemic presentation is uncommon. In most cases, the karyotype is aberrant and often characterized by complex abnormalities. Recently, recurrent chromosomal gains and losses have been documented in PTCL-NOS by comparative genomic hybridization.

Methods: We herein report a case of a 68-year-old woman with an unusual presentation of PTCL-NOS.

Results: The patient presented with a 2-month history of widespread palpable erythematous rash. She complained about pruritus, unresponsive to treatment with antihistamines and corticosteroids. The physical examination revealed supraclavicular lymphadenopathy. Her complete blood count showed a white-cell count of 30.000/µL with 95% lymphocytes, a hemoglobin level of 14.4 gr/dL, and a platelet count of 151.000/µL. Peripheral blood smear revealed numerous atypical intermediate-large sized mature lymphoid cells with Sezary-like flower-shaped nucleus and a small number with cerebriform nucleus. Biochemical analysis indicated increased serum LDH and uric acid.
Flow cytometry detected a population of mature T-cells CD3+/CD2+/CD4-CD8-/ TCRαβ+/CD7-/CD25-/CD30-/CD10-CD56- and clonal TCR gene rearrangement with Vb2 epitope being expressed in 94% of the T cells. Clonality was also confirmed by PCR. The neoplastic lymphocytes were negative for HTLV1. Karyotype was complex: 49,X,del(X)(q24),+del(1)(p13),t(2;6)(q31;q27),+4,+21[10]/46,XX[10]. CT scans were significant for generalized lymphadenopathy and hepatosplenomegaly.

We performed lymph node, bone marrow, and skin biopsies. Lymph node histology revealed diffuse infiltrates with effacement of the normal architecture. A mixture of intermediate and large atypical lymphocyte was noted. Lymphocytes expressed CD3, CD5, CD57, MUM, and CD2(+/-), but were negative for CD4, CD8, CD7, CD56, CD30, CD25, TCL1, and bcl6. Bone marrow involvement was 50% and skin was also infiltrated.

PTCL-NOS diagnosis was established and the patient received CHOP. However, disease progression with worsening of her leukocytosis was noted after the first cycle.

Conclusions: We report a case of CD4/CD8 double negative PTCL-NOS. To our knowledge, this is the first reported case harboring t(2;6)(q31;q27) translocation and manifesting leukocytosis with characteristic morphology resembling Sézary syndrome cells.

PFP-25

Hepatosplenic T cell Lymphoma. Role of flow cytometric immunophenotyping
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Introduction: HSTCL is a rare subset of T cell lymphoma typically characterized by young age at presentation, hepatosplenomegaly, cytopenias, and absent or minimal lymphadenopathy. Leukemic manifestations of the disease with bone marrow and/or peripheral blood involvement makes acute leukemia a close differential, especially when the cells have a blastic morphology. Thus, flow cytometric immunophenotyping (FCI) remains the cornerstone for the exact characterization of this entity.

Material and methods: A retrospective analysis of all the consecutive cases of HSTCL diagnosed over a six years period (June 2013 to May 2019) was performed. Clinical and laboratory details were retrieved from the hospital electronic medical records. FCI was done using a stain-lyse-wash protocol with 6 & 8 color panel. The morphological and flow cytometry data of all the cases were reviewed.

Results: A total of 12 cases of HSTCL were diagnosed during the period, which constituted ~1.8% of all non-Hodgkins lymphoma (NHL) diagnosed over this period. The median age of presentation was 24 years (range 16-30 years) with M: F ratio of 2:1. Anemia, thrombocytopenia, and hepatosplenomegaly were present in all the patients. Leukocytosis was noted in 50% (6/12) cases and 75% (8/12) cases had circulating tumor cells in peripheral blood ranging from 10-85%. A blastoid morphology was appreciated in six (50%) cases in the peripheral blood. Bone marrow biopsy displayed a diffuse and interstitial pattern of infiltration in 58.3% cases as compared to the classical sinusoidal pattern in only 41.7% cases. Immunophenotypically, the cells consistently expressed CD7 (100%), CD2 (100%), CD 3(91.7%); and gamma delta T cell receptor (100%). CD5, CD4, and CD8 were expressed in two (1.66%) cases and one (0.83%) case respectively. CD56 was expressed in 5(41.8%) cases. Interestingly, one of the cases was negative for surface CD3. Absence of all immaturity markers (CD34, CD99, CD1a, TdT), a bright CD45 expression and a gamma delta TCR expression along with the typical clinical presentation, supported this case as HSTCL.

Conclusion: Leukemic presentation and ‘blastic’ morphology of HSTCL may be a diagnostic challenge to oncologists and pathologists alike. A high index of suspicion, cognizance of this variant and use of FCI is mandated to establish an accurate diagnosis.
Metastatic lobular carcinoma of the Breast presenting as myeloma - a potential pitfall in CD138 immunoreactivity in non-haemopoietic disease

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Introduction: The specificity of CD138 reactivity is not confined to haematological neoplasms, particularly myeloma, but is expressed in a spectrum of non-haematopoietic malignancies.

Case Presentation: A 55 year old woman was referred for investigation of multiple lytic lesions (CT scan), lymphadenopathy, hypercalcaemia and hip pain. Her serum and urine were negative for monoclonal bands or raised free light chains, her free light chain ratio was normal. Local (district general hospital) review of the bone marrow aspirate and trephine was stated as most consistent with plasma cell myeloma. Trephine biopsy was reported as showing a significant infiltrate of plasma cells staining strongly for CD38 and CD138. The immunohistochemical staining of immunoglobulin light chains were inconclusive. Upon receipt of the referral material at the SIHMDS, based on the clinical history and presentation information a plasma cell neoplasm panel in addition to T-, B-, NK, myeloid immaturity assessment was processed. Immunophenotyping demonstrated no obvious clonal T or B-cell population or features of immaturity. 0.1% of all total TNC were CD138+/CD38+/CD19+/CD56-/CD117- polyclonal plasma cells. Additionally, a population noted to be CD138+/CD38~/CD19-/CD56-/CD117- with inconclusive light chains were detected, representing 0.8% of TNCs. Due to the negative CD38, cytoplasmic assessment of the non-glycosylated reversibly-palmitylated type II transmembrane protein p63 was evaluated using VS38c (as performed in post daratumumab therapy). VS38c was positive (52%) with moderate staining intensity. Our assessment of the BM morphology was that of normal appearing trilineage haematopoiesis, but infiltration by a population that resembled plasma cells or non-haematological neoplasm cells. These cells had eccentric nuclei and blue cytoplasm, many appeared to cluster. No cyogenetic or molecular testing was performed. Due to the clinical presentation and unusual phenotype (discordant for myeloma), local slides and tissue blocks were then sent for second opinion to the SIHMDS. Following integrated review, the marrow showed infiltration by carcinoma composed of dissociated plasmacytoid cells positive for CD138. These were also positive for cyclin D1, cytokeratin AE1/3, GATA3 and ER; negativity for immunoglobulin light chains, CD56, CD20 and CD79a. Integrated review concluded that this was metastatic breast carcinoma – lobular type.

Conclusion: High clinical suspicion of myeloma based on clinical or morphological features can be misleading. This case highlights that not all non-haematopoietic neoplasms are CD56 positive, that CD138/CD38 are not pathognomic for myeloma and that morphology alone is not sufficient to reach a diagnosis. A clear integrated approach, lateral thinking, and careful consideration of non-haematopoietic neoplasms must be employed.

Increased CD27+ Memory B-Cells in Poor Responder Rheumatoid Arthritis Patients Treated with Rituximab

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Background: Rituximab (RTX) is being increasingly used in the treatment of several autoimmune diseases, including Rheumatoid Arthritis (RA). RTX induces a deep depletion of all peripheral B cell subsets (memory and naïve B-cells). During the B cell repopulation phase, occurring approximately after 3 months of RTX administration, B-precursors and naïve cells reappear. Several studies have shown that relapsing RA patients are characterized by a relative expansion of memory B cells during the B cell repopulation phase. The aim of this study was to quantify the memory B cell compartment in AR patients with different disease activity scores, evaluated by DAS28, during RTX treatment.

Methods: 27 AR patients taking RTX cycles were studied. Nine showed high-to-moderate activity risk (median DAS28: 4.8) and 18 subjects showed low activity risk or were in remission at the end of treatment (median DAS28: 2.69). After 3 months from the last RTX infusion, B-cell subsets (precursors, naïve, memory B cells and plasma cells) were quantified in peripheral blood by flow cytometry, using 8 markers (CD3, CD4, CD8, CD19, CD20, CD27, CD38 and CD45). B naïve cells were identified as CD19+ CD20+ CD27+, B memory cells as CD19+ CD20+ CD27+, plasmacells as CD19+ CD38+ CD27++ CD20- and B precursors as CD19+ CD38++ CD20- CD27-. One million total cell events were the target collection to ensure high-resolution analysis and an adequate B cell subsetting.
Percent and absolute values were calculated for each subset, and 10 healthy subjects were included as normal control group (NC).

**Results:** The virtual absence of B cells was defined as <0.1 B cells/µL. In the responder group, 3/18 cases showed absolute B cell levels <0.1 cell/µL vs only in 1/8 of the non responder group. Median B cells/µL were higher in responders (69 vs 7.5), whereas Memory B cells% were significantly higher in non responders (46.5 vs 8, p<0.05) and similar to the NC. No differences were noted for percent and absolute naïve B cells, memory B cells/µL, CD38+ memory B cells% and plasmacells between the two groups.

**Conclusion:** We used a sensitive and easily applicable flow cytometric 8-color panel for an accurate and standardized identification and enumeration of peripheral blood B cell subsets to monitor AR patients under Rituximab treatment. As reported also by other studies, higher levels of memory B-cells were found in non responder AR patients treated by RTX, approaching those of healthy individuals.

**PFP-28**

**Detection of antigen-specific CD4+ T cells by flow cytometry using a whole blood assay.**

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**Introduction:** Quantifying levels of antigen-specific CD4+ T cells can be useful in the diagnostic work up of patients with suspected immunodeficiency. Methods for detection of microbial exposure to T cells are available but are laborious and time-consuming. The so-called 'OX40' assay, a flow cytometry method based on detection of CD25 and OX40 (CD134) surface membrane expression of activated CD4+ T cells, is a rapid and robust whole blood technique that is concordant when compared with more traditional methods of antigen-specific T cell identification.

**Methods:** We collected heparinized blood from healthy donors and used the ‘OX40’ assay to detect antigen-specific T cell responses to Cytomegalovirus, Varicella Zoster virus, Herpes Simplex virus, Candida albicans, tetanus toxoid, and diphtheria toxoid. Whole blood of the healthy controls were stimulated with antigen preparations of the above mentioned microorganisms and proteins. After stimulation during 44-48 hr cells were harvested, stained and acquired by flow cytometry.

**Results:** From a total of 20-30 healthy controls the T cell antigen-specificity to a range of recall antigens and commonly encountered pathogens were determined. Reference ranges were calculated as the 5th and 95th percentiles. The healthy control ranges for specific antigens were used to test the clinical utility in a number of patients with a diagnosed immunodeficiency.

**Conclusions:** The present study shows some clinical validation and establishment of healthy reference ranges for the detection of antigen-specific CD4+ T cell responses to recall antigens and a number of common encountered viruses. The assay is simple and easy to standardize in comparison with alternative assays for detection and monitoring of antigen-specific T cells.

**PFP-30**

**The effect of TNF-α on diabetic dendritic cells derived from bone marrow. An in vivo study**

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**Introduction:** Tumour Necrosis Factor α (TNF-α), is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a range of responses, leading to necrosis or apoptosis. Diabetic patients show high TNFα plasma levels and a direct correlation was found by our group between TNF-α and proinsulin (PI) expression on dendritic cells (DCs). DC are involved in immune response against external pathogens, secreting TNF-α upon infection.

**AIM:** We evaluated the effect of TNF-α and Glucose (Gluc) stimulus on PI expression in bone marrow-derived DCs from diabetic patients, with/without treatment with the inhibitor Adalimumab (ADA), a human anti-TNF-α antibody.
Methods: Bone marrow from diabetic patients with foot lesions (n=6) were processed to obtain BMNC, that were seeded and cultured for 9 days in RPMI supplemented with 10% FBS, GM-CSF (50ng/mL), IL-4 (50ng/mL), 1% glutamine, and 1% penicillin. At day 7, cells were challenged for 24h with TNF-α 10ng/mL, and Glucose 30mM, and at day 8 cultures were supplemented with ADA 10µg/mL for 24 hours. After the treatment cells were counted and analyzed by flow cytometry to evaluate the expression of PI, CD14, and DC markers.

Results: At day 8, both glucose and TNF-α treatments reduced cell number respect to untreated cells (80±12, glucose and 106±34, TNF-α vs 147±22, WT p<0.01). We found a significant reduction of CD14+ cells and an increased number of DC in culture in standard medium respect to T0 (7.7±2.7 vs 0.4±0.7; 4.6±2.3 vs 24.8±8.9, respectively, both p<0.001). In glucose treated cells we found an increase of proinsulin and DC markers expression respect to untreated cells (0.16±.09 vs 0.74±0.46 and 0.03±0.03 vs 0.43±0.35 respectively, p<0.001), whilst no changes were induced by TNF-α treatment. Following TNF-α and ADA treatments, PI expression (Gluc:0.74±0.46; GlucTNF-α:30 0.21±0.18; GlucTNF-α+ADA:0.22±0.19 p<0.01) and PI coexpression with DC markers (Gluc:0.43±0.35; GlucTNF-α: 0.065±0.04; GlucTNF-α+ADA:0.0235±0.03 p<0.05) were reduced versus Glucose stimulus alone.

Conclusion: We found an increase of PI and reduced DC under glucose treatment, similarly to what we had yet showed. TNF-α and ADA treatments do not affect the expression of PI in DC, and protect the cells from glucose-mediated apoptosis. Further studies are needed to establish the real biological significance of PI in innate immune system.

PFP-31

Myeloid-derived suppressor cells and cancer: identification and quantification in peripheral blood samples
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Introduction: Myeloid-derived suppressor cells (MDSC) are a heterogenous population of immature myeloid cells that have potent immunosuppressive activity, being one of the multiple parameters analyzed in patient’s Immunogram, a complex tool useful in predicting the response to immunotherapy. There are three different subsets of MDSC: polymorphonuclear MDSC (PMN-MDSC), which are phenotypically similar to neutrophils, monocytic MDSC (M-MDSC), phenotypically similar to monocytes, and, finally, immature or early-stage MDSC (e-MDSC), a small population that consists in a mix of MDSC with a more immature phenotype. In cancer patients (CP), comparatively to healthy individuals, MDSC are present in higher levels in circulation, which contributes for tumor progression. The aim of this work was to identify and quantify the MDSC present in cancer patients, by flow cytometry (FC), and also to study the mechanisms responsible for their immunosuppressive activity.

Methods: We used whole blood (WB) from 31 patients with solid or hematological neoplasias, while 11 samples from healthy donors (HD) were used as control. For FC analysis, samples were stained with the following antibodies: CD15, CD45s, Lineage-2, LOX-1, CD33, HLA-DR, CD11b, CD14.

Results: The amount of PMN-MDSC and M-MDSC was obtained in relation to neutrophils and monocytes, respectively, and as described in the literature PMN-MDSC were CD11b+ CD14- CD15+ CD33+ LOX-1+ and M-MDSC were CD11b+ CD14+ CD15- CD33+ HLA-DRlow. In comparison with the HD group, in the CP group were obtained percentages of PMN-MDSC 12 times higher (CP: 0.695%; HD: 0.054%) and percentages of M-MDSC 2 times higher (CP: 1.540%; HD: 0.909%). Within the total of MDSC, it was obtained a higher percentage of PMN-MDSC in the CP group, while a higher percentage of M-MDSC was obtained in the HD group.

Conclusions: The results show that MDSC are indeed increased in cancer patients. However, the identification and quantification of these cells was difficult given the lack of specific markers. For the study of the immunosuppressive activity of these cells, it is ongoing the sorting of the PMN-MDSC and M-MDSC for evaluation of the expression, by qRT-PCR, of some enzymes overexpressed on these cells, such as arginase-1 (ARG-1), indolamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS). Also, the loss of CD3 zeta chain on T lymphocytes is being assessed.
Precision Evaluation of the BD OneFlow™ Tubes on the BD FACSLyric™

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Introduction: BD OneFlow reagents are provided in a ready-to-use, dried, single-test tube that allow for direct specimen staining, eliminating the need for antibody pipetting, reducing operational mistakes and manual workload. It provides a clinical solution for leukemia and lymphoma immunophenotyping with improved laboratory efficiency and standardized instrument setup. The BD FACSLyric system provides clinical users a CE-IVD solution to standardized setup and simplifies the user workflow, helping to ensure reproducible results across instruments. Currently BD OneFlow tubes are available on the BD FACSCanto II. The BD FACSLyric is a standardized clinical system that is widely used by CE-IVD customers providing clear, reproducible results across instruments. In this study we examine the total system precision, repeatability and reproducibility of BD OneFlow assays on the BD FACSLyric system.

Methods: The BD FACSLyric settings were setup every day using BD CS&T beads. Three BD FACSLyric cytometers of 12C configurations 4-3-5 were used by three operators performing two runs each (AM and PM) per day. One cellular control lot was used as the study specimen throughout the study, and a combination of controls were used to assess all the markers captured within the OneFlow reagent tubes. Three lots of reagent for each assay were stained in duplicates. A total of n=180 data points were collected over the course of the study for BD OneFlow LST, BD OneFlow B-CLPD T1, BD OneFlow ALOT, BD OneFlow PCD and BD OneFlow PCST. Two separate controls were used for BD OneFlow PCST and BD OneFlow PCD tubes, and 12 replicates were stained daily. A total of 360 data points were collected over the five day testing period, however only 180 data points were used for analysis for each marker.

Results: Each sample was analyzed using identical gating pertaining to the assay, and the results were statistically analyzed and evaluated. The results showed the SD < 5.0 and %CV < 15.0 for each analyzed marker for all the BD OneFlow assays.

Conclusions: BD OneFlow reagents on the BD FACSLyric total systems precision was shown to be SD < 5.0 and %CV < 15.0 for each marked marker.

Disclaimer: BD OneFlow™ tubes on BD FACSLyric™ is under development and is not available for sale.
All the samples were evaluated on FACSLyric and FACSCanto II systems and the population for each marker was compared qualitatively for its absence/presence, position, and location. Seven samples were acquired for BD OneFlow LST BD OneFlow B-CLPD T1, and BD OneFlow PCD; whereas, BD OneFlow PCST and BD OneFlow ALOT had six and five samples each respectively.

**Results:** All the acquired samples showed identical populations on both FACSCanto II and FACSLyric for OneFlow reagents.

**Conclusions:** Results for BD OneFlow reagents on the FACSCanto II and FACSLyric were comparable.

**Disclaimer:** BD OneFlow™ tubes on BD FACSLyric™ is under development and is not available for sale.

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**Factors determining whether diffuse large B cell lymphoma samples are detected by flow cytometry**

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**Introduction:** Flow cytometry is an integral part in the workup for mature B-cell neoplasms (MBN). However numerous cases of diffuse large B-cell lymphoma (DLBCL), a common type of MBN, are not detected by flow cytometry. We investigated factors that increase the likelihood of DLBCL being missed in flow cytometry. We hypothesized that samples with fibrosis or necrosis were more likely to be not detected by flow cytometry.

**Methods:** We identified confirmed cases with a final diagnosis of DLBCL, for which a sample was submitted for flow cytometry in the previous 10 years. Histopathology, flow cytometry, genetic and clinical data were collected for each of these DLCBL cases.

**Results:** Initial analysis confirmed that among diagnosed MBN cases, DLBCL was the most common type to be missed by flow cytometry. Retrospective analysis identified a total of 135 cases with a confirmed diagnosis of DLBCL. In 22 (16%) of these cases, a lymphoma population was not detected by flow cytometry. A DLBCL sample was more likely to be missed by flow cytometry if microscopy revealed Epstein-Barr virus positivity or absence of MYC expression. However the presence of fibrosis or necrosis by morphology was not significantly associated with increased likelihood of failure to detect DLBCL by flow cytometry. Samples were more likely to be missed if flow cytometry demonstrated high CD4 and CD8 T-cell percentages, which may reflect the failure of the analysis to detect abnormal B cells. Finally, a high peripheral blood platelet count was significantly associated with failure to detect DLBCL by flow cytometry.

**Conclusions:** The study identifies several factors that increase the likelihood of DLBCL being missed by flow cytometry. However, fibrosis or necrosis in the lymphoma tissue was not significantly associated with failure to detect DLBCL.

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**Detection of minimal residual disease in chronic lymphocytic leukemia using t-SNE**

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**Introduction:** Accurate detection of small aberrant populations requires the acquisition of a few million events. Improvements in analysis strategy may contribute to increased sensitivity and reliability in MRD results. Single-cell data are often examined in two dimensions at a time in the form of a scatter plot. However, a pairwise viewpoint could miss biologically meaningful multivariate relationships that cannot be discerned in two dimensions. High-dimensional flow cytometry datasets can be analyzed in an automated and more unbiased manner than possible through bivariate gating. Algorithms like t-SNE can be used to visualize the data in intuitive low dimensional representation. viSNE has been previously used for the analysis of 8 color flow cytometry ALL MRD samples and t-SNE for the analysis of 8 color flow cytometry AML MRD. In both studies, it was capable of accurately identify MRD populations comprising representing as little as 0.001% of viable cells in clinical samples. As far as we are concerned, this strategy has not been previously described for CLL MRD analysis.
**Methods:** Eight peripheral blood CLL MRD samples were retrospectively analyzed using t-SNE (FlowJo®) and results were compared with those obtained using conventional gating strategy (Infinicyt®). Each sample CD19+ CD3- lymphocytes were analyzed using t-SNE. Samples were prepared with bulky lysis protocol as previously described and stained according to ERIC’s 6 markers core panel with the addition of CD3 and CD45 as optional markers. To determine normal t-SNE pattern, 3 normal peripheral blood were processed, stained and analyzed according to CLL MRD samples protocol.

**Results:** Two samples were MRD negative by conventional and t-SNE analysis. One MRD positive sample had the same quantification when analyzed by both methods (0.61%). For the remaining 5 samples, the difference between results from both methods were not clinically significant: 0.04% versus 0.05%; 0.06% versus 0.09%; 0.23% versus 0.31%; 1.26% versus 1.61% and 5.54% versus 5.20% using conventional gating strategy and t-SNE analysis, respectively (two-tailed P < 0.0001).

**Conclusion:** Although MRD monitoring is a specialized task and should be performed by those who are proficient in this task, t-SNE maps analysis significantly clarify the distinction between normal and leukemic cells and mitigate the risk of incorrect interpretation. We found that t-SNE may also be a tool to automate and facilitate CLL MRD analysis.

**PFP-36**

**Demonstration of Equivalence between OneFlow-specific tube settings on the BD FACSLyric™ compared to OneFlow setup on the BD FACSCanto II™**

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**Introduction:** The BD FACSLyric™ system provides clinical users a CE-IVD solution to standardised setup and simplified user workflow, helping ensure reproducible results across instruments. BD OneFlow™ reagents, currently released on the BD FACSCanto II™ requires more regular instrument setup using CS&T beads, BD OneFlow™ set up beads and BD FC beads™. The purpose of this study was to create a streamlined approach using tube settings on BD FACSLyric™ to demonstrate its equivalence with the OneFlow setup on the BD FACSCanto II.

**Method:** Spherotech Rainbow beads were acquired on six BD FACSCanto II™ and PMT voltages (PMTV’s) adjusted to within range. BD FC beads™ were acquired on six BD FACSCanto II™ and target MFI recorded and averaged. BD FC beads™ were then acquired on a BD FACSLyric™ and PMTV’s adjusted to averaged target MFI identified on the six BD FACSCanto II™ instruments. To ensure forward and side scatter met the EuroFlow guidelines, whole blood was lysed and PMTV’s adjusted. Tube settings were created, ported to ten BD FACSLyric™ instruments and tested against the BD FACSCanto II™ using BD FC beads™ and stained BD OneFlow ALOT™ tubes with whole blood from healthy subjects and acute leukemic patients. FCS files from both the BD FACSCanto II™ and BD FACSLyric™ were analysed for MFI difference and expressed as percentage of FACSCanto II acquired data.

**Results:** Data from BD FC beads™ and whole blood stained BD OneFlow™ ALOT tubes show that OneFlow specific tube settings on the BD FACSLyric™ are comparable to the OneFlow setup on the BD FACSCanto II™. MFI variance for markers in BD OneFlow ALOT™ tubes were equivalent between platforms for healthy and diseased stained samples. Furthermore, when assessing B cells and T cells using FACSuite software, FACSCanto & FACSLyric ALOT data overlapped, illustrating MFI comparison.

**Conclusion:** We demonstrated and confirmed that the BD FACSLyric setup with OneFlow specific tube settings are equivalent to BD FACSCanto II™ setup and are reproducible across BD FACSLyric™ instruments.

**Disclaimer:** BD OneFlow™ tubes on BD FACSLyric™ is under development and is not available for sale.
Factors influencing kappa/lambda identification by flow cytometry
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Introduction: B cells clonality by multiparametric flow cytometry (MFC) is based on identification and evaluation of light chain immunoglobulins restriction. In some cases, however, the separation between kappa and lambda B cells is not clear, and the identification of populations of B neoplastic cells among normal B cells may be difficult. In these cases, use of intracytoplasmic labeling or incubating at 37C may overcome this. However, the cause of this methodological difficulty is not yet quite clear.

Methods: During 6 consecutive months we evaluated all cases that required incubation or cytoplasmatic labeling to determine K/L differentiation. A total of 62 cases, using an 8 color panel, were included. For all these cases any acute or chronic health condition was considered as potential interference. As controls, 62 random cases performed in the same day where no complementation was needed were also investigated for health issues. Also, 60 HIV+ cases were compared to 90 HIV- to evaluate if HIV interferes in the test. All cases of lymphomas and multiple myeloma were excluded. Qui-square was used to compare groups.

Results: Of the 62 cases needing additional technics to separate K/L, 18 (30%) presented with “inflammatory” diseases: 5 autoimmune, 2 HIV+, 2 acute CMV, 2 AIHA, 1 acute EBV, 3 neoplasia (1 myelodysplastic syndrome, 1 CML, 1 color cancer), and 1 aplastic anemia. Among controls, only 8 (13%), presented with any inflammatory disease (p=0.03). Of interest, we retrospectively identified 60 HIV+ patients with peripheral blood MFC performed in past 5 years and compared them to 90 HIV- randomly selected controls. HIV was strongly associated with need to complementary studies (HIV+ x HIV-= 50% x 4,5%, p<0.001). After incubation or cytoplasmatic labeling, all cases included were non-clonal.

Conclusion: These findings suggest that patients with diseases with certain inflammatory features may possess some factor with may difficult a clear K/L separation using standard surface flow cytometry techniques. We could speculate that reactive immunoglobulins or some medications, for example, could be the responsible factor. These findings are importante in order to: 1- do not consider normal B cells as neoplastic, and; 2- realize that the finding of this technical problem should alert to some unrecognized clinical problem. Also, HIV+ patients are specially predisposed to this situation, but the mechanism remains unknown.

IL7 receptor (CD127) expression on blast cells in patients with Acute leukemia
Ekaterina Zakharko, Yanzhima Balzhanova, Darya Drokova, Kira Matokhina, Elena Rybkina, Vera Troitskaya, Elena Parovichnikova, Valentina Dvirnyk
National Research center for Hematology, Moscow, Russian Federation

Introduction: IL7/IL7 receptor (IL7R) signaling plays an important role in B- and T-cell lineage development and supposedly has contradictory influent on ontogenesis. Aim of this study was to evaluate expression IL7R on blast cells in patients with newly diagnosed acute leukemia (AL).

Methods: Eight-color flow cytometry (FC) FACS Canto II BD Bioscience was used for analyses of bone marrow (BM), peripheral blood (PB) and cerebrospinal fluid (CSF) blast cells expression of CD127 (α-chain of IL7R) in time of first examination of de novo AL patient. Anti-CD127 PerCP antibody (BD Bioscience) was used for this study.

Results: There are 48 newly diagnosed AL patients were included in this study. The age median was 36 years; male/female ratio was 25/23 consequently. There were 24 AML; 16 B-ALL; 8 T-ALL patients. In this group median of blast cells in the BM was 54%; 77%; 86% consequently. Expression of the IL7R (CD127) was measured 14/24 AML; 10/16 B-ALL; 4/8 T-ALL (mean: 9,4%; 9,3%; 12,4% consequently); from them expression of CD127 was > 20% (24%-52%) in 3/14; 2/10; 1/4 cases.

All 48/48 patients had blasts in the PB with median 26%; 41,6%; 56% consequently, in 16/24 of AML; 11/16 of B-ALL; 5/8 of T-ALL cases CD127 was investigated (mean 6,7%; 8,6%; 8,7%), it’s expression was >20% (20,5-55%) in 1/16; 3/11; 1/5 patients.

Flow cytometry assessment of CSF revealed blast cells in 15/24 AML; 5/16 B-ALL; 5/8 T-ALL patients; in 12/15; 3/5; 3/5 cases the CD127 was evaluated (mean 2,4%; 17,7%; 18% consequently) and considered >20% in 0/15 AML; 1/3 B-ALL (37%) and 1/3 T-ALL (35%), interestingly that BM and PB blasts were CD127 negative in B-ALL patient but CD127 positive in T-ALL.
There were only 4 patients, whose BM and PB blasts were simultaneously CD127 positive (1 AML; 2 B-ALL; 1 T-ALL). 3/4 (75%) of them have been resistant to therapy and 1/4 (25%) had remission only after 2d Induction Remission course. While patients without simultaneous CD127 expression on blasts of BM and PB had 6.0% of therapy resistance and 6.0% of late remission achievement.

**Conclusion:** α-chain of IL7R has low expression on blast cells of AL (about 50% and less). Blast cells of different body fluid or tissue (BM, PB, CSF) could have immunophenotypic difference including CD127 expression. Patients with simultaneous CD127 positivity of BM and PB blasts seem to have worse the therapy response, what has to be approved by future investigations.

**PFP-39**

**Challenges and Advances in Evaluation of Vitreous Humor by Flow Cytometry**

Laiz Bento, Rodrigo Barroso, Rodolfo Correia, Andressa Vaz, Daniela Schimidell, Eduardo Pedro, Flavia Sousa, Nadila Millan, Marília Passaro, Nydia Bacal

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**Introduction:** Conventional cytomorphology (CMP) is the gold standard diagnosis for intraocular lymphoma. However, CMP has limited sensitivity and is observer-dependent. Flow cytometry (FC) has been used as a powerful technique with high sensitivity and specificity for the detection of hematologic neoplasms. We present the challenges and the advances to evaluate vitreous humor (VH) specimens by FC.

**Methods:** From 2015 to 2018 our group evaluated 6 VH samples by FC. Euroflow Small Sample Tube–SST (CD8FITC+LAMBDAFITC/CD56PE+KAPPAPE/CD4Percp-y5.5/CD19PE-Cy7/CD3APC+CD14APC/CD38APC-H7/CD20V450/CD45V500). This tube was used as a screening strategy in 5 samples and in 1 sample we used a dry reagent personalized tube called Duraclone (KAPPA+CD4FITC/LAMBDA+CD8PE/CD3+CD14ECD/CD33PC5.5/CD20+CD56PEC7/CD34APC/CD19A700/CD10A750/CD5PB/45KO). Five samples were collected in Transfix and 1 was collected in RPMI. All samples were centrifuged (1100rpm), washed (3x) with 5% of serum bovine, stained, acquired in BD FACS Canto II (Becton Dickinson) and Navios Flow Cytometer (Beckman Coulter) and analysed in Infinicyt (Cytognos) software.

**Results:** Our study detected 1 case that had infiltration of Non-Hodgkin Lymphoma B CD10+ monoclonal to Kappa, using the dry technology reagent (DuraClone). This sample had 40 cells/mm³, a volume of 1mL was collected in Transfix. No phenotypic abnormalities were identified by FC in the other 5 samples that had on average volume of 1mL and undetectable cells by Neubauer chamber counting. However, even in these 5 samples, we could detect monocytes, CD3 T-lymphocytes, and the subtypes CD3/CD4 and CD3/CD8. All the cell death was excluded through the forward scatter and side scatter strategy.

**Conclusions:** The major challenge of VH study by FC is low cellularity in a low sample volume and increase cell death. In our routine, we have been applied advances in technical procedures, equipment, software and fluorochromes. Improvements such as standardization of technical procedures to better sample concentration in one tube, the use of Transfix to preserve cell viability and the use of 10-colours dry reagent have allowed the standardization and increase sensitivity and specificity even at lower cell concentrations samples. FC is a powerful and sensible technique for cell detection even in extremely low count samples and has become an important tool to provide information about the nature of the cellular infiltrate of vitreous specimens.
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ADV - ADVANCES IN CYTOMETRY

ADV-01

Evaluation of automated LST preparation compared to manual procedure

Hiep phuc Dong
Oslo University Hospital, Oslo, Norway

Introduction: Manual sample preparation is a time-consuming and risk-prone procedure. The Flow Cytometry Unit at Dept. of Pathology, Norwegian Radium Hospital, Oslo University Hospital, has long experience in manual preparation of peripheral blood (PB) and bone marrow (BM) samples. In this study we have evaluated an automated sample preparation instrument FlowStainer® manufactured by InstruNor Corporation, and compared its performance to the manual procedure.

Methods: The panel analyzed consisted of the modified Leukocyte Screening Tube (LST) from EuroFlow Consortium containing the following antibodies: CD20+CD4, CD45, CD8+Immunoglobulin light chain lambda (Ig\(\lambda\)), CD56+immunoglobulin light chain kappa (Ig\(\kappa\)), CD5, CD19+T-cell receptor gamma delta (TCR\(\gamma\delta\)), CD3 and CD38. A small series of 15 peripheral blood and 15 bone marrow samples was prepared and stained using both the manual and automated procedure. The samples were analyzed for aforementioned antibodies using flow cytometry, and the percentages of antigen expression on leukocytes using both methodologies were compared. In addition, preparation time for both methods was compared. There are no financial bindings between supplier of equipment and the Flow Cytometry Unit at the Oslo University Hospital.

Results: The percentages of antigen-expressing leukocytes using both methodologies were comparable and no significant differences were observed. Slightly higher cell debris was observed in automated stained samples compared to manual procedure. However, this did not affect the interpretation of the results. The hands-on preparation time using the FlowStainer® was not shorter than manual handling. However, the automation procedure leaves technicians more free time to perform other tasks and reduces errors.

Conclusions: This study shows that use of the FlowStainer® in sample preparation is equivalent to manual procedure. In addition to reduction of errors, the method also provides more free time for the lab personnel focusing on other tasks in the lab.

ADV-02

Standardized and Streamlined QC for ClearLLab 10C Application

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Beckman Coulter, Miami, U.S.A.

Introduction: The ClearLLab 10C Application is used for qualitative identification of cell populations by multiparametric immunophenotyping on Navios and Navios EX flow cytometers. These reagents are used as an aid in the differential diagnosis of hematologically abnormal patients having, or suspected of having, chronic leukemia, acute leukemia, non-Hodgkin lymphoma, myeloma, myelodysplastic syndrome (MDS), and/or myeloproliferative neoplasms (MPN). The application has four 10-color Panels in dry format.
The quality control workflow was developed for the 10C Application and is done in addition to Flow-Check™ Pro QC, including Standardization and Compensation via AutoSetup Wizard, and Daily QC monitoring of settings and process. Instrument Standardization uses Flow-Set™ Pro Fluorospheres (FSP), and compensation setup uses ClearLLab Compensation Kit (Comp Kit) stained ClearLLab Compensation Beads (Comp Beads). The Comp Kit contains ten tubes each with one antibody conjugated to one of ten fluorochromes in a dry format. For Daily QC, ClearLLab Control Cells (CCs), Normal and Abnormal, were developed as process control reagents, together with running FSP, to monitor the established instrument settings and processing. This abstract’s aim is to present the standardized and streamlined QC workflow.

Methods: Through the AutoSetup Wizard, FSP establishes the instruments' voltages, then single-color stained Comp Beads generate a compensation matrix, and a specimen is run as verifier tubes to check and finetune the compensation matrix for each of the four Panels. The established instrument settings and compensation matrix were monitored after the AutoSetup, by running the FSP and stained compensation beads using the established settings to assess variability and demonstrate stability. Acceptance criteria were established for the FSP X-mode values for Daily QC monitoring of the instrument settings, and assay ranges for each marker contained in the CCs were also established based on the system variability for daily process control.

Results and Conclusion: A standardized and streamlined QC workflow was established for the 10C application, including AutoSetup and Daily QC Monitoring. Standardization and Compensation via AutoSetup is not required daily and is performed at the initial setup and repeated only when daily QC fails, after instrument service, or when switching FSP lots. Daily QC monitors the settings using FSP against the target ranges, and monitors the process using CCs against the Assay Values using Kaluza C protocols with lot-specific assay values and Levey-Jennings plots. The Beckman Coulter products and service marks mentioned herein are trademarks or registered trademarks of Beckman Coulter, Inc. in the United States and other countries.

ADV-03 (also presented as Poster Flash Presentation # PFP-35)

Detection of minimal residual disease in chronic lymphocytic leukemia using t-SNE
Felipe Magalhaes Furtado, Camila Santos Nobre, Fernanda de Oliveira Resende, Anna Carolina Monteiro de Castro, Alessandra Lopes Barbosa, Lidia Freire Abdalla Nery, Rafael Henriques Jacomo, Sabin Medicina Diagnostica, Brasilia, Brazil

Introduction: Accurate detection of small aberrant populations requires the acquisition of a few million events. Improvements in analysis strategy may contribute to increased sensitivity and reliability in MRD results. Single-cell data are often examined in two dimensions at a time in the form of a scatter plot. However, a pairwise viewpoint could miss biologically meaningful multivariate relationships that cannot be discerned in two dimensions. High-dimensional flow cytometry datasets can be analyzed in an automated and more unbiased manner than possible through bivariate gating. Algorithms like t-SNE can be used to visualize the data in intuitive low dimensional representation. viSNE has been previously used for the analysis of 8 color flow cytometry ALL MRD samples and t-SNE for the analysis of 8 color flow cytometry AML MRD. In both studies, it was capable of accurately identify MRD populations comprising representing as little as 0.001% of viable cells in clinical samples. As far as we are concerned, this strategy has not been previously described for CLL MRD analysis.

Methods: Eight peripheral blood CLL MRD samples were retrospectively analyzed using t-SNE (FlowJo®) and results were compared with those obtained using conventional gating strategy (Infinicyt®). Each sample CD19+ CD3-lymphocytes were analyzed using t-SNE. Samples were prepared with bulky lysis protocol as previously described and stained according to ERICs 6 markers core panel with the addition of CD3 and CD45 as optional markers. To determine normal t-SNE pattern, 3 normal peripheral blood were processed, stained and analyzed according to CLL MRD samples protocol.

Results: Two samples were MRD negative by conventional and t-SNE analysis. One MRD positive sample had the same quantification when analyzed by both methods (0.61%). For the remaining 5 samples, the difference between results from both methods were not clinically significant: 0.04% versus 0.05%; 0.06% versus 0.09%; 0.23% versus 0.31%; 1.26% versus 1.61% and 5.54% versus 5.20% using conventional gating strategy and t-SNE analysis, respectively (two-tailed P < 0.0001).

Conclusion: Although MRD monitoring is a specialized task and should be performed by those who are proficient in this task, t-SNE maps analysis significantly clarify the distinction between normal and leukemic cells and mitigate the risk of incorrect interpretation. We found that t-SNE may also be a tool to automate and facilitate CLL MRD analysis.
ADV-04

Flow cytometric determination of a modified DNA base 5-hydroxymethyluracil at single-cell level as a new valuable research and diagnostic tool
Lidia Gackowska, Anna Labejszo, Anna Helmin-Basa, Małgorzata Wiese-Szadkowska, Jaroslaw Czyz, Daniel Gackowski
Nicolaus Copernicus University in Torun, Collegium Medicum, Bydgoszcz, Poland

Introduction: 5-hydroxymethyluracil (5-hmUra) was originally identified as an oxidatively modified DNA base derivative. Recent evidence suggests its formation from thymine in reaction catalyzed by TET proteins or by 5-hydroxymethylcytosine deamination by activation-induced cytidine deaminase. We hypothesize that the latter mechanism is particularly likely in leukemic lymphocytes B in chronic lymphocytic leukemia (CLL) patients. Current “gold standard” in 5-hmUra determination is liquid chromatography with tandem mass spectrometry. Despite many advantages it has one limitation—it is not able to measure compounds at single-cell level. Our main goal was therefore to develop and optimize method for the 5-hmUra content assessment with using flow cytometry - the only one technique allowing such determinations.

Methods: Peripheral blood were collected into tubes containing EDTA and TransFix. At first, samples were stained with direct conjugated anti-human-CD45 antibodies, for identification cell populations. Based on available literature data and own experience, we initially tested three procedures: a)protocol with using of different concentrations of HCl, b)immunofluorescent staining kit of incorporated bromodeoxyuridine, c)protocol using saponin solution and cell exposure to high temperature. The best results were obtained after using high temperature and this protocol was the starting point for further standardization. To control thermal denaturation of double helix to expose nucleobases, different temperatures and incubation’s time of heating were tested. Since the 5-hmUra epitope may also be present in RNA a test with using an RNase solution was carried out. Due to the lack of anti-5-hmUra antibody recommended for flow cytometry, the unconjugated one proven for histochemical staining was selected together with the compatible, recommended for flow cytometry, secondary antibody labeled with Alexa Fluor 647. Both antibodies were titrated. The samples were analyzed with using of BD FACSCantoII flow cytometer. The content of 5-hmUra was calculated as fluorescence intensity fold change over negative control. To provide further proof that observed signal comes from genomic DNA, nuclear localization we performed a study using a confocal fluorescence microscope.

Results: We have optimized flow cytometric method for assessment of 5-hmUra content at single-cell level, with the simultaneous extracellular staining of CD45 antigens. Using our protocol we carried out the determinations in CLL and non-CLL individuals and we showed a significantly higher 5-hmUra content in the group of CLL, which corresponded with the results obtained with using of liquid chromatography with tandem mass spectrometry.

Conclusions: In the future, our optimized method can become an useful research and diagnostic tool in assessing of DNA modification.

ADV-05

Flow cytometric assessment of Reactive Oxigen Species in MDS hematopoietic stem and progenitor cells
Thea Bensi, Valentina Giai, Michela Salvio, Roberto Guaschino, Marco Ladetto, Maria Matilde Ciriello
Santi Antonio e Biagio e Cesare Arrigo Hospital, Alessandria, Italy

Introduction: Myelodysplastic Syndromes (MDS) are clonal hematopoietic stem cell malignancies. Previous studies showed that reactive oxygen species (ROS) play a role in the pathogenesis and clinical evolution of MDS, contributing to hematopoietic stem and progenitor cells (HSPC) genetic instability. Our study aims to analyze ROS levels in MDS hematopoietic stem cells (HSC), common myeloid progenitors (CMP), granulocyte macrophages progenitors (GMP) and megacaryocyte-erythrocyte progenitors (MEP).

Methods: 38 MDS and 27 normal bone marrow (NBM) were collected. In order to avoid extra stress to cells, samples were run for analysis 1 hour after bone marrow aspiration, with no further CD34+ cells enrichment procedure. ROS levels were assessed using 2',7'-dichlorofluorescin diacetate and quantified by flowcytometry as mean fluorescence intensity (MFI): ROS values in lymphocytes were used to normalise ROS levels. Multiparameter flow cytometry was used to identify HSC, CMP, GMP and MEP, using CD34, CD38, CD45RA, CD123 and CD90 MoAbs.
**Results:** Among Lineage negative (Lin-) CD34+CD38+ cells, for MDS with no blast excess (MDS no BE), MDS with blast excess (MDS-BE) and normal bone marrow (NBM) CMP were 42%, 26.8% and 35.7% respectively (MDS no BE vs MDS BE p:0.003); GMP were 17%, 29.5% and 18.7% for MDS-no BE, MDS-BE and NBM (MDS no BE vs MDS BE p:0.017). No differences between MDS and NBM in MEP. ROS levels were higher in GMP than in the other progenitors, regardless the sample group (p< 0.01). In IPSS low risk MDS, CMP and GMP had higher ROS levels compared to NBM (2.36 and 3.17 vs 1.81 and 2.58, p: 0.025 and p:0.041). HSC Lin- CD34+CD38- cells were more elevated in MDS than in NBM (2.5% vs 1.3%, p:0.038). MDS-BE showed a higher HSC ratio compared to NBM (3.5% vs 1.3%, p:0.023). We found a direct correlation between ROS levels in CMP, GMP and MEP of low and intermediate-1 (int-1) risk MDS and absolute neutrophil count (ANC), the correlation was especially strong in GMP (p:0.02). In our study, overall survival was not influenced significantly by ROS levels, but “long survivors” showed higher ROS values.

**Conclusion:** Flow cytometry is an essential tool for ROS measurement and our results show ROS as a player in low and int-1 risk MDS. The correlations between ROS levels and clinical data suggest that the MDS HSPC are subject to stress as they struggle to maintain an effective hematopoiesis. Future studies should focus on how to reduce oxidative stress in HSPC.

**ADV-06**

**Analysis of novel AML immunophenotypic markers using a 12 color panel on BD FACSLyric**

Olga Weinberg1, Jean Oak2, Marisa Juntilla2

1Boston Children's Hospital, Newton center, U.S.A.
2Stanford University Medical Center, Palo alto, United States of America

**Introduction:** Acute myeloid leukemia (AML) is an aggressive hematological malignancy characterized by clonal proliferation of immature myeloid cells. Multiparameter flow cytometry (MFC) immunophenotyping provides critical information for AML diagnosis, classification, and monitoring. AML with monocytic differentiation poses a challenge due to lack of common stem cell marker expression and significant overlap with background monocytes. The use of new immunophenotypic markers in AML is limited by the number of parameters available to the average clinical flow cytometry lab. There is a need for new monocytic markers to be included in clinical routines to increase the value of MFC not only as a diagnostic but also prognostic tool for monitoring of MRD and for development of drugs for targeted therapy. The goal of this study is to evaluate novel AML markers in diagnostic AML samples using a single 12 color AML panel on a Lyric.

**Methods:** A one-tube 12 color AML panel included antibodies to the surface markers CD45, CD34, CD11c, CD13/CD33, CD38, CD64 ILT3, CD87, CD135, CD371, CD133, and a live/dead exclusion dye was used to analyze 8 AML samples and 3 normal controls. Expression of markers was graded from 0-1 in each case and correlation between markers was assessed using Spearman analysis using Prism.

**Results:** De novo AML cases were included in this study with cytogenetic abnormalities including t(15;17), t(8;21), add10p11.2, inv 3, complex karyotype and 2 normal karyotypes. Next generation sequencing results are available in 5 cases and showed presence of NRAS, PTPN11, CEBPA, NPM1 and RUNX1 mutations. The highest expressed markers in these samples included CD64, CD371, CD87, CD133 and CD135. Monocytic markers were more frequently seen in normal karyotype AML. A trend between expression of CD64 and ILT3 is present (p=0.065) and CD11c with CD133 (p=0.071).

**Conclusions:** We demonstrate performance, including setup and compensation of a novel one-tube 12 parameter AML panel. When performed on a FACSLyric, this panel results in workflow efficiency, time saving, and sample preservation. There is a useful pattern of heterogenous expression of these monocytic markers across different genetic subtypes of AML. Our results have implications for utilization of these markers and this panel in both AML diagnosis and AML MRD setting.
ADV-07 (also presented as Poster Flash Presentation # PFP-17)

Development of a dried-down, multicolor reagent solution for enhanced flow-cytometric applications

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BD Biosciences, San Jose, U.S.A.

Introduction: Multicolor flow cytometry is a powerful tool to identify, analyze and enumerate multiple cell populations phenotypically. As part of its custom reagents program, BD offers dried-down reagent cocktails for a diverse range of flow cytometry applications. The dried-down cocktails provide enhanced reagent stability, simplify the assay workflow, enhance operational efficiency and enable assay standardization across instruments, operators and testing sites.

Methods: The emergence of BD Horizon Brilliant™ dyes has resulted in significant demand for these dyes in multicolor reagent panels. However, cocktailing multiple BD Horizon Brilliant™ dyes result in unwanted dye-dye interactions. To address this issue, BD has developed a technology that enables the delivery of multiple BD Horizon Brilliant™ dyes in a single-use, dried-down format. We designed a 7-color panel (CD3, CD4 CD45A, CD25, CD127, CD15s, CD161) for the identification of Tregs and the characterization of the different Treg subsets (naive, effector and transitional) in a given sample. Additionally, the panel allows the assessment of the different functional subsets of Tregs, including the IL-17-producing Tregs (CD161`). Additionally, the potentially immunosuppressive CD15s ` Tregs.

Results: As part of assessing the feasibility of the reagent-drying technology, we compared the performance of the 7-color Treg panel in a dried-down state and as a liquid cocktail using the BD FACSLyric™ flow cytometer. Our results show that the dried-down cocktail is free of unwanted dye-dye interactions and is equivalent to the liquid cocktail in terms of resolution of the different functional Treg subsets. Specifically, the percent-positive cells measured for the different Treg subsets in a given sample are comparable when using the dried-down cocktail or the liquid cocktail. This data demonstrates the feasibility of the reagent-drying technology and sets the stage for its application in diverse areas of flow-cytometry-based research.

Conclusion: The development of the dry down technology enables manufacturing of dried-down reagent cocktails containing up to five BD Horizon Brilliant™ reagents.

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ADV-08 (also presented as Poster Flash Presentation # PFP-38)

IL7 receptor (CD127) expression on blast cells in patients with Acute leukemia

Ekaterina Zakharko, Yanzhima Balzhanova, Darya Drokova, Kira Matokhina, Elena Rybkina, Vera Troitskaya, Elena Parovichnikova, Valentina Dvirnyk
National Research center for Hematology, Moscow, Russian Federation

Introduction: IL7/IL7 receptor (IL7R) signaling plays an important role in B- and T-cell lineage development and supposedly has contradictory influence on ontogenesis. Aim of this study was to evaluate expression IL7R on blast cells in patients with newly diagnosed acute leukemia (AL).

Methods: Eight-color flow cytometry (FC) FACS Canto II BD Bioscience was used for analyses of bone marrow (BM), peripheral blood (PB) and cerebrospinal fluid (CSF) blast cells expression of CD127 (α-chain of IL7R) in time of first examination of de novo AL patient. Anti-CD127 PerCP antibody (BD Bioscience) was used for this study.

Results: There are 48 newly diagnosed AL patients were included in this study. The age median was 36 years; male/female ratio was 25/23 consequently. There were 24 AML; 16 B-ALL; 8 T-ALL patients. In this group median of blast cells in the BM was 54%; 77%; 86% consequently. Expression of the IL7R (CD127) was measured 14/24 AML; 10/16 B-ALL; 4/8 T-ALL (mean: 9,4%; 9,3%; 12,4% consequently); from them expression of CD127 was > 20% (24%-52%) in 1/14; 2/10; 1/4 cases. All 48/48 patients had blasts in the PB with median 26%; 41,6%; 56% consequently, in 16/24 of AML; 11/16 of B-ALL; 5/8 of T-ALL cases CD127 was investigated (mean 6,7%; 8,6%; 8,7%), it’s expression was >20% (20,5-55%) in 1/16; 3/11; 1/5 patients. Flow cytometry assessment of CSF revealed blast cells in 15/24 AML; 5/16 B-ALL; 5/8 T-ALL patients; in 12/15; 3/5; 3/5 cases the CD127 was evaluated (mean 2,4%; 17,7%; 18% consequently) and considered >20% in 0/15 AML; 1/3 B-ALL (37%) and 1/3 T-ALL (35%), interestingly that BM and PB blasts were CD127 negative in B-ALL patient but CD127 positive in T-ALL.
There were only 4 patients, whose BM and PB blasts were simultaneously CD127 positive (1 AML; 2 B-ALL; 1 T-ALL). 3/4 (75%) of them have been resistant to therapy and 1/4 (25%) had remission only after 2d Induction Remission course. While patients without simultaneous CD127 expression on blasts of BM and PB had 6,0% of therapy resistance and 6,0% of late remission achievement.

**Conclusion:** α-chain of IL7R has low expression on blast cells of AL (about 50% and less). Blast cells of different body fluid or tissue (BM, PB, CSF) could have immunophenotypic difference including CD127 expression. Patients with simultaneous CD127 positivity of BM and PB blasts seems to have worse the therapy response, what has to be approved by future investigations.

**ADV-09**

**Quality control for procedure of laboratory developed testing workflow**

Motoi Kinishi, Tomohiro Tsuji, Yasuaki Tsuruoka, Satoshi Yoneda, Hiroo Tatsutani
Sysmex Corporation, Kobe, Japan

**Introduction:** So far, the IVD applications like TBNK / CD34 are provided by Flow Cytometry (FCM) manufactures. Then, they also provide the Quality Control (QC) material and the software functions for QC management and Report. However, almost FCM laboratories (Labs) do the test based on the Laboratory Developed Test (LDT), and do the QC based on the Standard Operation Procedure (SOP) that is managed by each FCM Lab. The conventional FCM software doesn’t have the QC management function for LDT, so original QC chart is created, and the QC results are managed in each Labs. It is complicated for the FCM testing workflow. Therefore, we developed the “Panel Setup / QC” function, it includes the management and the report function of QC for LDT and decrease complicated steps and improve the FCM testing Workflow.

**Methods:** “1. Adjust the Gain/Photomultiplier voltage to the optimized value for analysis by using the QC material based on SOP in Lab.”, “2. Create the compensation table.”, “3. Save the measurement conditions as the panel information.”, “4. Create the QC file for LDT and set the baseline and the upper/lower limits for each channel.”, “5. Measure the QC material in daily.”, “6. Report the QC chart.”

**Results:** About QC, Adjustment of Gain/PMTV and Compensation, running sample, we evaluated the difference in the case of between the manual operations and using the “Panel Setup/QC” function. The usage materials are “Sample: CD Chex(Streck)”, “Reagents: Kombitest 6 color cocktail (Exbio)”, “Beads for compensation: CompTrol beads with CD8 antibody with the different fluorochromes”. We assume the TBNK 6 color of LDT panel. The steps for FCM testing Workflow are decreased in 56.5% by using the “Panel Setup/QC” function (there are 23 steps for the manual operations, but only 11 steps by using it). And, about daily sample measurement (if user complete to create the panel), we realize to shorten 32 minutes by using it (the manual operation is 64 minutes, but only 32 minutes by using it).

**Conclusions:** We can realize the followings for LDT by developing the “Panel Setup/QC” function. “1. Management of QC results by using Levey-Jennings chart.”, “2. Creating and output reports.”. As a result, we decrease the complicated steps for FCM testing Workflow.

**ADV-10**

**Bright SuperNova V428 Polymer dye for flow cytometry applications**

Arunkumar Easwaran, Massimiliano Tomasulo, Boi Hoa San, Mukesh Mudgal, Sergei Gulnik
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**Introduction:** A variety of fluorescent molecules are used for antibody labeling in flow cytometry, including small organic dyes, phycobiliproteins, and quantum dots. While these dyes have worked well in the past, they come up short in addressing modern needs. The first is the desire for more colors that can be excited by each laser, allowing for a higher degree of multiplexing. And, the second is the demand for brighter dyes that will increase the dynamic range of flow-cytometer measurements, which is especially important for the detection of low-abundance antigens. To address these needs, we designed a new 405 nm-excitable polymer dye, called SuperNova V428. This polymer dye is composed of a collection of small organic-dye molecules that are connected in one chain by a system of conjugated double bonds, resulting in extraordinary brightness. Such polymers are very efficient in both light harvesting and energy transfer to acceptor dyes, thus allowing for the generation of a variety of tandem dyes that are all excitable by one laser.
**Methods:** SuperNova V428 was synthesized using Suzuki coupling reactions on modified dihydrophenanthrene derivatives. The purified polymer was characterized using proton NMR and GPC. To assess the performance in flow cytometry, we conjugated SuperNova V428 to different antibodies and compared these with other commercially available small organic- and polymer-dye conjugates.

**Results:** SuperNova V428 has molecular weight (Mn) ranged between 70-80K, determined using polystyrene standards. The polymer exhibited excellent photophysical features, with a molar extinction coefficient of 2.5 million Lmol$^{-1}$cm$^{-1}$ and a fluorescence quantum yield of 0.6 in phosphate buffer. Using a CytoFLEX LX flow cytometer (Beckman Coulter, Brea, CA), we found that SuperNova V428 antibody conjugates exhibited 4-5x the brightness of violet-excitable coumarine-based fluorescent-dye conjugates, and had a brightness equal to or better than commercially available polymer-dye conjugates.

**Conclusion:** We believe that SuperNova V428 is an important addition to the growing collection of next-gen polymer dyes, and that it will help researchers to take maximal advantage of modern flow-cytometer technology. The CytoFLEX is for research use only. Beckman Coulter, the stylized logo, and the Beckman Coulter product and service marks mentioned herein are trademarks or registered trademarks of Beckman Coulter, Inc. in the United States and other countries. All other trademarks are the property of their respective owners.

ADV-11

Flow cytometric analysis of SuperNova V428 polymer dye conjugates

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**Introduction:** There is a persistent demand for brighter dyes that increase the dynamic range of flow-cytometry measurements, which is especially important for the detection of low-abundance antigens. While synthetic organic dyes exhibit relatively low molar absorption coefficients, polymeric dyes have received much attention as useful alternatives due to their higher brightness. However, unlike synthetic organic dyes, the current variety of polymeric dyes is limited by design challenges, such as the selection of a proper polymeric backbone, the introduction of solubilizing moieties and functional groups for conjugation, and the fine-tuning of excitation and emission wavelengths. In this context, we have designed and developed a novel 405 nm-excitable polymer dye, called SuperNova V428, which exhibits both an exceptionally high molar extinction coefficient (2.5 million Lmol$^{-1}$cm$^{-1}$) and a good quantum yield (0.6 in PBS).

**Methods:** SuperNova V428 was conjugated to antibodies using conventional techniques, such as NHS or maleimide chemistry. We evaluated the performance of several SuperNova V428 antibody conjugates in immunophenotyping cells by flow cytometry.

**Results:** We found that CD19-SuperNova V428-labeled B cells exhibited a much higher signal-to-noise ratio (S/N) than a violet-excitable coumarine-based fluorescent-dye conjugate, and performed better than commercially available polymer-dye conjugates. Similarly, we compared the detection of peripheral-blood CD4+ human Tregs, labeled using different antibody conjugates targeting the low-abundance CD25 antigen, and found that SuperNova V428 dramatically improves the detection of Tregs, with a S/N greater than the other polymeric dye conjugates.

**Conclusion:** SuperNova V428 is a new addition to the polymer-dye family that exhibits higher brightness in comparison to organic-dye and commercially available polymer-dye conjugates. Due to their high brightness, we demonstrated that SuperNova V428 antibody conjugates even provide superior performance for the detection of low-abundance antigens by flow cytometry. The CytoFLEX is for research use only. Beckman Coulter, the stylized logo, and the Beckman Coulter product and service marks mentioned herein are trademarks or registered trademarks of Beckman Coulter, Inc. in the United States and other countries. All other trademarks are the property of their respective owners.
ADV-12 (also presented as Poster Flash Presentation # PFP-33)

Evaluation of BD OneFlowTM reagents on BD FACSLyricTM to BD FACSCantoTM II system for immunophenotyping of leukemia and lymphoma

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Introduction: BD OneFlow reagents are provided in a ready-to-use, dried, single-test tube that allow for direct specimen staining, eliminating the need for antibody pipetting, minimizing operational mistakes and reducing manual workload. It is a complete clinical solution for leukemia and lymphoma applications with improved laboratory efficiency and standardized instrument setup. The BD FACSLyric system provides clinical users a CE-IVD solution to standardized setup and simplifies the user workflow, helping to ensure reproducible results across instruments. BD OneFlow reagents, currently released on the BD FACSCanto II require more regular instrument setup using BD OneFlow beads and BD FC beads. The purpose of our study was to demonstrate equivalency of BD OneFlow reagents on BD FACSLyric system when compared to BD FACSCanto II using clinical samples.

Method: Each sample was stained in replicates and acquired on the BD FACSCanto II and BD FACSLyric. Prior to acquisition, the BD FACSCanto II was setup using BD FACSDiva™ CS&T IVD beads, BD FC beads 8-color kit for the BD OneFlow assays and BD OneFlow setup beads with lot-specific mean fluorescence intensity (MFI) target ranges. Application settings were created using BD FACSDiva software. The BD FACSLyric was set up using the BD CS&T IVD beads, BD FC beads 7-color kit and BD FC beads 5-color kit. All the samples were evaluated on FACSLyric and FACSCanto II systems and the population for each marker was compared qualitatively for its absence/presence, position, and location. Seven samples were acquired for BD OneFlow LST BD OneFlow B-CLPD T1, and BD OneFlow PCD; whereas, BD OneFlow PCST and BD OneFlow ALOT had six and five samples each respectively.

Results: All the acquired samples showed identical populations on both FACSCanto II and FACSLyric for OneFlow reagents.

Conclusions: Results for BD OneFlow reagents on the FACSCanto II and FACSLyric were comparable.

Disclaimer: BD OneFlow™ tubes on BD FACSLyric™ is under development and is not available for sale.

ADV-13

8 color EuroFlow panels standardization towards BD FACSMelody Cell Sorter

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Introduction: EuroFlow standardized flow cytometry protocols have been key in achieving reproducibility of results among different laboratories, improving reports and clinical outcomes. Clinical laboratories need to improve isolation and concentration of pathological cells for genetic test sensitivity, for that purpose we incorporated a BD FACSMelody™ cell sorter in our oncohematology laboratory. The objective of this work is to demonstrate the standardization of the setup and samples results run in the BDFACSMelody instrument compare to the reference system BD FACSCantoll™.

Methods: Both instruments Flow Cytometer FACSCantoll 3 laser 8 colors and Cell Sorter FACSMelody 3 laser 9 colors where standardized using BDOneFlow™ Setup Beads, BDOneFlow FC Beads for compensation and BD CS&T Beads for control and automated daily adjustment. Median fluorescence intensity (MFI) from all the detectors where recorded periodically through 5 months, analyzing for each detector the dispersion of the data over time, median and coefficient of variation (CV%). 24 peripheral blood (PB) samples (14 normal and 10 abnormal) where stained with BDOneFlow LST dry tube and acquired in both instruments. The merge of all the normal samples from both instruments was analyzed by Infinicyt Software 2.0 Automated Population Separator (APS), the same analysis was performed on the merge of each abnormal sample in both instruments.
Results: The MFI results for BDOneFlow Setup beads in each detector of BDFACSMelody showed an improved CV% for 7 of 8 fluorescence compare to BDFACS Cantoll, PerCP MFI was equivalent in both instruments, for all the detectors MFI results were inside the target value interval. Normal PB stained with BDOneFlow LST showed similar MFI between both instruments, the APS analysis of the normal samples acquired in both flow cytometers showed 100% results inside the first Standard Deviation (SD) for each population B Lymphocytes, T Lymphocytes CD4 and CD8, NK Lymphocytes. The APS evaluation of the merge of each abnormal sample population from both cytometers showed in each case the comparison to be in the in the first SD, using the FACSCantoll results as the reference. Conclusion: The current data show the stability and reproducibility of MFI and CV% results for both instruments over 5 months, demonstrating the standardization of the BDFACSMelody using the BDOneFlow setup reagents. Lymphoid population from normal and abnormal samples showed equivalence between instruments, with dispersions of each compared population inside the first SD.

ADV-14 (also presented as Poster Flash Presentation # PFP-32)

Precision Evaluation of the BD OneFlow™ Tubes on the BD FACSLyric™
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Introduction: BD OneFlow reagents are provided in a ready-to-use, dried, single-test tube that allow for direct specimen staining, eliminating the need for antibody pipetting, reducing operational mistakes and manual workload. It provides a clinical solution for leukemia and lymphoma immunophenotyping with improved laboratory efficiency and standardized instrument setup. The BD FACSLyric system provides clinical users a CE-IVD solution to standardized setup and simplifies the user workflow, helping to ensure reproducible results across instruments. Currently BD OneFlow tubes are available on the BD FACS Cantoll. The BD FACSLyric is a standardized clinical system that is widely used by CE-IVD customers providing clear, reproducible results across instruments. In this study we examine the total system precision, repeatability and reproducibility of BD OneFlow assays on the BD FACSLyric system.

Methods: The BD FACSLyric settings were setup every day using BD CS&T beads. Three BD FACSLyric cytometers of 12C configurations 4-3-5 were used by three operators performing two runs each (AM and PM) per day. One cellular control lot was used as the study specimen throughout the study, and a combination of controls were used to assess all the markers captured within the OneFlow reagent tubes. Three lots of reagent for each assay were stained in duplicates. A total of n=180 data points were collected over the course of the study for BD OneFlow LST, BD OneFlow B-CLPD T1, BD OneFlow ALOT, BD OneFlow PCD and BD OneFlow PCST. Two separate controls were used for BD OneFlow PCST and BD OneFlow PCD tubes, and 12 replicates were stained daily. A total of 360 data points were collected over the five day testing period, however only 180 data points were used for analysis for each marker.

Results: Each sample was analyzed using identical gating pertaining to the assay, and the results were statistically analyzed and evaluated. The results showed the SD < 5.0 and %CV < 15.0 for each analyzed marker for all the BD OneFlow assays.

Conclusions: BD OneFlow reagents on the BD FACSLyric total systems precision was shown to be SD < 5.0 and %CV < 15.0 for each marker analyzed.

Disclaimer: BD OneFlow™ tubes on BD FACSLyric™ is under development and is not available for sale.
The significance of detection of immunophenotypic aberrances in patients with AML t(8;21) with low blast cell count
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Introduction: Acute myeloid leukemia (AML) with t(8;21) is one of the AML with recurrent genetic abnormalities were the diagnosis of Acute leukemia has to be established without regard to blast cell count. This group has characteristic morphological and immunophenotypic features, which could be useful for differential diagnosis in case of low blast cells count (<20%). Aim of this abstract to represent cytological and flow cytometry (FC) data of patients with AML t(8;21) with emphasizing in cases with low blast cells count.

Methods: cytological (Leica MD 3000), multicolor FC (FACS Canto II BD Bioscience), standard cytogenetic analysis and FISH (Fluorescence In Situ Hybridization) investigation of bone marrow (BM) aspirate were performed in patients with AML, who received treatment in National Research center for Hematology in 2016-2018 years.

Results: there were 484 AML patients included in this study. 20/484 (4,1%) were diagnosed as AML t(8;21) according to cytogenetic investigation. Characteristically immunophenotypic features were revealed in 20/20 AML t(8;21): aberrant expression of CD19 along was found in 6/11 cases; CD56 along – in 3/20 cases; the simultaneous CD19 and CD56 co-expression in 11/20 cases.

There were 5/20 patients with blast cell count by cytological evaluation <20% (10,8% - 17,6%). After cytological investigation 3/5 had a preliminary diagnosis as Myelodysplastic syndrome with excess blasts 2 (MDS EB2); 2/5 as Myelodysplastic/myeloproliferative neoplasms (MDS/MPN) group: 1 – Chronic myelomonocytic leukemia and 1 – MDS/MPN, unclassifiable. But during flow cytometry analyses the common AML t(8;21) features were found: aberrant expression of CD19 and CD56 together in 2/5 cases; CD19 only in 1/5 cases; CD56 only in 1/5 cases, what help to suppose t(8;21) in this kind of blasts. Cytogenetic analysis approved these surmise, and the diagnosis of AML t(8;21) was established in spite of low blast cells count.

Conclusion: detection of typical immunophenotypic aberrances (such as CD19 or/and CD56 co-expression) on myeloid blast cells allows to suppose AML with t(8;21), what could be important for differential diagnosis with neoplasms of MDS, MDS/MPN group.

Precision Evaluation of the BD OneFlow™ Tubes on the BD FACSLyric™
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Introduction: BD OneFlow reagents are provided in a ready-to-use, dried, single-test tube that allow for direct specimen staining, eliminating the need for antibody pipetting, reducing operational mistakes and manual workload. It provides a clinical solution for leukemia and lymphoma immunophenotyping with improved laboratory efficiency and standardized instrument setup. The BD FACSLyric system provides clinical users a CE-IVD solution to standardized setup and simplifies the user workflow, helping to ensure reproducible results across instruments. Currently BD OneFlow tubes are available on the BD FACS Canto II. The BD FACSLyric is a standardized clinical system that is widely used by CE-IVD customers providing clear, reproducible results across instruments. In this study we examine the total system precision, repeatability and reproducibility of BD OneFlow assays on the BD FACSLyric system.

Methods: The BD FACSLyric settings were setup every day using BD CS&T beads. Three BD FACSLyric cytometers of 12C configurations 4-3-5 were used by three operators performing two runs each (AM and PM) per day. One cellular control lot was used as the study specimen throughout the study, and a combination of controls were used to assess all the markers captured within the OneFlow reagent tubes. Three lots of reagent for each assay were stained in duplicates. A total of n=180 data points were collected over the course of the study for BD OneFlow LST, BD OneFlow B-CLPD T1, BD OneFlow ALOT, BD OneFlow PCD and BD OneFlow PCST. Two separate controls were used for BD OneFlow PCST and BD OneFlow PCD tubes, and 12 replicates were stained daily. A total of 360 data points were collected over the five day testing period, however only 180 data points were used for analysis for each marker.
**Results:** Each sample was analyzed using identical gating pertaining to the assay, and the results were statistically analyzed and evaluated. The results showed the SD < 5.0 and %CV < 15.0 for each analyzed marker for all the BD OneFlow assays.

**Conclusions:** BD OneFlow reagents on the BD FACSLyric total systems precision was shown to be SD < 5.0 and %CV < 15.0 for each marker analyzed.

Disclaimer: BD OneFlow™ tubes on BD FACSLyric™ is under development and is not available for sale.

**ADV-17 (also presented as Poster Flash Presentation # PFP-16)**

**EuroFlow-guided comparative evaluation of technical features and performance of newly-available high-end cytometers for leukocyte immunophenotyping**

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**Introduction:** Flow cytometry (FC) is currently rapidly expanding in its (pre)clinical applications, particularly for immune monitoring. This requires novel technical features, such as analysis of more cells, less sample volume, less time, more colors, better resolution. Our aim was to critically evaluate the performance of several instruments as valid alternatives for current reference instruments.

**Methods:** Four different >20-color high-standard flow cytometers recently available on the market were tested to evaluate vendors’ claims and identify critical factors impacting on data quality; they were evaluated in parallel in two EuroFlow centers (LUMC and USAL). The aim of the study is beyond recommending equipment purchasing. Therefore technical details linking to the vendor’s identity were excluded. The following parameters were assessed (March, 2018 – June, 2019): optical configuration and actual number of fluorochromes detectable simultaneously; overtime behavior (over-time instrument quality-checks; several months-intervals); acquisition stability (45-minute long-runs); carryover; abort rates; effect on data quality of sample concentration (four concentrations, three technical replicates) and sample input rate (depending on system) using both beads and cells. For cell-based tests, normal peripheral blood samples were stained using multi-color marker combinations (16 to 25 colors) for leukocyte characterization (designed per individual cytometer).

**Results:** All tested cytometers claimed the usage of >20 fluorescent parameters (n=21-27). However, considering redundancy in detectors, signal interaction/spread and fluorochrome availability, on average only 84% (76-92%) of the fluorescent parameters (n=19-25) could be used simultaneously. All tested cytometers were stable overtime, but for 2/4 cytometers stability of signal during long-runs (Height and Area) was not optimal. In 2/4 systems a significant effect (absolute coefficient of variation – CVs of signal of dim and bright peaks >5%) of flow rate >60µL/min (not of concentration) was documented in bead-based assays. Staining indexes (SI) variability related to each marker-detector pair at different speed and concentration were calculated in cell-based assays. Variations in intra-assay %CV of SI within +/-15% were considered acceptable (i.e. no significant impact on data quality). This information, together with abort rate was used to calculate the maximum acquisition speed (4.700-38.000 events/second). The maximum speed value claimed by the companies (30.000-100.000 events/second) was confirmed in 1/4 cytometers.

**Conclusions:** Keeping up with the new requirements in clinical flow cytometry, instrument replacement becomes a challenge. The current study highlights the necessity to investigate whether the manufacture’s technical claims of the new instruments meet the eligibility criteria and requirements, as needed to further progress towards routine clinical FC.
Factors influencing kappa/lambda identification by flow cytometry
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Introduction: B cells clonality by multiparametric flow cytometry (MFC) is based on identification and evaluation of light chain immunoglobulins restriction. In some cases, however, the separation between kappa and lambda B cells is not clear, and the identification of populations of B neoplastic cells among normal B cells may be difficult. In these cases, use of intracytoplasmic labeling or incubating at 37°C may overcome this. However, the cause of this methodological difficulty is not yet quite clear.

Methods: During 6 consecutive months we evaluated all cases that required incubation or cytoplasmatic labeling to determine K/L differentiation. A total of 62 cases, using an 8 color panel, were included. For all these cases any acute or chronic health condition was considered as potential interference. As controls, 62 random cases performed in the same day where no complementation was needed were also investigated for health issues. Also, 60 HIV+ cases were compared to 90 HIV- to evaluate if HIV interferes in the test. All cases of lymphomas and multiple myeloma were excluded. Qui-square was used to compare groups.

Results: Of the 62 cases needing additional technics to separate K/L, 18 (30%) presented with “inflammatory” diseases: 5 autoimmune, 2 HIV+, 2 HCV+, 2 acute CMV, 2 AIHA, 1 acute EBV, 3 neoplasia (1 myelodysplastic syndrome, 1 CML, 1 color cancer), and 1 aplastic anemia. Among controls, only 8 (13%), presented with any inflammatory disease (p=0.03). Of interest, we retrospectively identified 60 HIV+ patients with peripheral blood MFC performed in past 5 years and compared them to 90 HIV- randomly selected controls. HIV was strongly associated with need to complementary studies (HIV+ x HIV-: 50% x 4,5%, p<0.001). After incubation or cytoplasmatic labeling, all cases included were non-clonal.

Conclusion: These findings suggest that patients with diseases with certain inflammatory features may possess some factor with may difficult a clear K/L separation using standard surface flow cytometry techniques. We could speculate that reactive immunoglobulins or some medications, for example, could be the responsible factor. These findings are importante in order to: 1- do not consider normal B cells as neoplastic, and; 2- realize that the finding of this technical problem should alert to some unrecognized clinical problem. Also, HIV+ patients are specially predisposed to this situation, but the mechanism remains unknown.

Automation of workflow in a clinical laboratory: Integration of Pre-analytical set-up using the BDFACSDuet™ and analysis on the BDFACSlyric™ cytometer
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Introduction: Manual set up and processing of flow cytometry is an error prone process which utilises a significant amount of scientific staff time. In addition, manual work, including steps such as antibody selection, pipetting, and transfer of primary and secondary tubes have been identified as potential sources of error in flow cytometry. Here we investigate parallel set up of (a) manual processing and (b) using an integrated system made of a sample preparation unit and a flow cytometer.

Methods: An observational study was conducted at a National Health Service acute care clinical laboratory in collaboration with a lean specialist for the purposes of workflow analysis. The laboratory workflow for diagnosis of primary immunodeficiency (CD4 and TBNK) was mapped for 2 days, over 15 hours. Videotape was reviewed and analyzed to measure the two methods of laboratory time spent testing samples.

Results: In processing a batch of 10 samples, hands on time was reduced by 46%, with a batch of 20 samples hands on time was reduced by 69%. Individual processing steps were reduced by 71% and critical error prone steps were totally eliminated.

Conclusion: This observational study quantified manual work resulting in substantial cost to the laboratory in indirect costs and opportunities for error. The results suggest that, in addition to reducing the potential for error, minimizing unnecessary manual steps in the flow cytometry workflow can reduce laboratory indirect costs and improve efficiency.
ADV-20

Multicenter validation of clinically relevant acute myeloid leukemic stem cell flow cytometric analyses

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Introduction: Leukemic stem cells (LSC) are supposed to be the driving force in the occurrence of relapses in acute myeloid leukemia (AML). Therefore, implementation of LSC measurements is warranted to facilitate accurate risk stratification. Previously, we published the composition of a one tube flow cytometric assay characterized by the presence of 7 important single markers as well as a cocktail of 6 markers in one fluorescence channel. Recently, the assay was tested in a prospective large cohort of patients and showed that LSC frequency had prognostic value additive to well-known AML risk factors, including minimal residual disease. As the tube is an affordable, easy and broadly-applicable approach for LSC detection it could easily be incorporated in large multi-institutional studies. Here we present the validation experiments of the assay in several large AML research centers both in Europe and the United States.

Methods: To test the validity and applicability, nine laboratories contributed with a range of flow cytometers (BD FACSCanto II, BD LSRFortessa, BC Gallios and BC Navios). Four frozen diagnostic AML samples together with antibody mixtures were sent to participating laboratories. Protocols were provided and laboratories were asked to perform analysis accordingly. Raw data and analyses were compared for accuracy. Additional validation of the gating strategy was performed on 10 flow cytometry data files. The antibodies for this validation were provided by Becton Dickinson.

Results: The technical variability within instruments and sample processing is relatively small and correlations between different instruments were high ($R_{\text{pearson}}$>0.91, $p$=<0.001). Multi-center testing introduced variation in reported LSC percentages but this was below the clinical relevant threshold and variation in inter-operator gating was small. Clear criteria for optimal gating resulted in all laboratories being able to perform LSC assessment of the validation set after only 2 feedback rounds. Participating centers were unanimously able to distinguish LSC$^{\text{high}}$ ($>0.03\%$ LSC frequency) from LSC$^{\text{low}}$ ($<0.03\%$ LSC frequency) diagnostic files despite inter-laboratory variation in reported LSC percentages contributed by differences in the selected hierarchy of LSC aberrant markers. Reported values in LSC$^{\text{high}}$ samples were within accepted 0.5log error. Although the observed error for LSC$^{\text{low}}$samples was greater at around 1 log, this did not influence the stratification in different groups

Conclusion: We show that the one-tube LSC assay is highly reproducible between multiple flow cytometry laboratories. These results together with the high prognostic impact of LSC load at diagnosis in AML patients renders the one-tube LSC assessment a good marker for future risk classification.

ADV-21

Association of t(8;21) Acute Myeloid Leukemia and Systemic Mastocytosis: is it just a chance?

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Introduction: The association of t(8;21) Acute Myeloid Leukemia (AML) and Systemic Mastocytosis it’s rare but has been described. The associated mastocytosis is not always diagnosed concurrently with the AML. The exact relationshineep between neoplastic mast cells and the leukemic clone remains obscure. We describe a case recently brought to our attention. A 57-year old man was diagnosed with t(8;21) AML in March 2019.
Methods: On Flow cytometric analysis we characterized blasts (52%) with following phenotype: the blasts expressed CD13, CD33, CD34, CD38, CD117, HLA-DR, cytoplasmic myeloperossidase and CD19. At smear examination the blasts showed M2-FAB morphology. Cytogenetic molecular analysis showed t(8;21). No mast cells were highlighted at onset. The patient achieved a morphologic remission with induction chemotherapy (daunorubicine, cytarabine and etoposide).

Results: Flow cytometric analysis of bone marrow in June showed 1% of blasts but a clear population of mast cells had appeared (10%), with aberrant phenotype such CD25 and CD2 positivity. At smear examination mast cells presented atypical morphology, often with vacuoles and many of them seemed to explode. The bone marrow FISH analysis resulted positive for t(8;21) to the extent of 12% and molecular analysis of AML1-ETO rearrangement was positive in RT-PCR.

Conclusion: Regarding the origin of the mast cells burden in AML-SM several studies suggest that the mast cells may be derived from the leukemic clone. The prevalence of SM associated with t(8;21) AML is currently unknown. Even if t(8;21) is a prognostically favourable factor in AML, the association AML-SM get worse the outcome. Further studies of the mutational status of cKit gene will be necessary for the evaluation of risk disease assessment and for the setting of intensive therapeutic regimes, such as allogenic transplantation.

ADV-22 (also presented as Poster Flash Presentation # PFP-36)

Demonstration of Equivalence between OneFlow-specific tube settings on the BD FACSlyric™ compared to OneFlow setup on the BD FACS Canto II™

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2BD Biosciences, San Jose, U.S.A.

Introduction: The BD FACSlyric™ system provides clinical users a CE-IVD solution to standardised setup and simplified user workflow, helping ensure reproducible results across instruments. BD OneFlow™ reagents, currently released on the BD FACS Canto II™ requires more regular instrument setup using CS&T beads, BD OneFlow™ set up beads and BD FC beads™. The purpose of this study was to create a streamlined approach using tube settings on BD FACS Lyric™ to demonstrate its equivalence with the OneFlow setup on the BD FACS Canto II.

Method: Spherotech Rainbow beads were acquired on six BD FACS Canto II™ and PMT voltages (PMTV’s) adjusted to within range. BD FC beads™ were acquired on six BD FACS Canto II™ instruments and target MFI recorded and averaged. BD FC beads™ were then acquired on a BD FACS Lyric™ and PMTV’s adjusted to averaged target MFI identified on the six BD FACS Canto II™ instruments. To ensure forward and side scatter met the EuroFlow guidelines, whole blood was lysed and PMTV’s adjusted. Tube settings were created, ported to ten BD FACS Lyric™ instruments and tested against the BD FACS Canto II™ using BD FC beads™ and stained BD OneFlow ALOT™ tubes with whole blood from healthy subjects and acute leukemic patients. FCS files from both the BD FACS Canto II™ and BD FACS Lyric™ were analysed for MFI difference and expressed as percentage of FACS Canto II™ acquired data.

Results: Data from BD FC beads™ and whole blood stained BD OneFlow™ ALOT tubes show that OneFlow specific tube settings on the BD FACS Lyric™ are comparable to the OneFlow setup on the BD FACS Canto II™. MFI variance for markers in BD OneFlow ALOT™ tubes were equivalent between platforms for healthy and diseased stained samples. Furthermore, when assessing B cells and T cells using FACSuite software, FACS Canto & FACS Lyric ALOT data overlapped, illustrating MFI comparison.

Conclusion: We demonstrated and confirmed that the BD FACS Lyric setup with OneFlow specific tube settings are equivalent to BD FACS Canto II™ setup and are reproducible across BD FACS Lyric™ instruments.

Disclaimer: BD OneFlow™ tubes on BD FACS Lyric™ is under development and is not available for sale.
The importance of Flow Cytometry in the advancement of translational science
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Introduction: Translational research has allowed improving the diagnosis and follow-up of hematological diseases and malignancies. However, there are some classical approaches used for research and clinical practice that remain practically unchanged, whereas current advancements in flow cytometry (FCM) technology can be easily implemented to obtain more precise measurements. Here, we discuss three different strategies used for: a) doublet discrimination (DD); b) FCM quantification of granulocytic alkaline phosphatase (GAP) activity; and c) CD34+ cell counting according to recommendations of the ISHAGE. First, DD ensures only single cells are counted with the exception of a large range of cell sizes on a linear scale, which can make it difficult to discriminate pathological large cells. Second, semi-quantitative quantification of the GAP activity is still based on observer-dependent color-intensity classification. And third, absolute CD34+ cell counting by the single-platform protocol is associated with the well known “vanishing counting beads phenomenon”. The studies presented here are aimed at better understanding how to improve these approaches for the analysis of a) highly enriched samples and pathological large cells; b) quantification of GAP activity; and c) true volumetric counting of CD34+ stem cells.

Methods: Peripheral blood samples were used for DD (n=25) and GAP activity (n=29) studies, and mobilized peripheral blood (MPB) for CD34 study (n=58). Leukocytes were stained with Vybrant DyeCycle™ Violet Stain (Thermo Fisher) for DD, and GAP studies. Samples for GAP study were incubated with Alkaline Phosphatase Live Stain (Thermo Fisher). MPB was prepared following ISHAGE guidelines. Samples were acquired on the Attune™ Acoustic Focusing Flow Cytometer (Thermo Fisher), Attune™ NxT Flow Cytometer (Thermo Fisher) and Cytomics FC500 (Beckman Coulter).

Results: The Bland-Altman test showed agreement between fluorescence and scattering methods. Pair wise comparisons between fluorescence and scattering methods showed that the slope was near one (95% CI, 0.89-0.93, P-value=0.0001) (study a). In study b), differences between the classic index and the new implemented one, were statistically significant (95% CI, 17.38-45.95, P-value<0.0001). The Bland-Altman test showed agreement between both methods. In study c), the Bland-Altman test and linear regression showed that volumetric CD34+ counting was comparable with current ISHAGE recommendations (R²=0.9925, n=56).

Conclusions: Our results demonstrate the applicability of newly implemented FCM methods and show the advantages of the introduced strategies. The transference of research advancements to the clinic is a crucial step to improve diagnosis and follow-up of hematological diseases and malignancies.
HEM - HEMATOLOGY-ONCOLOGY

HEM-01

Clinical features, Laboratory Characteristics, and Response to Therapy in Patients with Acute Undifferentiated Leukemia
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Introduction: Acute undifferentiated leukemia (AUL), has been recognized as a unique entity by the WHO classification of myeloid neoplasms since 2008. It is characterized by the lack of expression of either myeloid or lymphoid lineage markers and by expression of CD56 and other immature T-cell markers. Studies have reported that AUL is associated with a poor prognosis; however, cytogenetic and molecular characterization of this subgroup of patients is lacking, as are the long-term outcomes of induction therapy and stem cell transplantation (SCT). The goal of this study was to assess clinical and laboratory features of AUL patients including response to initial therapy.

Methods: Parameters assessed at diagnosis included: blood counts, bone marrow (BM) blast cell percentage, phenotypic characteristics of BM blasts, molecular mutations and translocations, as well as cytogenetic abnormalities. Immunophenotyping of BM cells at diagnosis was performed using eight-color flow cytometry. Multi-parameter Cytognost acute orientation tube (ALOT) and the combination of the following antigens were used: CD34/CD117/CD14/CD56/CD36/CD123/HLA-DR/CD45/CD64/CD11c. Data were acquired with FACScount flow cytometer and analyzed with Infinicyt software. Myeloperoxidase staining by fluorescence-activated cell staining was considered negative if <3% of blast population stained positive. Data on BM response to initial therapy and the 18-month outcome following the diagnosis of the first patient in this cohort were assessed.

Results: One hundred and fifteen cases of acute myeloid leukemia were diagnosed at the Rambam Health Care Campus between 11/2016 and 5/2018; 15 (1.4%) of them were defined as AUL. CD56 positivity was demonstrated in 8 (50%) cases, 5 of which were also CD7 positive. None of the patients was found positive for either NPM1 or FLT3 mutations. Likewise, no core binding leukemias were diagnosed in this cohort. 66% (10/15) of the BM samples demonstrated cytogenetic and/or FISH abnormalities, with 26% complex karyotype cases, and 20% chromosome 7 aberrancies. All but two patients were treated with AML-oriented therapies. None of the patients achieved remission with induction therapy regardless of whether it included a chemotherapy backbone (82% of patients) or a hypomethylating agent.

Conclusions: The present study confirms the poor response to therapy of AUL patients. The high percentage of complex karyotype abnormalities, the lack of NPM1 mutations, and the expression of T/NK cell immaturity markers could partly account for the established poor outcome.

HEM-02 (also presented as Poster Flash Presentation # PFP-20)

MRD detection in Mantle cell Lymphoma using multicolour Flow cytometry in a clinical Laboratory setting
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Introduction: Mantle cell Lymphoma (MCL) represents 3-10% of mature B cell neoplasia with an incidence in Europe of 0.45 per 100,000 persons. MCL is characterised by immunophenotyping (D19+,CD5+,sIgM+,CD20+,CD23-,CD79b+,CD62L+), over expression of Cyclin D1 and the t(11;14) translocation. Recent advances in treatment have improved CR rates however low levels of residual disease is a significant cause for disease relapse. In addition to the molecular methods currently used; PCR of clonal IgH rearrangements and detection of the t(11;14) translocation, multicolour flow cytometry methods have bee shown to have similar sensitivities.

Method: This study validated for use in the clinical laboratory an 8 colour single tube MRD assay described by Chovancov et al using (CD19,CD5,CD20,CD23,CD62L,CD200,CD45 & CD3) with a sequential gating strategy and compared the results for treated MCL patients to those obtained by molecular techniques.
Results: Our results showed that this multicolour flow cytometry assay is able to reach a robust sensitivity of 0.025%. A comparison between this method and current molecular methods was carried out on 10 MCL patients post treatment, with very good correlation observed.

Conclusion: Our results show that this MRD assay using multicolour flow cytometry is suitable for testing treated Mantle cell Lymphoma patients and is suitable for use in the clinical laboratory setting.


HEM-03 (also presented as Poster Flash Presentation # PFP-06)

IDENTIFICATION OF HIGH RISK IMMUNOPHENOTYPIC PROFILE CORRELATING WITH PRIMARY INDUCTION FAILURE, SHORTER OVERALL AND DISEASE-FREE SURVIVAL IN NPM1-MUTATED AML

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Introduction: Although sharing a driver gene mutation, NPM1-mutated (NPM1mut) acute myeloid leukemia (AML) is a clinically heterogeneous disease with variable outcome; to date a comprehensive description of the immunophenotypic landscape in NPM1mut AML has not been reported; our aim was to investigate the association between specific immunophenotypic patterns and clinical outcome in this AML subset.

Methods: We identified 64 NPM1mut AML cases treated between 2010 and 2018 with intensive chemotherapy. Flow cytometry was performed on diagnostic bone marrow samples (BM) in order to fully characterize the immunophenotypic profiles of leukemic populations. We focused our attention on myeloid cells in the CD45dim SSClow blast area. Based on CD34 expression we identified two main cell subsets: CD34+/CD45dim versus CD34−/CD45dim. The two subgroups were analyzed by using a comprehensive antigen panel. Parameters were expressed as percentage of positive cells and/or as mean fluorescence intensity; 6BM samples from healthy donors were used as controls to set normal references.

Results: Cluster analysis led to the identification of 3 immunophenotypic clusters (C1, C2, C3). Distinctive features of C1 were expression of immature antigens (CD117, HLA-DR) on both CD34+/CD45dim and CD34−/CD45dim cell compartments as well as asynchronous CD11b expression and cross-lineage CD7 and CD25 expression. In C2, CD34−/CD45dim population showed an acute promyelocytic leukemia-like phenotype (CD117+/−, HLA-DR−) albeit with low SSC and CD13dim. In C3 the leading trait was differentiation to monocytic lineage, with low frequency of aberrant antigen expression. Survival analysis by cluster revealed significant differences in terms of DFS and OS (P=0.006; P=0.05, respectively). Patients belonging to C1 had a higher relapse risk and a relatively poor outcome when compared to patients classified as C2 and C3. Since the latter categories had a substantially superimposable outcome, we compared patients from C1 to the rest of the population as a unique entity: C1 cluster correlated with significantly shorter survival. Along with FLT3-ITD and age, C1 phenotype emerged as an independent predictor for DFS and OS. While CR rates after first induction cycle were comparable between C1 and Non-C1 patients, C1 was enriched with patients not achieving CR after 2 or 3 induction cycles (36% vs 11%; P=0.016; OR 2.024). In multivariate analysis, a C1 profile was the sole predictive factor for not achieving a CR anytime.

Conclusion: Our analysis led to the identification of 3 distinctive immunophenotypic profiles significantly correlating with clinical outcome. C1 cluster enucleated patients at high risk of refractory disease and shorter DFS and OS.
HEM-04 (also presented as Poster Flash Presentation # PFP-25)

Hepatosplenic T cell Lymphoma. Role of flow cytometric immunophenotyping
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Introduction: HSTCL is a rare subset of T cell lymphoma typically characterized by young age at presentation, hepatosplenomegaly, cytopenias, and absent or minimal lymphadenopathy. Leukemic manifestations of the disease with bone marrow and/or peripheral blood involvement makes acute leukemia a close differential, especially when the cells have a blastic morphology. Thus, flow cytometric immunophenotyping (FCI) remains the cornerstone for the exact characterization of this entity.

Material and methods: A retrospective analysis of all the consecutive cases of HSTCL diagnosed over a six years period (June 2013 to May 2019) was performed. Clinical and laboratory details were retrieved from the hospital electronic medical records. FCI was done using a stain-lyse-wash protocol with 6 & 8 color panel. The morphological and flow cytometry data of all the cases were reviewed.

Results: A total of 12 cases of HSTCL were diagnosed during the period, which constituted ~1.8% of all non-Hodgkin lymphoma (NHL) diagnosed over this period. The median age of presentation was 24 years (range 16-30 years) with M: F ratio of 2:1. Anemia, thrombocytopenia, and hepatosplenomegaly were present in all the patients. Leucocytosis was noted in 50% (6/12) cases and 75% (8/12) cases had circulating tumor cells in peripheral blood ranging from 10-85%. A blastoid morphology was appreciated in six (50%) cases in the peripheral blood. Bone marrow biopsy displayed a diffuse and interstitial pattern of infiltration in 58.3% cases as compared to the classical sinusoidal pattern in only 41.7% cases. Immunophenotypically, the cells consistently expressed CD7 (100%), CD2 (100%), CD3 (91.7%); and gamma delta T cell receptor (100%). CD5, CD4, and CD8 were expressed in two (1.66%) cases and one (0.83%) case respectively. CD56 was expressed in 5(41.8%) cases. Interestingly, one of the cases was negative for surface CD3. Absence of all immaturity markers (CD34, CD99, CD1a, TdT), a bright CD45 expression and a gamma delta TCR expression along with the typical clinical presentation, supported this case as HSTCL.

Conclusion: Leukemic presentation and ‘blastic’ morphology of HSTCL may be a diagnostic challenge to oncologists and pathologists alike. A high index of suspicion, cognizance of this variant and use of FCI is mandated to establish an accurate diagnosis.

HEM-05 (also presented as Poster Flash Presentation # PFP-04)

Validation of a10-color 21-monomoclonal antibody screening panel for multi-parametric diagnostic Immunophenotyping
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Introduction: The rapid and sensitive multi-parameter detection, even at a single cell level, renders flow cytometry (FCM) to a powerful tool to distinguish malignant cells from normal cells. In particular, detection of MRD or characterization of very small cell populations (e.g. in cerebral spinal fluid and hypoplastic bone marrow) that can only be analysed by a one-tube determination, needs high-level multi-color analyses. Due to the limited number of PMT’s in most widely used flow cytometers for clinical purposes, a stacking of conjugates in one color is needed to enlarge the number of parameters to detect aberrant populations. Therefore we developed recently a screening panel of 10 colors containing 21 MoAbs directed against target antigens to detect malignancies in all lineages.

Method: After identification and selection of target antigens and an extensive search for suitable MoAb clones, all MoAbs were titrated to find the correct concentration. Subsequently, compensation of spectral overlap and extensive balancing of the panel were performed to establish suitable and correct combinations of MoAbs within one color and preservation of their capacity to separate and identify cell populations. This resulted in the development of a 10-color 21 MoAb screening panel. To test the feasibility of this panel a validation study was performed together with our standard 6-tube 5-color MoAb screening panel.
Results: To validate our new 10-color 21-MoAb screening panel, forty-two bone marrow and peripheral blood samples from normal individuals and patients with various hematologic malignancies were screened with both panels using a Navios 3-laser 10-color instrument. KALUZA analysis software was used to compose protocols for sample evaluation tested with both panels. The capacity to detect subpopulations of all lineages and malignant aberrancies was compared. This analysis resulted in comparable detection of malignancies with both screening panels.

Conclusion: This validation study of a one tube 10-color 21-MoAb panel showed that normal and malignant subpopulations in bone marrow and peripheral blood samples can be equally identified as with standardly in-use panels. By using one tube only, a very high resemblance in detection of subpopulations of all lineages and malignant aberrations, despite the use of multiple combinations of MoAbs within the same color, can be found. The key issue to create such a high-level powerful screening tool is the performance of a correct balancing of the panel and the use of correct analysis protocols.

HEM-06 (also presented as Poster Flash Presentation # PFP-23)

Comparison of anti-Kappa and Lambda light chains antibodies from Dako and Beckman Coulter

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Background: Each mature B lymphocyte expresses surface immunoglobulins bearing only one class of light chain, either Kappa or Lambda. Assessment of immunoglobulins light chains expression on B-cells by flow cytometry is the hallmark of B-cell malignancies studies. Indeed, while in healthy individuals the ratio of kappa+ to lambda+ B cells is roughly 3:2, this ratio is skewed by the expansion of clonal malignant cells. Kappa/Lambda flow cytometry assays can sometimes be challenging for laboratories, as results interpretation may be difficult. It is especially the case when non-specific staining occurs using non-optimized protocols or reagents. Dako's rabbit polyclonal anti-kappa and lambda antibodies are widely known as the gold standard, delivering best-in-class performances. In this study we compared Dako's antibodies to Beckman Coulter's new goat polyclonal anti-Kappa and Lambda antibodies, conjugated to FITC and PE.

Methods: Both suppliers' reagents were compared 15 times in various conditions. The Stain/Wash/Lyse protocol recommended by the International Clinical Cytometry Society (ICCS) was used in three variants, incorporating 1, 2 or 3 sample washes. 5 whole blood samples were processed with the three variants. Comparison between conjugated antibodies was based primarily on the assessment of the staining index, calculated as (MFI positive population – MFI negative population)/(2xSD of negative population).

Results: Performances of both supplier reagents are similar. Beckman Coulter’s antibodies provided a greater staining index when using all non-B lymphocytes as a negative population. Restricting the negative population to B-cells when calculating the staining index, Dako’s antibodies delivered a better staining index on average. Performing a single washing step of the samples did not allow to gate and discriminate positive and negative subsets. In contrast, two washes greatly facilitated the distinction and three washes offered a clear separation of Kappa+ and Lambda+ populations, no matter the supplier.

Conclusion: Each laboratory must perform their own protocol optimization and validation, as there are no real consensus protocol for Kappa and Lambda light chains assessment. It is particularly important to determine the number of washing steps required to deliver accurate and trusted results. Either Beckman Coulter or Dako anti-Kappa and Lambda antibodies could be used in this process, as performances are overall similar.
Acute myeloid leukemia with 3q26/MECOM (EVI1) gene rearrangement displays distinct immunophenotypic profiles with frequent CD7 expression and absent myeloperoxidase

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Introduction: Acute myeloid leukemia (AML) with inv(3)/t(3;3);GATA2-MECOM is a distinct subtype of high risk AML in the World Health Organization (WHO) classification with characteristic morphologic and clinical features. The MECOM rearrangement may also result from variant translocations such as t(3;21) or t(3;12) where it partners with other genes. We hypothesized that the immunophenotypic profiles of blasts in AML with MECOM rearrangement are distinct and shared by all variant translocations (3q26;v).

Methods: We retrospectively reviewed 62 consecutive cases of AML diagnosed during Jan. 2014 to Jan. 2019 with MECOM rearrangement confirmed by fluorescence in situ hybridization (MECOM/EVI1 dual color, breakapart probe, Leica/Kreatech Inc.). Each case was analyzed with 8 color flow cytometry on FACSCanto II instruments (BD Biosciences) using a panel of antibodies against CD2, surface CD3, cytoplasmic CD3, CD4, CD5, CD7, CD13, CD14, CD15, CD19, CD22, CD25, CD33, CD34, CD36, CD38, CD13, CD145, CD117, CD123, HLA-DR, MPO and TdT. The expression pattern of each marker was assessed by the “difference from normal” approach using FCS Express software (De Novo).

Results: The study group included 31 de novo, 21 following MDS/CMML (n=17) and 10 therapy related. The blasts typically expressed CD7, CD34, CD117 and HLA-DR with increased CD13 and CD123, but decreased CD33 and CD38, absent or rare MPO events and negative for TdT. This profile was seen in 25/62, 40% of cases, and in another 26/62 (42%) of cases with mild (1-3 markers) deviations from it. Total or partial CD7 or CD5 expression were observed in 39/62, 63% and 9/62,15%, respectively. Four cases coexpressed CD2 and CD7. Additionally, CD56 and CD25 were positive, often partially, in 19/62, 31% and 15/62, 25%, respectively. The blasts usually lacked or expressed dim/partial CD36 and CD64, except in two cases of monoblastic leukemia. None had significant cytoplasmic CD3 expression. CD7 expression was seen across all 3q26 translocation variants: inv3/t(3;3) (21/34, 62%), t(3;12) (5/5, 100%), t(2;3) (4/5, 80%), other variants (7/9, 78%), but was least seen in t(3;21) (2/8, 25%). Two cases expressed CD19, both t(3;21).

Conclusions: AML cases with MECOM gene rearrangement, including most with variant translocations, share distinct immunophenotypic profiles with frequent CD7 expression and lack of MPO, suggesting that they can be grouped in the category of AML with inv3(t(3;3) in the WHO classification. The immunophenotype, with markers of lineage infidelity, suggests the blasts arise from primitive hematopoietic precursors or highly dysplastic clone(s), correlating with the aggressive clinical features.
**Conclusions:** Based on the expression of CD34 and CD117, BPDCN can be categorized into three different stages of maturation. As both markers were expressed in our case the immunophenotype of the plasmacytoid dendritic cells corresponds to immature entity. Considering different treatment protocols for BPDCN and AML, it is crucial to differentiate between these two entities.

**HEM-09 (also presented as Poster Flash Presentation # PFP-21)**

**Radar plots facilitate quick evaluation of the lymphoid cell compartment using 10-color 15-antibody panel**

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**Introduction:** We have previously validated a 10-color 15-antibody lymphoid subpopulation screening panel in two laboratories (Toronto, Canada and Lund, Sweden) using harmonized settings (IntJLab Hematol. 2017;39;Suppl 1:76-85). We have now developed radar plots (RP) to visualize B- and T-cell subsets with the aim to quickly detect abnormal patterns.

**Methods:** In Toronto, list mode files of previously reported samples (n=695) were retrospectively re-analyzed. In Lund, 655 consecutive samples were re-analyzed blindly and categorized as normal or abnormal. The results were then compared with the previously issued reports. RPs were also applied to 20 additional samples with known abnormal T-cell population.

**RP analysis:** After removal of dead cells and debris, a “lympho/mono” gate was applied to the CD45/side scatter (SSC) plot. B cells were gated as CD19+ or CD20+ lymphocytes with exclusion of plasma cells. A six-parameter RP showed: kappa and lambda horizontally from east to west and CD20/CD10 respectively CD5/CD23 vertically north and south. In normal blood samples, a clear separation of kappa and lambda positive B-cells was seen, while in bone marrow or lymph nodes CD10+ B-cells were also present. T cells were gated as “lympho/mono” with exclusion of CD14+/SSCintermediate/high and CD19+ cells. A six-parameter RP displayed CD4 and CD8 horizontally from east to west and CD5/CD10 and CD57/CD38 vertically north and south, respectively.

**Results:** In Toronto, all 458 cases with normal B-cell and T-cell populations were correctly identified using RPs. B-cell populations with a typical CLL/SLL phenotype were detected in 145/146 cases (sensitivity: 99%). Other CD5+ B-LPD were detected in 29/30 cases. CD5+ monotypic B-cell populations were detected in 4 cases. CD5-/CD10-monotypic B-cell populations were detected on RPs in 45/48 cases. In Lund, a concordance was found in 370 cases reported as normal. RP were difficult to evaluate in 30 cases due to a low total lymphocyte count. In 17 cases, an abnormal RP was found but cases were reported as normal after additional studies. In 173 cases, concordant results concerning an abnormal B-cell population were found. In 25 samples, a small abnormal B-cell population (<1%) was reported but it was not easily seen on RPs. In 43 cases with a reported abnormal T-cell population, it was identified on the RP in 37 samples (86%). In 6 cases, abnormal T-cells were found only in additional analyses.

**Conclusions:** The proposed RPs can potentially improve turn-around time as they quickly differentiate between normal and aberrant lymphoid patterns.
Eosinophilia and flow cytometry (FC) related studies: a Spanish multicentre experience
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Introduction: Non-haematopoietic conditions and haematopoietic neoplasms (HN) may be associated to eosinophilia. This work describes the FC protocols applied for the study of samples requested for eosinophilia and aims to determine its diagnostic yield.

Methods: A group of Spanish clinical FC laboratories answered a survey to investigate their current workflow when “eosinophilia” was the reason for consulting.

Results: Between 2016 and 2018, the 9 centres that joined the study received a total of 60,687 immunophenotyping studies. Eosinophilia was the reason for request in 266 samples: 210 (0.34%) peripheral blood, PB; 55 (0.09%) bone marrow, BM and 1 urine. The frequency of this request per year among laboratories ranged between 0.05-1.74% in PB, and 0.07-0.7% in BM. Only 17 patients had serial evaluations. Median eosinophil count (218 cases had available information) was 1,200/mm³ (<100-33,563).

After reception, 46 samples (17.3%) were discarded for FC study because eosinophilia wasn’t confirmed. A sequential strategy for staining was used in 8/9 centres. All included CD3, CD4, CD8 and CD45 in their screening panel, 7 added CD5 and B cell marker, and 2 centres studied CD7. To characterise abnormal T-cell populations, laboratories designed heterogeneous home-made panels, with agreement on 12/36 reagents in >50% laboratories.

Definitive clinical diagnosis was available in 150/220 cases (68.2%), mostly reactive conditions (n=102/150, 68%). In the remaining 48 cases (42 patients), eosinophilia was associated with myeloid malignancies (5 myeloid neoplasms with PDGFRA/PDGFRB rearrangement, 2 acute leukemias, 2 myelodysplastic syndromes, 1 systemic mastocytosis); lymphoid malignancies (6 T-NHL, 5 B-CLL, 2 Hodgkin’s disease, 2 clonal gammopathies, 1 NK malignancy); eosinophils disorders (14 hypereosinophilic syndrome, 5 lymphocyte-variant hypereosinophilia); and other entities (2 autoimmune thrombocytopenia, 1 hyper-IgE syndrome).

FC detected abnormal T-cell populations in 18 cases: 5 lymphocyte-variant hypereosinophilia, 1 angioimmunoblastic T-NHL, 1 large granular lymphocytic leukemia and 3 reactive conditions. Eight cases lacked conclusive diagnosis. Median percentage of abnormal T-cells as referred to total lymphocytes was 12.5% (0.02-50%).

Conclusions: A FC screening test for all lymphoid populations seems a reasonable approach to a “eosinophilia” request because most of them are finally catalogued as reactive. In this series, lymphomas were infrequent, with a similar FC detection of B-CLL and T-NHL. Further studies are necessary to determine whether eosinophilia associated with B-CLL is reactive or a manifestation of a synchronous myeloid malignancy. FC was crucial to diagnose lymphocyte-variant hypereosinophilia but follow-up and TCR molecular studies are often needed to determine the clinical significance of abnormal T-cell populations.
Flow cytometry challenge for measurable residual disease (MRD) monitoring in B-acute lymphoblastic leukemia (B-ALL) patients targeted against immunotherapy (TI)

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INTRODUCTION: TI with Blinatumomab (Bln) or Inotuzumab ozogamicin (IO) are a salvage option B-ALL patients with MRD, in which high rates of MRD negative can be achieved, allowing a bridge to transplantation and longer survival. However, some patients relapse with a phenotype lacking CD19 or CD22 expression can be found in blast cells. Those escape variants have also been described under CAR-T cells therapy and have to be considered in flow cytometry immunophenotyping (FCM) analysis when monitoring MRD. We propose to review the monitoring of MRD by FCM of patients with B-ALL treated with TI in our center and who relapsed.

METHODS: FCM monitoring were performed in bone marrow aspirate (BMA) samples, using 8-colour combinations of monoclonal antibodies adapted to the TI received and diagnosis phenotype. Stained cells were measured in FACSCanto II cytometer equipped with the FACSDiva software and analyzed with the Infinicyt software with a sensitivity (S) 10^-4.

RESULTS: Between 2015 and 2018, 2 B-ALL patients relapsed after TI, but only one showed immunophenotypic changes. The patient was a 72-year-old woman diagnosed of common B-ALL who started PETHERMA-LAL-OLD-07 protocol. BMA at the end of induction was in morphological complete response (CR) with MRD positive (0.12%). After 3rd consolidation cycle (C), MRD persisted (0.04%), so she started Bln and achieved MRD negative (S 10^-4) after 1stC. However, after 3rdC, a new CD19- blast cell population was detected (0.07%), with a lower expression of CD20, while the other markers were similar to the diagnostic. In normal B cells (0.05%) the intensity of CD19 expression was preserved. Blast cells increased after 4thC (2.9%) with an associated morphological relapse (21%). She started IO and reached CR and MRD negative (S 10^-4) after 1stC. Treatment was stopped after 4thC due to upper gastrointestinal bleeding, and in the BMA previous to the next C programmed, CD19- CD20dim CD34+ blast cells were detected again (0.55%). All blasts lost CD22 expression, and only 9% maintained the CD10 expression, and the rest lost it. The patient was not candidate to any other therapy, she started palliative treatment and died 2 months later.

CONCLUSIONS: From our experience, changes in immunophenotype of B-ALL blast cells are frequent after TI, due to loss of CD19 and CD22 expression. Therefore, FCM analysis in patients treated with TI demands a high degree of expertise and attention, to avoid the loss of detection of new populations that could emerge with different phenotype.

HEM-12 (also presented as Poster Flash Presentation # PFP-22)

Optimization and evaluation of a 10-color antibody panel for flow cytometric immunophenotyping of B-cell lymphomas

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Introduction: Flow cytometric immunophenotyping (FCI) is widely used in cytopathology to distinguish between reactive lymphocytic proliferations (RLP) and B-cell lymphoma (BCL). The aim of this study was to evaluate and introduce a new 10-color antibody panel for the diagnosis of B-cell lymphoma in our routine laboratory work.

Methods: 48 samples (39 fine needle lymph node aspirations biopsies, 6 surgical excision lymph node biopsies and 3 samples of pleural effusion) were analyzed with an existing 4-color antibody panel (tube 1: CD3 FITC/CD19 PE/CD45 PerCp-Cy5.5/CD20 APC; tube 2: SmIg FITC/SmIgA PE/CD19 PerCp-Cy5.5/CD10 APC; tube 3: FMC7 FITC/CD23 PE/CD19 PerCp-Cy5.5/CD5 APC) and a new 10-color antibody panel (SmIgk FITC /SmIgA PE/CD5 PerCp-Cy5.5/CD19 PE/Cy7/CD20 APC/CD3 APC-Cy7/CD23 APC-R700/FMC7 V450/CD45 V500c/CD10 BV605). The percentages of positive cells and mean fluorescence intensity (MFI) values for all markers were determined and correlated (Spearman correlation). The cytopathology diagnoses for all samples were based on morphological examination and FCI data and compared to the final diagnoses which were set by histology examination. This study was approved by The National Medical Ethics Committee of the Republic of Slovenia (0120-574/2017/3).
Results: Statistically significant correlations for the percentages of positive cells and MFI values were observed for all B-cell markers and CD3 (p<0.05). There were no differences between the immunophenotypes of malignant cells and the diagnoses determined with 4-color and 10-color antibody panels. 10 cases had a final diagnosis of RLP and 38 of BCL (7 follicular lymphomas, 6 mantle cell lymphomas, 10 chronic lymphatic leukemias, 7 diffuse large B-cell lymphomas, 6 marginal zone lymphomas and 2 composite lymphomas). In 42 cases (87.5%) the cytopathology diagnosis matched the final diagnosis. In 4 cases (8.3%) a diagnosis of BCL was established but the lymphomas were wrongly classified. In 2 cases (4.2%) a diagnosis suspicious for BCL was given.

Conclusions: Our study showed that the new 10-color antibody panel is suitable for everyday routine use for the diagnosis of BCL. As only one tube is required for the analysis, the new 10-color antibody panel will ensure a more reliable diagnosis of BCL, especially for the samples with few cells.

HEM-13

Immunophenotyping of UK NEQAS PCM Samples Using a One-tube 12-color Antibody Panel on the BD FACSLyric™
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Introduction: Flow cytometry is one of the most powerful tools used for the classification and analysis of plasma cell neoplasms. Flow cytometric applications rely on standardized multicolor antibody reagents and instrument systems to generate reliable and reproducible data comparable across laboratories. The newly developed 12-color BD FACSLyric instrument combines faster acquisition with measurement of higher number of total cells allowing for the determination of low concentration aberrant phenotypes and characterization of phenotype profile for the abnormal cells. BD FACSLyric™ system simplifies cytometer standardization by enabling a one-step daily instrument setup and QC with automatic daily optimization of fluorescence compensation. Biological assays with the same tube or assay settings are easily transported across different BD FACSLyric instruments for multisite studies. In this study we demonstrate Immunophenotyping using a one-tube, 12-color antibody panel on the BD FACSLyric for the Plasma Cell Myeloma (PCM) samples provided by the UK National External Quality Assessment (UK NEQAS).

Methods: The 12-color BD FACSLyric was set up using the BD CS&T IVD beads and BD FC beads. Normal bone marrow and UK NEQAS PCM samples were surface- and intracellular-stained using a one-tube, 12-color panel containing CD38 FITC, CD56 PE, B2-Microglobulin PerCP-Cy5.5, CD19 PE-Cy7, CyAnti-Kappa APC, CyAnti-Lambda APC-H7, CD45 V450, CD138 V500-C, CD28 BV605, CD27 APC-R700, CD117 BV711 and CD81 BV786. The four backbone markers (CD38, CD138, CD45 and CD19) were selected to identify and distinguish plasma vs abnormal cells. Other eight markers were used to confirm clonal nature of plasma cells and to characterize/classify normal/reactive vs aberrant plasma cells. This 12-color antibody panel combined all unique markers in the two EuroFlow panels: PCST and PCD.

Results and Conclusion: We analyzed 3 normal bone marrow and 2 UK NEQAS PCM samples on the BD FACSLyric using the one-tube 12-color antibody panel to obtain immunophenotyping profiles. The percentages and MFI of plasma cells were calculated and compared between normal bone marrow and PCM samples. Relative to robust mean of abnormal cell percentages from peer laboratories, Z scores of the two PCM samples on the BD FACSLyric were 0.45 and -0.18, respectively. We demonstrated that UK NEQAS PCM samples can be analyzed on the 12-color BD FACSLyric with standardized setup using a one-tube 12-color reagent panel to generate consistent results as compared with peer laboratories.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.
Analysis of content of 5-hydroxymethyluracil - potential specific biomarker of leukemic cells in patients with chronic lymphocytic leukemia

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Introduction: Although there are several recurrent molecular abnormalities present in chronic lymphocytic leukemia (CLL), none is specific and therefore immunophenotyping still plays a central role in the diagnosis of CLL. The current diagnostic criteria have some limitations affecting reproducibility, in particular relating to flexibility in the requirement for each marker to be present or absent as well as in the required expression level of each marker. In preliminary results we observed ca. 10-30 times higher level of 5-hydroxymethyluracil (5-hmUra) in DNA of peripheral blood nuclear cells of CLL patients, comparing to non-CLL individuals. The main goal of this study was to investigate the content of 5-hmUra in leukemic lymphocytes and residual cells in CLL patients in comparison to lymphocytes, granulocytes and monocytes in healthy control.

Method: The blood samples were stained with direct conjugated antibodies BV421 anti-human-CD45, for identification cell populations. Next, the cells were fixed, permeabilized and resuspended in PBS with calcium and magnesium ions and exposed to a high temperature 99 °C. Then, cells were inserted into dry ice for 10 minutes. Next step involved co-stained by indirect method with anti-human primary antibody anti-5-hmUra followed by compatible secondary antibody conjugated with fluorescent dyes rabbit anti-goat Alexa Fluor 647. The content of analyzed nucleobase calculated as fluorescence intensity fold change over negative control.

Results: Preliminary results indicate that higher content of 5-hmUra was observed in all tested cell populations in CLL compared to healthy individuals. It is noteworthy that a statistically significantly higher content of 5-hmUra was observed in leukemic lymphocytes in CLL comparison to lymphocytes in control group. During the 3-month follow-up of patients no difference in the level of 5-hmUra was noticed.

Conclusions: The significantly higher level of 5-hmUra in the leukemic lymphocytes in CLL compared to lymphocytes in control group suggest the involvement of these nucleobase in the pathogenesis of this type of leukemia. Therefore, further studies are needed on a larger group of patients to prove its epigenetic role. Furthermore, this research shed new light on the search for a potential epigenetic marker for CLL, which could be routinely marked in laboratories.

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HEM-15 (also presented as Poster Flash Presentation # PFP-26)

Metastatic lobular carcinoma of the Breast presenting as myeloma - a potential pitfall in CD138 immunoreactivity in non-haemopoietic disease

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Introduction: The specificity of CD138 reactivity is not confined to haematological neoplasms, particularly myeloma, but is expressed in a spectrum of non-haematopoietic malignancies.

Case Presentation: A 55 year old woman was referred for investigation of multiple lytic lesions (CT scan), lymphadenopathy, hypercalcaemia and hip pain. Her serum and urine were negative for monoclonal bands or raised free light chains, her free light chain ratio was normal. Local (district general hospital) review of the bone marrow aspirate and trephine was stated as most consistent with plasma cell myeloma. Trephine biopsy was reported as showing a significant infiltrate of plasma cells staining strongly for CD38 and CD138. The immunohistochemical staining of immunoglobulin light chains were inconclusive. Upon receipt of the referral material at the SIHMDs, based on the clinical history and presentation information a plasma cell neoplasm panel in addition to T-, B-, NK, myeloid immaturity assessment was processed. Immunophenotyping demonstrated no obvious clonal T or B-cell population or features of immaturity. 0.1% of all total TNC were CD138+/CD38+/CD19+/CD56-/-CD117- polyclonal plasma cells. Additionally, a population noted to be CD138+/CD38-/-CD19-/CD56-/-CD117- with inconclusive light chains were detected, representing 0.8% of TNCs. Due to the negative CD38, cytoplasmic assessment of the non-glycosylated reversibly-palmitlyated type II transmembrane protein protein p63 was evaluated using VS38c (as performed in post daratumumab therapy).
Ambiguous immunophenotypic data in Burkitt lymphoma: rare cases of discrepancies in diagnostics

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**Introduction:** Complete differential diagnostics of Burkitt lymphoma/leukemia (BL) is based on integration of flow cytometric, morphologic and cytogenetic features. The discrepancies in these laboratory findings may challenge the correct diagnostics. Flow cytometry (FCM) provides a rapid assessment of the leukemic blast immunophenotype in bone marrow or peripheral blood. The presence of surface IgM (slgM) alone or with the light chain restriction indicates a mature blast phenotype usually observed in BL. Nevertheless slgM expression is seldom observed in case of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). FAB L3 morphology of bone marrow blasts and MYC-gene rearrangements together with FCM data complete the classical complex of BL laboratory signs. In present study, we report 10 cases of these features discordance.

**Methods:** Between 2010 and 2016 10 pediatric BL patients (aged 1-18 years; median age 10 years; M:F 8:2) with discrepant immunophenotypic, cytomorphologic and cytogenetic features were studied. These children were diagnosed as BL because of clinical and pathological studies. FCM (6-8 color) was used for evaluation of CD10, CD19, CD20, CD22, CD24, CD34, CD45, CD58, CD38, iCD79a, NG2, CD13, CD33, CD117, CD15, immunoglobulins heavy/light chains expression by leukemic blasts in bone marrow. The conventional cytogenetic analysis included G-banded karyotyping and fluorescence in situ hybridization (FISH).

**Results:** Majority of cases (n=8) were negative for slgM but had typical rearrangements of the MYC gene, e.g. t(8;14)(q24;q32), t(8;22)(q24;q11) or t(14;18)(q32;q21). Two cases exhibited L2 blast morphology. Two cases were negative for MYC rearrangements or slgM expression. Two MYC rearrangements, t(8;14)(q24;q32) and t(14;18)(q32;q21) were found simultaneously in one slgM-negative patient. The L2 morphology was found in two patients, although MYC abnormalities typical for BL were detected. In one case, undifferentiated blasts were positive for slgM and negative for MYC rearrangements. The presence of L2 blasts in one case contradicted with either immunophenotypic or cytogenetic data. Among patients who did not have any data on extramedullary lesions, one slgM-negative case was diagnosed as BL according to cytogenetic and morphologic studies. Another case demonstrated discrepancies between the cytogenetic and immunophenotypic/morphologic findings.

**Conclusions:** The presented cases taken together with the published data demonstrate the importance of a thorough multidisciplinary approach in the assessment of laboratory results. Lack of sufficient set of laboratory data and leaning only on FCM results poses a risk of incorrect or delayed diagnosis and inadequate therapy.
CD5 Positive Diffuse Large B Cell Lymphoma: Immunophenotypic, Clinical, and Pathological Findings.
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Introduction: Diffuse large B cell lymphoma (DLBCL) is one of the most common non-Hodgkin lymphoma in western countries. Most of the DLBCL are CD5 negative with rare cases being positive. We report a case series of CD5+ DLBCL and their immunophenotypic, clinical, pathological, cytogenetic, and fluorescent in situ hybridization (FISH) findings.

Methods: Cases of DLBCL were searched from hospital pathology data base from January 2017 to June 2019. Pertinent clinical, immunophenotypic (flow cytometry and immunohistochemistry (IHC)), cytogenetic and FISH findings were collected. DLBCL were further subtyped into germinal or activated B cell subtype by Han’s algorithm using CD10, BCL6 and MUM1 immunohistochemistry.

Results: A total of 135 DLBCL cases were identified during above period. Most of the cases were CD% negative except 5 cases (3.7%). These cases predominantly occurred in elderly population (age ranged from 54 to 80 years with a mean of 65 years) with a male to female ratio of 3:2. All cases showed advanced disease at staging by diffuse bone marrow involvement, 3 cases relapsed in CNS with involvement of CSF and one case spread to peripheral blood. Even though patients received variety of chemotherapy and bone marrow treatment, two patients died within a year and three patients relapsed in a short time.

By flow cytometry analysis, all cases co-expressed CD5, CD19, CD20, CD22 (3 cases), CD38, HLA-DR and light chain restricted. One case each was positive for CD10 and CD52. None of the cases were positive for CD30. By IHC, 4 cases were activated and one case was germinal center B cell subtype.

Cytogenetic showed complex karyotype and one case was positive for BCL2 and c-MYC translocation by FISH.

Conclusion: CD5+ DLBCL cases present with advanced stage, associated with poor prognosis irrespective of variety of treatments and a higher CNS relapse. Hence, all DLBCL cases should be tested for CD5 by flow and or IHC in clinical practice.

Wide panel screening for new useful markers for MRD monitoring in pediatric BCP-ALL
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Introduction: There are several well-established antigen markers commonly used in flow cytometric (FC) diagnostics of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Apart from cell lineage confirmation, a number of other antigens is used to determine possible genetic abnormalities or facilitate subsequent monitoring of minimal residual disease (MRD). To enhance the sensitivity and specificity of MRD detection, new markers should be used, preferably in ≥10-color panel setting. The aim of our study was to select new candidate markers for MRD monitoring from 12 antigens tested at diagnosis. Here we present the preliminary results of the screening phase.
Methods: The study group consisted of 66 consecutive pediatric BCP-ALL patients treated in 14 centres of the Polish Pediatric Leukemia and Lymphoma Study Group. The bone marrow samples were processed and analysed centrally at Medical University of Silesia in Zabrze, according to sample preparation protocol recommended by EuroFlow. 12 new surface markers were screened at diagnosis: CD371, CD45RA, CD72, CD44, CD27, CD50, CD43, CD82, CD40, CD273, CD274 and CD74 in 10-color setting. The frequency of all markers at BCP-ALL diagnosis was determined, as well as their relative expression level. For this purpose, median fluorescence intensity (MFI) values of blasts were compared with the MFI of positive and negative reference populations, determined individually per marker. 4 levels of expression were distinguished: negative, dim (low), strong and overexpression.

Results: 5 of 12 antigens: CD72, CD273, CD82, CD45RA and CD43 showed the highest occurrence rate on leukemic blasts at BCP-ALL diagnosis (95%, 92%, 82%, 84% and 79% of cases, respectively). The expression levels were variable; in majority (68%) of cases expression level of CD82 was strong or higher than that observed on positive reference population. Conversely, CD72, CD273, CD45RA, CD43 showed dim expression in majority of cases. On the other hand, CD27 and CD274 were most often negative or showed dim expression in 9–26% of cases. CD371 was positive only in 3% of cases, however its expression level was strong in all cases. Finally, expression of CD40, CD50 and CD44 was heterogeneous and similar to that observed on normal lymphocytes.

Conclusion: At least 4 of the tested antigens: CD72, CD82, CD273 and CD371 are potentially useful markers for MRD monitoring in BCP-ALL, which require further investigation. Conversely, CD40, CD50 and CD44 exhibited the lowest performance as MRD markers.

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HEM-19 (also presented as Poster Flash Presentation # FFP-12)

Patients with hematogones after allogeneic stem cell transplantation for acute lymphoblastic leukemia show reduced GvHD severity and improved overall survival
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Introduction: Benign precursors of B lymphocytes, termed hematogones (HG), are observed in the regenerative state of hematopoiesis following chemotherapy or allogeneic hematopoietic stem cell transplantation (allo-HSCT). Recent reports suggest that emergence of HGs is associated with better outcomes following allo-HSCT. The aim of this study was to investigate the clinical and prognostic significance of HGs in clinical outcomes of patients with acute lymphoblastic leukemia (ALL) who underwent allo-HSCT.

Methods: The patient cohort included 52 ALL patients, 35 males and 17 females, with a median age of 34 years (16-60 years). Disease status at the time of transplantation was complete remission 1(CR1) in 40 patients, CR2 in 8 patients and refractory disease in 4 patients. The median number of CD34+ cells transplanted was 6.5 x 10^6/kg (range 2.24 to 13.2 x 10^6/kg). Graft source was peripheral blood stem cells (PBSC), bone marrow (BM) and umbilical cord blood (UCB) in 46, 5 and 1 patients respectively. Conditioning regimens consisted of total body irradiation/cyclophosphamide (TBI/CY) for 39 patients, busulfan (BU/CY) for 4, and fludarabine-based reduced-intensity conditioning for 8 BMT recipients, respectively. The median follow-up period for survivors after allo-HSCT was 390 days (range 25-2169). HGs were studied by flow cytometry analysis of bone marrow samples on day 30 and on day 90 in 37 and 38 patients respectively, while 30 patients had available data for both timepoints.

Results: HGs were confirmed in 24/37 (=64.8%) patients on day 30 and in 19/38 (=50%) on day 90. Among the HG-positive patients, the frequency of HGs did not exceed 1%. The median time of engraftment was 13 and 14 days for the neutrophils and platelets respectively. There was no significant relationship between the day of engraftment and the frequency of HGs. Due to few BM/UCB grafts and RIC conditioning regimens, no association with HGs could be made. Other factors such as recipient age, donor age and donor type or HLA-matching were not associated with HGs. Interestingly, only 1/24 patients with positive HGs on day 30 developed acute grade III-IV graft-versus-host disease (aGVHD) (p=0.02). Moreover, there was a trend for an advantage in overall survival (OS) in patients presenting HGs on day 90 sampling. (68% versus 54% 2-year OS, p=0.067).

Conclusions: The presence of HGs in ALL patients after allo-BMT shows a favorable impact on aGVHD severity and post-transplant overall survival. Longer follow up in larger cohorts may reveal additional correlations in graft-recipient interaction.
HEM-20 (also presented as Poster Flash Presentation # PFP-24)

PTCL-NOS with leukemic presentation resembling Sézary syndrome: a case report
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Introduction: Peripheral T-cell lymphomas not otherwise specified (PTCL-NOS) are a heterogeneous group of diseases involving lymph nodes and extranodal sites deriving from the clonal expansion of mature T-lymphocytes. The cell of origin is an activated mature CD4+ lymphocyte. The phenotype is usually CD4+/CD8-. PTCL-NOS accounts for approximately 4-7% of all non Hodgkin Lymphomas and 30-70% of all mature T-cell lymphomas. Most patients present with peripheral lymph node involvement. Lymphoma cells can be detected in the peripheral blood smear, but leukemic presentation is uncommon. In most cases, the karyotype is aberrant and often characterized by complex abnormalities. Recently, recurrent chromosomal gains and losses have been documented in PTCL-NOS by comparative genomic hybridization.

Methods: We herein report a case of a 68-year-old woman with an unusual presentation of PTCL-NOS.

Results: The patient presented with a 2-month history of widespread palpable erythematous rash. She complained about pruritus, unresponsive to treatment with antihistamines and corticosteroids. The physical examination revealed supraclavicular lymphadenopathy. Her complete blood count showed a white-cell count of 30.000/μL with 95% lymphocytosis, a hemoglobin level of 14.4 gr/dL, and a platelet count of 151.000/μL. Peripheral blood smear revealed numerous atypical intermediate-large sized mature lymphoid cells with Sézary-like flower-shaped nucleus and a small number with cerebriform nucleus. Biochemical analysis indicated increased serum LDH and uric acid. Flow cytometry detected a population of mature T-cells CD3+/CD4+/CD8-/ TCRαβ+/CD7-/CD25-/CD30-/CD10-/CD56- and clonal TCR gene rearrangement with Vb2 epitope being expressed in 94% of the T cells. Clonality was also confirmed by PCR. The neoplastic lymphocytes were negative for HTLV1. Karyotype was complex: 49,X,del(X)(q24),+del(1)(p13),t(2;6)(q31;q27),+4,+21[10]/46,XX[10]. CT scans were significant for generalized lymphadenopathy and hepatosplenomegaly.

We performed lymph node, bone marrow, and skin biopsies. Lymph node histology revealed diffuse infiltrates with effacement of the normal architecture. A mixture of intermediate and large atypical lymphocyte was noted. Lymphocytes expressed CD3, CD5, CD57, MUM, and CD2(+/-), but were negative for CD4, CD8, CD7, CD56, CD30, CD25, TCL1, and bcl6. Bone marrow involvement was 50% and skin was also infiltrated.

PTCL-NOS diagnosis was established and the patient received CHOP. However, disease progression with worsening of her leukocytosis was noted after the first cycle.

Conclusions: We report a case of CD4/CD8 double negative PTCL-NOS. To our knowledge, this is the first reported case harboring t(2;6)(q31;q27) translocation and manifesting leukocytosis with characteristic morphology resembling Sézary syndrome cells.

HEM-21 (also presented as Poster Flash Presentation # PFP-34)

Factors determining whether diffuse large B cell lymphoma samples are detected by flow cytometry
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Introduction: Flow cytometry is an integral part in the workup for mature B-cell neoplasms (MBN). However numerous cases of diffuse large B-cell lymphoma (DLBCL), a common type of MBN, are not detected by flow cytometry. We investigated factors that increase the likelihood of DLBCL being missed in flow cytometry. We hypothesized that samples with fibrosis or necrosis were more likely to be not detected by flow cytometry.

Methods: We identified confirmed cases with a final diagnosis of DLBCL, for which a sample was submitted for flow cytometry in the previous 10 years. Histopathology, flow cytometry, genetic and clinical data were collected for each of these DLCBL cases.
**Results:** Initial analysis confirmed that among diagnosed MBN cases, DLBCL was the most common type to be missed by flow cytometry. Retrospective analysis identified a total of 135 cases with a confirmed diagnosis of DLBCL. In 22 (16%) of these cases, a lymphoma population was not detected by flow cytometry. A DLBCL sample was more likely to be missed by flow cytometry if microscopy revealed Epstein-Barr virus positivity or absence of MYC expression. However, the presence of fibrosis or necrosis by morphology was not significantly associated with increased likelihood of failure to detect DLBCL by flow cytometry. Samples were more likely to be missed if flow cytometry demonstrated high CD4 and CD8 T-cell percentages, which may reflect the failure of the analysis to detect abnormal B cells. Finally, a high peripheral blood platelet count was significantly associated with failure to detect DLBCL by flow cytometry.

Conclusions: The study identifies several factors that increase the likelihood of DLBCL being missed by flow cytometry. However, fibrosis or necrosis in the lymphoma tissue was not significantly associated with failure to detect DLBCL.

HEM-22 (also presented as Poster Flash Presentation # PFP-01)

**Evaluation of myelodysplastic syndromes by flow cytometry. Experience of a center**

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**Introduction:** Myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal hemopathies characterized by ineffective hematopoiesis and cytopenias in peripheral blood, with risk of progression to acute myeloblastic leukemia. It mainly affects elderly individuals, with an annual incidence of 4-5 / 100,000 inhabitants. Diagnosis is based mainly on morphological and cytogenetic studies. Flow Cytometry (FC) is a sensitive technique that identify immunophenotypic alterations in cellular populations. The objective of this work is to evaluate the contribution of FC when there is suspicion of MDS or clinical data of one or more cytopenias.

**Methods:** Bone marrow (BM) samples from patients with one or more cytopenias and/or with suspicion of MDS from the Maciel Hospital in Montevideo, from January 2015 to June 2019 were analyzed. A five-color cytometer FC500 of Beckman Coulter was used. Labeling was carried out using the lysis and washing technique. A five-tube myeloid screening panel was used (Tube 1: CD4 / CD8 / CD3 / CD45 / CD19, Tube 2: HLADR / CD11B / CD45 / CD13 / CD117, Tube 3: HLADR / CD34 / CD38 / CD45 / CD117, Tube 4: CD64 / IREM2 / CD14 / CD56 / CD45, Tube 5: CD71 / CD117 / CD19 / CD34 / CD45). The fluorescents used were FITC, PE, ECD, PECy5 and PECy7. The monocyte, myeloid, erythroid, lymphoid, and immature precursors population were evaluated. The results were compared with the morphology (myelogram or BM biopsy), which is the gold standard for the diagnosis of MDS. This work complies with the Helsinki Declaration Principles.

**Results:** 62 patients between 17 and 83 years (median 65 years) were studied. In 28 (45.2%) the morphological diagnosis was MDS and in 34 (54.8%) the diagnosis was not conclusive with MDS. Considering these two groups, the following tendencies were observed by FC in the group of MDS: 1) greater frequency of hypogranularity of the granular series, 2) greater frequency of maturational delay of the granular, monocyctic and erythroid series, 3) greater frequency of atypia (CD56 expression) in the monocyte series, 4) maturational delay together with aberrant expression in the monocyte series, 5) association of monocyctic and granulocytic maturational alteration and atypia in monocytes.

**Conclusion:** FC phenotypic alterations of the MDS studied correlated with the dysplasia found by morphology, being a useful technique that contributes to the diagnosis. The trends found should continue to be evaluated in subsequent studies.
HEM-23

Comparative study between BD OneFlow™ PCST/PCD dry tubes and liquid reagent panel for plasma cell neoplasm immunophenotyping.

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Introduction: Taking into account the increase frequency of plasma cell disorders, the BD OneFlow™ system offers a solution with BD OneFlow™ PCST (Plasma Cell Screening Tube) and PCD (Plasma Cell Disorders), facilitating the staining procedure for bone marrow (BM) samples and greater immunophenotyping standardization in order to become a useful and reliable tool for the study of diseases such as Multiple Myeloma. The objective of this work is to compare the performance of OneFlow PCST/PCD with the same panels of liquid reagents for the immunophenotyping of plasma cells disorders in BM.

Methods: 50 BM samples from patients with plasma cell neoplasms diagnosis or follow-up where stained with BD OneFlow PCST and PCD dry tubes and the same panel with liquid reagent as a comparison method. All the samples were acquired with BD FACSCanto II™ flow cytometer standardized with OneFlow setup reagents. The analysis of all data was performed on merged data files using Infinicyt software 2.0. To compare the results of both staining systems we evaluated Median Fluorescence Intensities (MFI), Standard Deviation (SD) and distribution in normal and neoplastic populations, presence of aberrant markers and clonality.

Results: For each sample the lymphoid and plasmocytic populations obtained by both staining methods where merged and compared by Automatic Population Separator (APS), showing all the results within 1 SD, except in 9 samples were pathological clonal population in low concentration (<0,01%) were detected with OneFlow PCST/PCD but not with the liquid reagent staining. The immunophenotype for these 9 samples was kappa for 6 of them and lambda for the remaining 3. For the other 41 BM, a comparable staining of kappa and lambda cytoplasmic chains was achieved, in all the cases remnant normal plasma cell presented polyclonal populations and the abnormal cells showed the same light chain clonality in the analysis. Aberrant markers in plasma cells such as CD56, CD27 and CD28 presented a homogeneous MFI pattern and APS comparison within 1SD between both methods.

Conclusions: It was demonstrated that BD OneFlow PCST and PCD dry tubes solution is equivalent to the liquid panel and presents enhanced sensitivity for abnormal populations in low concentration (<0,01%). Showing a useful, reliable and effective solution for plasma cell disorders study.

HEM-24

Fluorescent Activated Cell Sorting application for oncohematology laboratory

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Introduction: Fluorescence Activated Cell Sorting (FACS) is a current efficient method for selecting and purifying cell populations allowing the recovery of cells in low concentration and sort more than one cell population simultaneously. High purity Sorted Cells are needed to enhance molecular techniques such as Fluorescent In Situ Hybridization (FISH) or molecular biology assay. Our objective is to demonstrate the utility of cell sorting for clinical samples.

Methods: All the samples have shown low infiltration in the cell population of interest to be studied. Sample staining was performed with different panels according to the population of interest, cell separation was performed by BD FACSMelody cell sorter in purity or enrichment mode. Bone marrow (BM) from Multiple Myeloma (MM) patients (n=27; x=5.1% pathological cells infiltration), BM with 2% of lymphoblasts from B Acute Lymphoblastic Leukemia (B-ALL) and with 96.1% of eosinophilia (n = 1), peripheral blood of patients with aberrant T-population expansion (n=3), BM from B Lymphoproliferative Syndrome(BLS) (n=1), BM of patient with mastocytosis (n=1, 0.03% mast cells). For MM and B ALL samples FISH test was performed on post sort cells. TCR rearrangement by molecular biology was performed on sorted and non-sorted T cell expansion. Allele specific oligonucleotide test was performed on BLS and mastocytosis samples.
**Results:** In all the samples (n=33) a higher purity population (>85%) was obtained to be evaluated by the appropriated molecular assay. In 23 of 27 MM patients TP53 deletion and rearrangements of IGH were studied by FISH (13% and 39% positive, respectively), in 15 MM patients Del1p/Gain1q was studied by FISH (47% presented some abnormality). In B-ALL, translocation t(5;14)(q31;q32) was studied by FISH with a negative result . The study of the c-kit oncogene in mastocytosis and myd-88 in lymphoplasmacytic lymphoma showed a positive molecular biology results (mutated). All T cell expansion showed a monoclonal population in the post-sort cells, while the result was not detectable in the original sample.

**Conclusion:** Our results demonstrate the clinical utility of cell enrichment by high-throughput FACS methodology, obtaining enhanced purity and quantity of defined cell population to apply further test. Although Cell Sorting is applied mainly to research application, it has significant potential in the clinical field, increasing the sensitivity for molecular techniques that contribute to the correct diagnosis and adequate treatment for oncohematology patients.

**HEM-25**

**Immunophenotypic characteristics of mast cells in mastocytosis. Clinical cases report**
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**Introduction:** Mastocytosis is a clonal, neoplastic proliferation of mast cells that accumulate in one or more organ systems. (WHO, 2017). Immunophenotypic characteristics of clonal mast cells are heterogeneous. Aberrant expression of CD25, CD2 antigens is one of the minor diagnostic criteria for systemic mastocytosis.

**Methods:** bone marrow (BM) or biopsy material of the tumor of 3 patients with mastocytosis were analyzed by multiparameter flow cytometry (FACS Canto II, BD Bioscience).

**Results:**

**Case 1:** woman, 34 years old. The Onset of the disease with weakness, sweating, fever, skin itch, weight loss, diarrhea, bleeding from a duodenal ulcer. After 2 months skin pigmentation, hepatosplenomegaly, portal hypertension, varicose veins of the esophagus, retroperitoneal lymphadenopathy were appeared. Peripheral blood: anemia, thrombocytopenia, blast cells 5%, basophils 18%. Bone marrow: 6% of blast cells, 41% of mast cells (atypical type II), 11% of basophils. C-KIT D816V mutation was manifested via PCR. Immunophenotypic characteristic of bone marrow cells by FC: Blasts (3%): CD45low+CD34+CD117+HLA-DR+CD13+CD33+; Mast cells (11%): CD203c+CD123+CD117(bright)+CD25(bright)+CD22+CD2+CD25+CD33+ HLA-DR+CD13+CD4+CD34-CD66b-; Basophils (11%): CD203c+CD123+CD13+CD33+CD117(low)+CD25-CD11b+CD22-CD34-CD66b-.

**Diagnosis:** Systemic mastocytosis with an associated hematological neoplasm (SMAHN): mast cell leukaemia (MCL) with associated myelodysplastic syndrome with excess blasts 1 (MDS EB 1).

**Case 2:** male, 22 years old. Beginning of disease: erythematous skin rash. After 1 month: weakness, weight loss, sweating, fever, hepatosplenomegaly, foci of destruction on 6 ribs on the right and Th7-rib biopsy (restriction kappa chain?) - B cells clonality (heavy chain) - chemotherapy – progression There were signs of hypersplenism. Splenectomy performed. Bone marrow cytology: mast cells make up 14,2% (atypical type II). FC: Mast cells (0,2%): CD45+CD117+CD2-CD25-. 

**Diagnosis:** Aggressive systemic mastocytosis.


**Blood:** neutrophilia, lymphopenia. BM (aspirate and trepan biopsy): mast cells were not detected.

**FC of tonsil’s biopsy:** Mast cells (64,4%): CD45+CD117+CD13-CD2-CD25- Immunohistochemistry: Mast cells CD45+CD117+CD2-CD25-CD30+tryptase+ C-KIT mutation was negative

**Diagnosis:** Mast cell sarcoma.

**Conclusion:** FC could be helpful in the diagnostic of mastocytosis. This method allows detecting even low count of mast cells. Immunophenotypic of mast cells can have different aberrances, which could be useful for clonal mast cell detection in the dynamic study.
Comparative evaluation of mast cell detection methods in the bone marrow of patients with myelodysplastic syndromes
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Introduction: The percentage of bone marrow mast cells (MC) might be elevated in some hematologic malignancies such as myelodysplastic syndromes (MDS). Previously we have shown that a flow cytometry scoring system including the MC percentage provided high sensitivity and specificity and an excellent discriminative power between MDS and non-MDS samples. In this study one of our objectives was to explore the optimal conditions for the measurement of MCs by flow cytometry. Furthermore, we investigated the association between percentage of MCs and bone marrow fibrosis, since MC plays a key role in fibrosis of different tissues.

Methods: Seven bone marrow samples collected in K₂-EDTA and Na-heparin were labelled and analysed by flow cytometry right after aspiration and on two consecutive days. In addition, in 35 patients the MC percentage was evaluated by flow cytometry and trephine biopsy was used to detect the grade of fibrosis by silver staining and to evaluate the MC percentage by CD117 and tryptase staining.

Results: The percentage of MCs were significantly increased in samples collected in K₂-EDTA not only at day-0 but also at day-1 or day-2 (p=0.03, p=0.016, p=0.047, respectively). Delayed sample handling did not influence significantly the percentage of MCs. The intensity of CD117 expression on MCs proved to be stable regardless of the type of anticoagulant. On the basis of silver staining, the distribution of patients regarding myelofibrosis (MF) was the following: MF-0 (n=6), MF-1 (n=20), MF-2 (n=8), and MF-3 (n=1). The percentage of MC detected by flow cytometry correlated with the percentage evaluated by tryptase or CD117 immunohistochemical staining (r=0.41, p=0.016 and r=0.38, p=0.028, respectively). Significant correlation was found between the grade of fibrosis and the percentage of MCs detected by flow cytometry (r=0.48, p=0.04).

Conclusions: The most suitable sample for MC detection by flow cytometry was the bone marrow aspirate collected into K₂-EDTA. The difference of MC percentage depending on the type of anticoagulant was not caused by technical factor, as the gating marker (CD117) intensity did not differ significantly between samples collected in K₂-EDTA or Na-heparin. An increased MC percentage was detectable, which correlated with the adverse fibrotic stage of the bone marrow. This raises the possibility that MC might have a role in the development of fibrosis in some MDS cases.

Assessment of BAFF-receptors’ expression in the peripheral B cell compartment by flow cytometry: Implications for anti-BAFF/ BAFF-R immunotherapy
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Introduction: The novel of novel immunotherapies targeting the B cell Activating Factor (BAFF)/ BAFF receptors (BAFF-R) is currently assessed in clinical trials involving patients with autoimmune diseases or hematological malignancies. The BAFF-R expression patterns within the B-cell compartment may define differential susceptibility to BAFF-inhibition and thereby generate differences in treatment efficacy. The current study aimed at flow cytometric (FC) evaluation of BAFF-R expression patterns, such as CD267 (transmembrane activator and calcium-modulator ligand interactor, TACI), CD268 (BLys receptor 3), and CD269 (B cell maturation antigen, BCMA) on circulating B cell subsets in normal subjects.

Methods: The study was conducted at the Dept. of IHB, LUMC, Leiden. Peripheral blood samples from healthy donors (n=3) were processed (staining following whole blood erythrocyte bulk lysis) for immunophenotyping by FC. Pe-Cy7 conjugated CD267, CD268 and CD269 antibodies (BioLegend) were tested each in a combination of 14 color/20 markers for in-depth characterization of the peripheral B cell compartment. A LSR-Fortessa (Becton Dickinson) cytometer and the Infinicyt (Cytognos) software were used for data acquisition and data analysis, respectively. The BAFF-Rs expression patterns (staining indexes against basophils) was measured on pre-germinal (immature, total naïve, CD5+ naïve), memory (unswitched and eight switched) B subsets and plasmablasts.
Results: CD267 was weakly expressed, if any, on all B cell subsets assessed in all subjects. CD268 was highly expressed on all pre-germinial and memory B cell subsets in all donors, but weakly expressed in plasmablasts in 2 out of 3 donors. The lowest variability (CV<5%) among the donors in terms of CD268 expression was recorded in total B cells, total pre-germinial, total naïve, naïve CD5+, total memory, and five memory B cell subsets (unswitched, IgG2+, IgG4+, IgA1+, IgA2+). CD268 staining indexes in the remaining cell subtypes (immediate, memory IgG1+, IgG3+, IgD-IgM, IgH+, and plasmablasts) were highly variable among donors. CD269 was not expressed in any of the B cell subsets/ donor tested, with two exceptions: on immature B cells in one donor and on plasmablasts of another donor.

Conclusions: The susceptibility to BAFF-inhibition may correlate with BAFF-R expression patterns. Therefore, validated laboratory techniques are required for pre- and post-therapy evaluation of patient treated with anti-BAFF(R). The current study reveals that the expression of the three different evaluated BAFF-Rs within the peripheral B-cell compartment is B-cell subset specific, but the recognized BAFF-R epitopes might be donor-dependent (polymorphic). Further technical developments relies on commercially available BAFF-R antibody clones against non-polymorphic epitopes and their fluorochrome-conjugates.

HEM-28

Intra-operator and inter-operator variability in flow cytometry-derived data analysis: Implications for translation of research into clinical practice

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Introduction: A large array of flow cytometry (FC) based methods have been developed for diagnosis and immune-monitoring in various medical fields. Proper assessment and revision of intra-assay variability is one of the key requirements for their ultimate validation and transition into routine clinical practice. The aim of the current study was to evaluate intra-operator and inter-operator variability in data output by manual analysis of FC standard (FCS) files. For unacceptable deviations, sources of inaccuracy were identified.

Methods: As a model we used data analysis of FCS files generated by a complex, 14-color/21-marker combination designed to characterize the B-cell compartment in peripheral blood samples (E. Blanco et al. JACI 2019). The LSR-Fortessa X-20 cytometer (Becton Dickinson) and the Infinicyt, version 2.0.1.e.000 (Cytognos) software were used for data acquisition and data analysis, respectively. Absolute counts were calculated in parallel tubes containing major lineage markers (B/T/NK/pan-leukocyte) and Perfect Count microsphere (Cytognos). The panel choice and data analysis were based on procedures previously developed and standardized by the EuroFlow Consortium. For the inter-operator comparison, four operators manually analyzed the data (three data files containing 1x10⁷ events). Coefficients of variability (%CV) in absolute counts of fifteen different B-cell subsets were calculated. Correlations (Pearson’s coefficients, r) were established between each operator and one reference expert and between pair-analysis of thirty data files (the same file analyzed twice several weeks apart) performed by one operator (intra-operator variability).

Results: %CV and r values indicative of acceptable reproducibility were <20% and within +/-0.9, respectively. The highest variability among operators and the poorest concordance between each operator and the reference expert were consistently recorded for the smallest cell subsets (IgG4+ memory B cells: 35-50 events and immature B cells: 80-200 events). A secondary source of unacceptable variability identified was from the least resolved B cell sub-populations, due to the continuous pattern of marker expression (CD5 and IgM). The intra-operator variability assessment generated similar conclusions, with the least concordant results for the same cell sub-populations.

Conclusions: Manual analysis of FC-derived data files from complex, multi-parameter combinations remains time consuming, labor-intensive, and susceptible to subjectivity in the absence of standardized gating strategies. With proper coordination of multi-center teamwork, standardized gating strategies for manual analysis and development of reference data bases and automated gating tools are expected to improve data reproducibility, which is required for a safe translation of research into clinical practice.
HEM-29 (also presented as Poster Flash Presentation # PFP-19)

Multiple myeloma minimal residual disease detection in stem cell apheresis product
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Introduction: Multicolor flow cytometry (MFC) is well-suited to study biological samples containing plasma cells. Multiparameter cytometry with evaluation of at least 8 markers in a single tube can identify abnormal plasma cell phenotypes at MRD levels if sufficient cell numbers are evaluated. Because the aberrant phenotypes of clonal plasma cells are readily distinguishable from normal plasma cells, flow MRD in applicable in virtually every MM patient. Autologous stem cell transplantation (ASCT) is part of first line therapeutic approaches for fit patients with multiple myeloma (MM). MRD negativity after ASCT has been previously shown by a few groups to be predictive of favorable progression free survival (PFS) and overall survival (OS). Although post ASCT MRD has been well studied, MRD by MFC in stem cell apheresis product has never been described. Our objective was to investigate whether it is possible to identify abnormal plasma cells in stem cell apheresis products. This study was approved by UNICEUB ethic board and was conducted according to the declaration of Helsinki.

Methodology: Up to three hours after collection, 1mL of peripheral blood stem cell apheresis product was processed according to bulky lysis previously described protocol. Two eight colors tubes were prepared using antibodies combinations including CD19 APC-H7, CD27 PerCP-Cy5.5, CD38 PE-Cy7, CD138 APC, CD45 V500, CD56 BV421, CD81 FITC, CD117 PE, KAPPA FITC and LAMBDA PE. Samples were acquired with a FACSCANTO II (BD Bioscence) and analyzed using Infinicyt (Cytognos) software.

Results: Six samples from different patients were studied. Number of events analyzed ranged from 6822250 to 8020156 (mean 7179334). Abnormal monoclonal plasma cells were identified in two samples: in one this population corresponded to 0.003% (205 events) and in the other to 0.01% (970 events).

Conclusion: The clinical significance of contaminating MM tumor cells in apheresis product is controversial. Previous studies (López-Pérez et al, 2001 and Galimberti et al, 2003) used gene scanning PCR to detect residual disease and had different findings according to its role in PFS and OS. We demonstrated that MM MRD assessment by MFC in peripheral blood stem cell apheresis product is possible. As previous studies used different techniques to detect MRD and were conducted at a different MM treatment era, we believe the role of contaminating MM tumor cells in apheresis product should be reassessed in clinical trials.

HEM-30

Fully automated sample processing for assessment of Multiple Myeloma and Acute Myeloid Leukaemia Minimal Residual Disease
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Introduction: The last decade has seen a significant increase in the volume of testing in clinical immunophenotyping laboratories. One reason for this is due to the need for measurement of minimal/measurable residual disease (MRD) for haematological malignancies. This testing not only increases sample numbers but also requires increased events to be acquired, more complex analytical methods and increased standardisation. To accommodate the rapidly increasing workload for our 2 highest volume MRD assays, Acute Myeloid Leukaemia (AML) and Multiple Myeloma (MM) our department sought out an automated solution which could be used for high sensitivity MRD assays (0.01%) in addition to routine clinical analysis. We identified the FlowStainer™ from InstruNor as the only commercially available solution and undertook a validation process.

Methods: Samples processed for minimal residual disease were set up using our current standard method which were in parallel run on the FlowStainer™. FCS files were analysed using our standard profiles in Infinicyt™ (Cytognos, Salamanca) and results compared in terms of both percentage of disease identified for both methods as well as comparing the median fluorescence obtained for each antigen.
Results: 21 Samples were run, 13 MM and 8 AML MRD. Out of 8 AML cases 6 tested positive (range 0.1%-1.54%) and 2 negative using our current cut off value of 0.1% with an R² value of 0.98. Of critical importance all samples remained in the sample risk group using both preparation methods. All 13 MM cases tested showed residual disease (Range 0.04%-35.91%) and all results reported as positive using current processes remained positive using the FlowStainer™ automated sample processor. When results were compared a R² value of 0.99 was obtained showing excellent correlation between methods. In addition to MRD level we correlated percentages of non-malignant plasma cells and B cells to ensure no selective population loss was occurring and all populations showed correlation >0.9. In addition to confirming the analytical utility of this automated preparation system we compared the processing time required for each batch of these tests using our existing method and the FlowStainer™. The hand’s on time was reduced from 60 minutes per test to approximately 5 minutes per batch.

Conclusions: We have been able to successfully validate for routine clinical use, a fully automated sample processing methods for assessment of MRD in both MM and AML.

HEM-31

Monoclonal antibodies against intracellular CD20 epitopes may improve the identification of B cells-subsets by flow cytometry in Rituximab treated patients
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Introduction: Newly established immunotherapies targeting B-cells in patients with hematological malignancies or autoimmune diseases require systematic assessment of treatment efficacy, minimal residual disease (MRD), and cell lineage regeneration. The aim of the current study was to optimize the characterization of the B-cell compartment by flow cytometry (FC) in patients treated with Rituximab (RTX), using a CD20 monoclonal antibody specific for an intracellular epitope.

Methods: Peripheral blood samples from healthy donors (HD, n=3) and RTX-treated patients (week 28 post-therapy, n=3) were processed (staining following whole-blood erythrocyte bulk lysis) for immunophenotyping. Two different CD20 clones (L26-specific for an intracellular epitope, A488/ThermoFischer and 2H7-specific for an extracellular epitope, PE-CFS94/BDBiosciences) were tested at the cell-surface and intracellularly in combination with CD45, T cell (CD3/CD5), basophilic (FceR1) and B cell markers (CD19, IgM, CD27, CD38). The LSR-Fortessa-X-20 (BD) and the Infinicyt software, version 2.0.1.e.000 (Cytognos) were used for data acquisition and data analysis, respectively.

Results: The expression of CD20-L26 (and CD20-2H7 as a control) was measured on pre-germinal (pre-G), naïve, (un)switched (USW/SW) memory B-cells and plasma blasts and compared (intracellular versus membrane) as staining indexes (SI) against basophils. CD20-2H7 expression was significantly reduced in all B cell subsets in RTX-versus HD-related samples (up to 7 fold-decrease in CD20-SI, with the greatest reduction on SW B-cells, from 40.5 to 5.7). CD20-L26-SI were significantly reduced in all B cell subsets in RTX-versus HD-related samples (up to 2.73 fold decrease, with the greatest decrease on SW B-cells, from 114.06 to 60.62). However, in comparison to the 2H7-clone approach the intracellular staining with L26 showed an important (yet not complete) restoration of the CD20 detection, with the lowest RTX versus HD fold-decrease (1.43) recorded in pre-G B-cells, from 76.2 to 53.4.

Conclusions: Expression of CD20 on all B cells is masked by RTX treatment. As a consequence, the post-therapy evaluation of those patients is problematic, since the level of CD20 expression (low/medium/high) together with its expression pattern (homogeneous/heterogeneous) is used for further dissection of the B cell compartment (when B cell lineage regeneration is assessed) or for detection of atypical B cells in hematological malignancies (MRD). Therefore the current study proposes the use of antibodies against intracellular CD20 epitopes to achieve better detection of clinically relevant B-cell subsets in samples from RTX-treated patients. Further technical improvements for targeting intracellular CD20 epitopes remain constrained by the limited choices of commercially available antibodies and conjugates.
HEM-32 (also presented as Poster Flash Presentation # PFP-05)

Immunophenotyping features in specific genetic subgroups with FLT3-ITD and NPM1 mutations in acute myeloid leukemias
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Introduction: In acute myeloid leukemia (AML) is recommended that all patients be tested for the presence of FLT3-internal tandem duplication (FLT3-ITD) mutations. and allelic ratio (AR) (FLT3-ITD/FLT3-WT) be determined in order to establish the genetic risk category. Previously reports shown that FLT3-ITD mutations induce a specific phenotype in leukemic blasts which is characterized by high levels of CD33 & CD123, and that CD33 & CD123 levels are dependent of FLT3-ITD AR levels.

Methods: Thirty-six FLT3-ITD AML cases without APL were included in the current analyses, of which 18 (50%) were positive for NPM1 mutations. Control group - 84 FLT3-ITD negative AML cases. Gating strategy was based on selecting leukemic blasts from the CD45/SSC gate, verified by CD117, CD34 and HLA-DR backgating. The antigen panel tested was: CD4, CD7, CD9, CD13, CD14, CD33, CD34, CD56, CD64, CD71, CD117, and CD123. Quantitative antigen expression was determined as the ratio between the geometric mean fluorescence intensities (MFIs) of leukemic blasts which were normalized to the MFIs of negative lymphocyte populations for the respective markers. FLT3-ITD DNA and RNA analyses were performed, under the same conditions, by capillary electrophoresis.

Results: Of the 36 FLT3-ITD cases, 26 patients had good quality RNA samples. Compared with the control group, FLT3-ITD cohort presented significantly higher CD7, CD33 and CD123 levels (Mann-Whitney U Test). Impact of FLT3-ITD levels on antigen expression was assessed by separation of patients into 2 groups according to: (1) AR <0.5 (15 patients) & AR ≥0.5 (21 patients), according to current AML guidelines; (2) RR < 0.8 (11 patients) & RR ≥0.8 (15 patients); (3) FLT3-ITD mRNA abundance RR/AR= 1 (5 patients) & RR/AR > 1 (20 patients), 1 excluded patient presented RR/AR ratio <1. There were no statistical differences between CD7, CD33 and CD123 levels for all sets of 2 groups (Mann-Whitney U Test). The correlation between CD7, CD33, CD123 MFI ratio values and the 3 FLT3-ITD quantitative parameter values was also tested, without significant correlations (Pearson’s correlation).

Conclusion: Presence of FLT3-ITD mutations induces a specific antigen profile in AML blasts, however the profile is essentially unchanged among the FLT3-ITD cohort regardless of the FLT3-ITD expression level. Our data does not correlate with previous reports in which both CD33 & CD123 expression were influenced by the FLT3-ITD AR. Possible reasons for these results could be due to differences in the statistical tests used, the different AR cut-off, or it could represent differences between patient cohorts.

HEM-33

Deletion of 17p in CLL is associated with overexpression of galectin-3
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Introduction: Galectins belong to the family of galactose-binding proteins known to play an important role in the processes of cell proliferation, differentiation, migration and neoplastic progression. In this study, we analyzed the expression of galectin-3 in chronic lymphocytic leukemia (CLL).

Methods: The expression of galectin-3 was analyzed by means of multiparametric flow cytometry in normal and pathological B-cells from peripheral blood and bone marrow samples of 67 patients with CLL. Cyrogenetic analysis was performed using interphase in situ hybridization technique.

Results: Pathological B-cells expressed significantly higher levels of cytoplasmic Gal-3 than normal B-cells. The highest levels of cytoplasmic galectin-3 were observed in patients with deletion of 17p, the prognostically poorest subgroup of CLL patients.
Conclusions: Our results demonstrate an association between 17p deletion and overexpression of galectin-3 and indicate a possible role of galectin-3 in CLL pathophysiology and its potential value as a prognostic marker and therapeutic target.

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HEM-34

Evaluation of sample quality as a preanalytical error in flow cytometry analysis

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Introduction: The intensity of chemotherapy and further therapeutic decisions in childhood acute lymphoblastic leukemia (ALL) depend on several prognostic factors, one of which is assessing minimal residual disease in the bone marrow (BM) on Day 15 of the treatment. Treatment response is regularly checked by examining peripheral blood (PB), BM and cerebrospinal fluid (CSF) samples at certain time points. Sample quality is crucial for the correct risk assessment.

Methods: We retrospectively analyzed flow cytometry results of BM (104 Day 15 BM samples, 90 Day 33 samples) and 26 CSF samples from children with ALL in different cohorts focusing on PB contamination and viable cell ratio among nucleated cells. PB contamination of a BM sample was defined as samples containing <2% erythroid precursors. CSF samples containing <30% of viable cells were considered inadequate for analysis and PB contamination was concluded if the CSF sample contained > 100 red blood cells / microliter (RBC/µL), based on data found in literature. We also compared viable cell percentages in 22 native and 29 stabilized CSF samples.

Results: Due to PB contamination 12.5% of Day 15 and 14% of Day 33 BM samples were inadequate for flow cytometry risk stratification. 45% of the CSF samples from children with ALL were PB-contaminated, malignant cells were found in 54% of them overall. 42% of the samples had <30% viable cell count, 4 of these having identifiable malignant cell population despite the low viable cell percentage. Significantly fewer CSF samples had to be considered inadequate for analysis in the subgroup of stabilized samples compared to native samples (Fisher's exact test, p=0.05).

Discussion: Poor sample quality can hamper risk stratification and further therapeutic decision in childhood ALL. For stabilizing CSF samples, fixatives should be used. Despite low viable cell count malignant cell populations may still be identified in a CSF sample.

HEM-35 (also presented as Poster Flash Presentation # PFP-08)

Candidate White Cell Counting (WCC) Reference Method by Flow Cytometry

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Introduction: The current reference method for WCC as stipulated by the International Council for Standardization in Haematology (ICSH) is a single channel semi-automated electronic counter using the aperture-impedance principle. However, analytical errors are inherent, including inaccurate displacement sample volume, recirculation of cells inside orifice tube, and coincidence events. Our aim is to derive a flow cytometric assay that is more accurate, precise and robust.

Methods: A cohort of ‘normal’ peripheral blood (PB) samples were analysed on EDTA whole blood collected <24 hours. Method 1 was based on the ICSH Reference Platelet method, 5uL of CD41 FITC, 5uL of CD61 FITC, 2uL of CD45 V500 were added to 5uL of PB and 100uL of PBS. Sample was incubated in the dark at room temperature (RT) for 15 minutes. A 1:2000 dilution was made and acquisition using fluorescence thresholding off FITC and V500. WCC was calculated by dividing the haematology analyser platelet count by ‘R’ (platelet events/white cell events).

Method 2 was based on the CD34 ISHAGE strategy, 2uL of CD45 V500 were added to 100uL of PB and incubated in the dark at RT for 15 minutes. Addition of 2mL NH4Cl followed by incubation in the dark at RT for 10 minutes. 100uL of FlowCount (Beckman Coulter) beads were added before acquisition on the cytometer. Similar methodologies were employed for Perfect-Count microspheres (Cytognos) and TruCount (Becton Dickinson) beads.
**Results:** Precision was determined in triplicate with a low (0.8), normal (5.1) and high (250) PB. Respectively, the CV were 1.7, 2.8 and 0.7 for method one, and 1.2, 1.1, 2.1 for method two. Accuracy was determined by using Cell-DYN CD29-Plus Control (Streck). Results were within the manufacturer’s acceptance limits for low (3.0±0.4), normal (7.0±0.8) and high (15.9±3.0). Linearity was achieved by serially diluting a high WCC to 133, 66, 16, 4, 1, 0.25.

Correlation of 10 patients between the Haematology Analyser (CellDyn Sapphire) WCC with method 1 yielded an $R^2$ value of 0.98, and an $R^2$ of 0.99 for method 2.

**Conclusions:** Performance specifications of the linearity and precision were excellent. Accuracy were within acceptance limits of the manufacturer’s reference ranges. Correlation was excellent between all three WCC techniques. Further patient accrual is ongoing and will be performed to determine the optimal method. Whilst a long term goal will be to utilize one of these methods in a multicenter study.

**HEM-36**

**Biphenotypic leukemic B-cell chronic lymphoproliferative disorders: report from a single Hellenic tertiary hospital**

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**Introduction:** Initial flow cytometric (FC) investigation of suspected leukemic B-cell chronic lymphoproliferative disorders (B CLPDs) commonly relies on assessment of immunoglobulin light chain expression by CD19+ cells. Extensive phenotypic evaluation is performed in cases with restricted light chain expression, leading to the detection of a single clone in the majority of cases. The aim of this study is to record the incidence and characteristics of B CLPD cases with 2 immunophenotypically divergent populations.

**Methods:** During a 5 year period, initial FC immunophenotyping of PB was performed during diagnostic evaluation of adult patients with a 5 color protocol of a CD23/CD5/CD45/CD20/CD19 combination on the grounds of the increased incidence of chronic lymphocytic leukemia (CLL) phenotype among leukemic B CLPDs. A more comprehensive phenotypic analysis was subsequently performed in cases with clinical and laboratory suspicion of a B CLPD.

**Results:** Approximately 3% (12 cases) of newly diagnosed B CLPDs had 2 phenotypically distinct populations. In 5/12 cases, the 2 B cell subsets displayed a different light chain restriction, usually associated with an abnormal CD5/CD20 expression, and were initially suspected by B lymphocytosis. 9/12 and 8/12 patients exhibited populations with dissimilar expression of CD20 and CD5, respectively. Additional phenotypic differences were recorded in subsequent analysis in 7/12 cases. According to the comprehensive immunophenotyping, 5/12, 4/12 and 3/12 of the patients displayed populations with a CLL, a CLL and a non-CLL phenotype, respectively.

**Conclusions:** In line with previous reports, our results indicate that biphenotypic leukemic B CLPDs are uncommon. Single light chain expression should not be the unique criterion in initial evaluation of patients with suspected B CLPD; evaluation of other phenotypic markers, especially CD20 and CD5, seems to be important. Studies integrating immunophenotypic with molecular and clinical findings could clarify the clinical significance of such cases.

**HEM-37**

**Using endothelial colony-forming cells as a non-invasive tool for assessing the anti-angiogenic and anti-proliferative effect on RNA Helicase inhibition**

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**Introduction:** Endothelial colony-forming cells (ECFCs) are a population of endothelial progenitor cells endowed with endothelial phenotype, clonal proliferative potential, and vasculogenic capacity in vitro and in vivo. They are increasingly used as a non-invasive strategy to study the endothelial compartment and cancer vasculogenesis.
We previously demonstrated that ECFCs isolated from the blood of patients with Kaposi’s sarcoma (KS), a lymphoangio proliferative tumor associated with infection by human herpesvirus-8 (HHV8), are HHV8-infected, show higher IL-6 production and higher proliferative and vasculogenic potential than control ECFCs, suggesting that ECFCs may be putative precursors of spindle cells, which are the typical KS tumor cells. As such, ECFCs obtained from KS patients may also represent a valuable tool for screening drug activity for KS treatment. Dead-Box ATPase dependent RNA helicase 3 (DDX3) is a multifunctional protein involved in all aspects of RNA metabolism, and a new class of antiviral and antitumoral compounds targeting DDX3 is under development. In this study, we aimed to assess the activity of DDX3 inhibition on ECFC proliferation and vasculogenic activity that would amplify the in vivo antitumoral effect and give new options for the treatment of KS.

Methods: ECFCs isolated from the peripheral blood of 5 cKS patients and 5 healthy donors were treated with a DDX3 inhibitor developed by First Health Pharmaceuticals for 24, 48 and 72 hours. At the end of the incubation periods, ECFCs were analyzed for the following parameters: cell viability, assessed by MTT assay; cell apoptosis, assessed by Annexin-V staining; cell proliferation, assessed by crystal violet assay; vasculogenic activity, assessed by the Matrigel assay.

Results: ECFCs obtained by healthy donors were sensitive to DDX3 inhibition as shown by significant dose-dependent increase of cell apoptosis, reduction of cell viability, proliferation and vasculogenic activity in presence of the DDX3 inhibitor. ECFCs obtained from cKS patients as well had sensitivity to the anti-proliferative action of the compound.

Conclusions: The DDX3 inhibitor was efficient in reducing in vitro the cell viability, proliferation and vasculogenic activity of ECFCs. The results of this study confirm the antitumoral activity of the compound observed in other models, providing new evidence of a potential effect against neo-angiogenesis in solid tumors as well as against KS, paving the way to further investigations and developments.

HEM-38

Curcumin reverses multidrug resistance in Chronic Lymphocytic Leukemia cell models in combination with antileukemic drugs

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Introduction: Multidrug resistance is one of the major problems in cancer management. The overexpression of ABC transporters decreases intracellular drug concentration and contributes to drug resistance. Hence, reversal of multidrug resistance may be a promising strategy to improve treatment outcomes. The aim of this study was to investigate the inhibitory effect of different drugs, used for managing Chronic Lymphocytic Leukemia ( CLL), on the ABCB1 transporter, and how the natural product curcumin can potentiate this effect.

Methods: I83 and Mec1 CLL cell lines were plated in RPMI at a concentration of 1x10^5 cells/mL. Mitoxantrone (MTX), a fluorescent drug which is a known substrate of ABCB1, was used to measure drug efflux. Efflux assays were performed with MTX alone or in combination with fludarabine (10µM), cytarabine (0.01µM), ibrutinib (10µM) and venetoclax (10nM), and with or without curcumin (5µM). Cells were incubated for 1 hour at 37°C and 5% CO2, and for one more hour after removing MTX. Cells were stained with DAPI to exclude dead cells and acquired on the Attune™ NxT Flow Cytometer (Thermo Fisher). MTX was excited at 638nm and its emission was collected using a 670/14 band pass filter to obtain Mean Fluorescence Intensity ( MFI ).

Results: MTX Relative Fluorescence Intensity (RFI) index was calculated accordingly to the following formula: [(MFI (Tested compound) – MFI (Negative control)) / MFI (Negative control)] x 100. Drugs tested differentially increased MTX MFI. Ibrutinib induced the highest effect, followed by curcumin (RFI index = 262% and 100% in I83; and 130% and 79% in Mec1, respectively). Fludarabine, cytarabine and venetoclax RFI index was lower (about 20% in I83 and 35% in Mec1, for all drugs). MTX MFI of each drug combined with Curcumin was compared with the drug alone. Curcumin increased MTX MFI in combination with fludarabine, cytarabine, ibrutinib and venetoclax (RFI = 62%, 70%, 12% and 60% in I83; and 21%, 27%, 7% and 16% in Mec1, respectively).

Conclusions: Ibrutinib and curcumin strongly reduced MTX efflux in both cell models. When combined with curcumin, ibrutinib had low effect, suggesting that its induced inhibition was close to the maximum. In contrast, fludarabine, cytarabine and venetoclax alone showed a low inhibitory effect that was strongly potentiated by curcumin. Since curcumin is a vegetal compound with low demonstrated toxicity, these results may indicate that it could be used to inhibit ABC transporters in combination with different antileukemic drugs, whilst decreasing drug dosage.
IMM-01

Altered immunophenotype of peripheral blood dendritic cells in patients with cardiomyopathy
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Introduction: Heart failure is one of the most common causes of death. The damage factor in heart failure may be cardiomyopathy and one of the mechanisms is a malfunction of the immune system. Abnormal levels of cytokines which induce more pro-inflammatory profile are observed in the patient with cardiomyopathy. The type of immune response is to a large extent determined by the activity of antigen-presenting cells (APCs), especially dendritic cells (DCs) which constitute a “link” between the nonspecific and specific responses. Here we wanted to find out if the phenotype of DCs is changed between dilated cardiomyopathy (DCM) patients and ischemic cardiomyopathy (ICM) patients and control group.

Methods: The patients with cardiomyopathy were divided into DCM (n = 11) and ICM (n = 15) patients. The control group consisted of healthy volunteers (n = 10). The DCs population was assessed by flow cytometry for the expression of HLA-DR and the lack of lineage mixture (Lin) expression. The DCs were evaluated for the expression of subpopulation markers CD11c (marker of mDCs), CD123 (marker of pDCs) and maturation markers such as CD83, CD86.

Results: Our results indicated a significantly higher frequency of DCs with a mature phenotype in both subpopulations (mDCs, pDCs) in patients compared with control group. There were no significant differences between DCM and ICM patients.

Conclusions: Present results may suggest that there is a connection between cardiomyopathy and the phenotype of DCs, but the essence of it is not clear. Predominance of mature DCs may indicate autoimmune origins of this disorders. Further tests are required.

IMM-02

Flow cytometry role in diagnostics of inherited platelet function disorders - Wiskott-Aldrich syndrome case report
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Introduction: Inherited platelet function disorders (IPFD) constitute a large and heterogeneous group of hereditary and/or congenital rare disorders of variable severity caused by numerous genetic defects. IPFD diagnostics is set as guidance from The Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH).

Methods: The platelet functional assays are graded into first step laboratory tests (blood smear, light transmission aggregometry (LTA), flow cytometry (screening), granules’ release assays) and second step laboratory tests (LTA(extension), flow cytometry (extension), granules’ content, serum TxB2, transmission electron microscopy, clot retraction, mixed tests (LTA/FC)) and third step laboratory tests (molecular genetics diagnosis, biochemical assays).

In our Flow Cytometry laboratory in Vilnius University Hospital Santariskiu Clinics Laboratory Medicine Center we evaluate the expression of platelet markers participating in Platelet adhesion (CD42a (GPIX), CD42b (GPlb), CD31 (GPia), CD36 (GP IV/IIib), GPVI and CD49b), Platelet aggregation markers (CD41a (GP Illb) and CD61 (GP IIIa)), Platelet activation (PAC-1), Platelet degranulation (CD63 and CD62P) and Platelet coagulant activity including stimulation with agonists (TRAP and ADP).

Results: Clinical history and physical examination: 2 years old boy was on follow-up from 3 months years old by hematologists for chronic immune thrombocytopenia. At 3 months he was diagnosed with atopic dermatitis. Boy grows and develops corresponding to his age. Current complains: fast-growing hematoma throughout the body. Internal organs show no pathology. CBC PLT:7,4 x10^9/l (thrombocytopenia), MPV 12,46 fl (borderline high), PCT 0,0092%, PDW 14,90; Platelet morphology not evaluated due to marked thrombocytopenia; APTT 25, 50 s (slightly reduced), PT/INR, fibrinogen – normal; Flow cytometry: decreased CD3+CD8+(502/mm3).
Comparing with healthy donor control significantly decreased expression of: CD42a (GPIX), CD42b (GPIb), CD31 (GPIa), CD41a (GP IIb) and CD61 (GP IIIa); decreased CD63 and CD62P expression after stimulation with TRAP. Other marker (CD36 (GP IV/IIIb), GPVI, CD49b, PAC-1 after stimulation with ADP, annexin V ) expression was normal. FC results have triggered next-generation sequencing (NGS) testing for Wiskott-Aldrich syndrome (WAS) gene. NGS: The pathogenic variant of the WAS gene (NM_000377.2) was determined in the hemi-zygotic state of c.134C> T (P. (Thr45Met), rs132630273).

Conclusions: Diagnosis - Wiskott–Aldrich syndrome (WAS). It is an X-linked inherited disorder characterized by the classic triad of: severe immunodeficiency, microthrombocytopenia, and eczema. All laboratory tests may be helpful to confirm or deny IPFD diagnosis, flow cytometry being one of the screening tools to trigger molecular tests such as NGS, which are essential to confirm the accurate diagnosis.

IMM-03

Freezing Blood Samples for Deferred Immunophenotyping
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Introduction: Previous work presented by the Canadian Clinical Research Organisation (CRO), Caprion, found that whole blood samples stored in Cytomark’s TransFix/EDTA Vacuum Blood Collection tubes (TVT) are suitable for immunophenotyping after being frozen at -80°C. Caprion also observed that a competing product, Streck’s Cyto-Chex Blood Collection Tubes (BCT), were not useable after storage at -80°C.

Methods: A preliminary feasibility study was performed to determine whether Caprion’s findings could be replicated. This study used the TVTs and BCTs to compare the immunophenotype of fixed peripheral whole blood samples at time zero, and after freezing at -80°C for 7 days. The structural integrity of the frozen TVTs and BCTs were also examined. Optimisation of storage conditions was further investigated to improve immunophenotyping after freezing.

Results: TVTs showed the best percentage differences in cell counts from the control with an average lymphocyte cell recovery of 77% compared to 26% for the BCTs. All three BCTs cracked during the freezing process while all of the TVTs remained intact. Storage optimisation experiments showed tubes frozen at an angle and thawed at 37°C performed best overall.

Conclusions: These preliminary results raise the possibility that whole blood samples for immunophenotyping may be stored at -80°C for extended periods beyond 7 days. Further studies will aim to demonstrate the upper limit of time the sample will remain stable and suitable for immunophenotyping. Experiments will also be conducted to examine the suitability for use in phosphoflow assays.

IMM-04

Validating the use of TransFix to stabilise CSF for flow cytometry immunophenotyping of haematological malignancies
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Introduction: Cells within cerebrospinal fluid (CSF) samples are typically low in number and degrade quickly, prompting the necessity for urgent analysis; which may not always be possible. Here, we set out to validate TransFix® as a means of stabilising CSF and to validate antibody panels for TransFix-stabilised samples.

Methods: Fresh CSF samples were split into two aliquots and analysed fresh (within 30 minutes) or placed in TransFix®/EDTA. Stabilised aliquots were stored at 4 - 8°C for 72 hours prior to analysis. Cells were concentrated by centrifugation, labelled, washed, and acquired within 30 min on a FACS Canto II. Cells in fresh and stabilised samples were compared in terms of forward and side scatter (FSC, SSC), for median fluorescence intensity (MFI), and coefficient of variation for all antibodies used. Twenty-three antibodies were tested, each on at least three separate CSF samples.
Results: All Cells in TransFix-stabilised CSF samples showed reduced FSC. Mono-myeloid cells also had a greater FSC spread, and B-ALL cells showed increased SSC MFI. However, none of the changes seen in stabilised samples posed difficulties when gating populations. Similarly, we found that for many of the antibodies tested, gates required adjusting because the signal from stabilised cells was weaker. This did not affect relative population sizes or qualitative description of abnormal populations. Cell yields were found to be higher in TransFix-stabilised samples after 72 hours than in fresh CSF. Critically, the clinical interpretation of data from fresh and TransFix-stabilised samples stored for 72 hours was the same.

Conclusions: Flow cytometry assay validation criteria are difficult to meet for rare and pauci-cellular samples. We deem the data collated in this study sufficient to accept the tested assays fit for purpose on both fresh CSF and TransFix-stabilised CSF samples stored for 72 hours.

IMM-05

Infliximab regulates circulating regulatory B cells and dendritic cells expressing CD200 in children with Crohn’s disease
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Introduction: Tumor necrosis factor α (TNF-α) is targeted by inhibitor such as infliximab which neutralizes this cytokine. Although there are a few studies showing induction of regulatory T (Treg) cells by infliximab, the effect of infliximab on regulatory B (Breg) cells and CD200R1/CD200 axis in children with Crohn’s disease (CD) remains poorly understood. The aim of study was to investigate the effect of infliximab treatment on expression of the inhibitory molecules CD200R1, their ligand CD200 on dendritic cells (DCs), and proportions of circulating Breg and Treg cells.

Methods: Pediatric patients with CD before and after infliximab treatment (after 3 doses of infliximab and after one year of infliximab treatment) were included. Aged-matched healthy children served as a control group. The percentage of CD24^{high}CD38^{high} and CD24^{high}CD27^{+} Breg cells, naive memory, and activated Treg cells, CD11c^{+} myeloid DCs (mDCs) and CD123^{+} plasmacytoid DCs (pDCs) expressing CD200R1, CD200, CD80 and CD86 were evaluated in the peripheral blood by eight colours flow cytometry.

Results: The proportions of Breg cell subsets (particularly the CD24^{high}CD38^{high}), mDCs and pDCs expressing CD200 were lower while Treg cells were higher in CD patients than control group. The treatment with infliximab (after 3 doses of infliximab) resulted in marked increase in the proportions of Breg cells and mDCs expressing CD200 along with reduction of Treg cells and CD86 expression on DCs. After one year of infliximab treatment the percentages of these cells were similar in CD patients and control group.

Conclusions: Our results demonstrated that CD in children is associated with reduction of circulating Breg cells pool and dysregulation in CD200R1/CD200 axis. Thus, the percentage of Breg cells along with the expression of CD200 on DCs could be markers of the response to treatment with infliximab in children with CD.

IMM-06

Suppressed immune profile in children with type 1 diabetes in combination with celiac disease
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Introduction: Cytokines, chemokines, acute phase proteins (APP), adipocytokines and matrix metalloproteinases (MMP) are involved in different pathophysiological processes of inflammatory character. The role of the different immune markers and the peripheral immunoregulatory milieu in children diagnosed with type 1 diabetes (T1D) in combination with celiac disease (CD) is not fully understood and is not well studied. The purpose of the present study was therefore to acquire more knowledge and to gain deeper understanding on peripheral immunoregulatory milieu in children with T1D and/or CD.
Methods: The study included children diagnosed with T1D in combination with CD (n=18), children with T1D (n=27) or CD (n=16), and reference children (n=42). Blood samples were collected, and serum stored in -80°C until analysis, avoiding multiple freeze-thaw cycles. The inflammatory cyto/chemokines (IL-1β, -5, -6, -8, -9, -10, -13, -15, -17A, -22, -25, -33, IFN-γ, TNF-α, G-CSF, MCP-1, MIP-1α, MIP-1β), diabetes related immune markers (visfatin, resistin), APP (procalscitonin (PTC), ferritin, tissue protein activator, fibrinogen, serum amyloid A) and matrix metalloproteinases (MMP-1, -2, -3) were analyzed with Luminex technique using BioPlex assays. Hierarchical cluster analysis was used to identify similarities/differences in immune profiles between children with double diagnosis and children with single diagnosis and reference children. Mann-Whitney U test was used for comparison of the different diagnosis groups within the clusters and whole cohort, respectively.

Results: The largest cluster included 75% of the participants and the diagnose distribution in the cluster were very similar to the distribution in the whole study cohort. The remaining 25% were divided in two smaller clusters representing 15.5% and 6.5% respectively. The major finding of this study showed that children with double diagnosis had (1) lower serum levels of IL-22, MCP-1, PCT, visfatin and MMP-1 compared to children with T1D; and (2) lower serum levels of the APC associated chemokine MIP-1α compared to reference children, observed in the main cluster. Most of these observations were also seen in the whole cohort.

Conclusion: Our observations indicate decreased serum levels of IL-22, MIP-1α, MCP-1, PCT, visfatin and MMP-1 in children diagnosed with T1D in combination with CD. These results indicate a suppressed immune profile including Th17 cytokines, chemokines, acute phase proteins, diabetes-related and matrix metalloproteinase immune markers. Functional studies of the involved immune cells (CD4+ Treg, CD8+ Treg, NK-cells and dendritic cells) could contribute to elucidate the heterogeneous immunological processes in children with more than one autoimmune disease.

IMM-07 (also presented as Poster Flash Presentation # PFP-28)

Detection of antigen-specific CD4+ T cells by flow cytometry using a whole blood assay.
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Introduction: Quantifying levels of antigen-specific CD4+ T cells can be useful in the diagnostic work up of patients with suspected immunodeficiency. Methods for detection of microbial exposure to T cells are available but are laborious and time-consuming. The so-called ‘OX40’ assay, a flow cytometry method based on detection of CD25 and OX40 (CD134) surface membrane expression of activated CD4+ T cells, is a rapid and robust whole blood technique that is concordant when compared with more traditional methods of antigen-specific T cell identification.

Methods: We collected heparinized blood from healthy donors and used the ‘OX40’ assay to detect antigen-specific T cell responses to Cytomegalovirus, Varicella Zoster virus, Herpes Simplex virus, Candida albicans, tetanus toxoid, and diphtheria toxoid. Whole blood of the healthy controls were stimulated with antigen preparations of the above mentioned microorganisms and proteins. After stimulation during 44-48 hr cells were harvested, stained and acquired by flow cytometry.

Results: From a total of 20-30 healthy controls the T cell antigen-specificity to a range of recall antigens and commonly encountered pathogens were determined. Reference ranges were calculated as the 5th and 95th percentiles. The healthy control ranges for specific antigens were used to test the clinical utility in a number of patients with a diagnosed immunodeficiency.

Conclusions: The present study shows some clinical validation and establishment of healthy reference ranges for the detection of antigen-specific CD4+ T cell responses to recall antigens and a number of common encountered viruses. The assay is simple and easy to standardize in comparison with alternative assays for detection and monitoring of antigen-specific T cells.
**IMM-08** (also presented as Poster Flash Presentation # PFP-31)

**Myeloid-derived suppressor cells and cancer: identification and quantification in peripheral blood samples**

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**Introduction:** Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells that have potent immunosuppressive activity, being one of the multiple parameters analyzed in patient's Immunogram, a complex tool useful in predicting the response to immunotherapy. There are three different subsets of MDSC: polymorphonuclear MDSC (PMN-MDSC), which are phenotypically similar to neutrophils, monocytic MDSC (M-MDSC), phenotypically similar to monocytes, and, finally, immature or early-stage MDSC (e-MDSC), a small population that consists in a mix of MDSC with a more immature phenotype. In cancer patients (CP), comparatively to healthy individuals, MDSC are present in higher levels in circulation, which contributes for tumor progression. The aim of this work was to identify and quantify the MDSC present in cancer patients, by flow cytometry (FC), and also to study the mechanisms responsible for their immunosuppressive activity.

**Methods:** We used whole blood (WB) from 31 patients with solid or hematological neoplasias, while 11 samples from healthy donors (HD) were used as control. For FC analysis, samples were stained with the following antibodies: CD15, CD45, Lineage, CD11b, CD14.

**Results:** The amount of PMN-MDSC and M-MDSC was obtained in relation to neutrophils and monocytes, respectively, and as described in the literature PMN-MDSC were CD11b⁺ CD14⁺ CD15⁻ CD33⁻ LOX⁻¹ and M-MDSC were CD11b⁺ CD14⁺ CD15⁻ CD33⁻ LOX⁻¹⁻²⁻. In comparison with the HD group, in the CP group were obtained percentages of PMN-MDSC 12 times higher (CP: 0,695%; HD: 0,054%) and percentages of M-MDSC 2 times higher (CP: 1,540%; HD: 0,909%). Within the total of MDSC, it was obtained a higher percentage of PMN-MDSC in the CP group, while a higher percentage of M-MDSC was obtained in the HD group.

**Conclusions:** The results show that MDSC are indeed increased in cancer patients. However, the identification and quantification of these cells was difficult given the lack of specific markers. For the study of the immunosuppressive activity of these cells, it is ongoing the sorting of the PMN-MDSC and M-MDSC for evaluation of the expression, by qRT-PCR, of some enzymes overexpressed on these cells, such as arginase 1 (ARG-1), indolamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS). Also, the loss of CD3 zeta chain on T lymphocytes is being assessed.

**IMM-09** (also presented as Poster Flash Presentation # PFP-27)

**Increased CD27+ Memory B-Cells in Poor Responder Rheumatoid Arthritis Patients Treated with Rituximab**

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**Background:** Rituximab (RTX) is being increasingly used in the treatment of several autoimmune diseases, including Rheumatoid Arthritis (RA). RTX induces a deep depletion of all peripheral B cell subsets (memory and naïve B-cells). During the B cell repopulation phase, occurring approximately after 3 months of RTX administration, B-precursors and naïve cells reappear. Several studies have shown that relapsing RA patients are characterized by a relative expansion of memory B cells during the B cell repopulation phase. The aim of this study was to quantify the memory B cell compartment in AR patients with different disease activity scores, evaluated by DAS28, during RTX treatment.

**Methods:** 27 AR patients taking RTX cycles were studied. Nine showed high-to-moderate activity risk (median DAS28: 4.8) and 18 subjects showed low activity risk or were in remission at the end of treatment (median DAS28: 2.69). After 3 months from the last RTX infusion, B-cell subsets (precursors, naïve, memory B cells and plasma cells) were quantified in peripheral blood by flow cytometry, using 8 markers (CD3, CD4, CD8, CD19, CD20, CD27, CD38 and CD45). B naïve cells were identified as CD19+ CD20+ CD27+; B memory cells as CD19+ CD20+ CD27+, plasmacells as CD19+ CD38++ CD27++ CD20- and B precursors as CD19+ CD38++ CD20- CD27+, respectively.
One million total cell events were the target collection to ensure high-resolution analysis and an adequate B cell subsetting. Percent and absolute values were calculated for each subset, and 10 healthy subjects were included as normal control group (NC).

**Results:** The virtual absence of B cells was defined as <0.1 B cells/µL. In the responder group, 3/18 cases showed absolute B cell levels <0.1 cell/µL vs only in 1/8 of the non responder group. Median B cells/µL were higher in responders (69 vs 7.5), whereas Memory B cells% were significantly higher in non responders (46.5 vs 8, p<0.05) and similar to the NC. No differences were noted for percent and absolute naïve B cells, memory B cells/µL, CD38+ memory B cells% and plasmacells between the two groups.

**Conclusion:** We used a sensitive and easily applicable flow cytometric 8-color panel for an accurate and standardized identification and enumeration of peripheral blood B cell subsets to monitor AR patients under Rituximab treatment. As reported also by other studies, higher levels of memory B cells were found in non responder AR patients treated by RTX, approaching those of healthy individuals.

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**IMM-10**

**Degranulation of NK cells as an additional laboratory assay in the diagnostics of patients with mutations in PSTPIP1 gene**

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**Introduction:** The degranulation assay of NK cells is a known and reliable method for the diagnostics of primary immunodeficiencies (PID) especially familiar hemophagocytic lymphohistiocytosis and others where abnormality in degranulation is caused by impaired reorganization of F-actin and defect of lytic granules polarization. These include Wiskott–Aldrich syndrome (WAS), DOCK8 deficiency, WIP deficiency, etc. Among others WAS protein is known to interact with proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1) - cytoskeletal adaptor that is implicated in autoinflammatory diseases: PAPA syndrome (pyogenic sterile arthritis, pyoderma gangrenosum, acne) or in the more severe form - PAMI (PSTPIP1-associated myeloid-related-proteinaemia inflammatory syndrome), frequently accompanied by severe cytopenia with or without pyogenic features. There is no understanding of the mechanism underlying phenotypic differences of PSTPIP1 defects.

**Methods:** We studied 6 patients (age 8-41 years) with identical p.Glu250Lys PSTPIP1 mutation 5 of whom had severe PAMI phenotype, and one - PAPA phenotype. Degranulation of NK cells from peripheral blood was by studied by their incubation with MHC-I-negative cell line K562 and subsequent flow cytometric assessment of CD107a expression.

**Results:** 4/5 PAMI patients had decreased NK degranulation. One PAMI and one PAPA patients had normal degranulation.

**Conclusions:** We propose that NK cells degranulation assay can be tested as a potential method for helping to distinguish patients with two phenotypes of PSTPIP1 defects. Our data provide a preliminary basis for further study of actin polymerization defects underlying PAMI phenotype.

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**IMM-11**

**Specific pattern of SAP protein expression in patients with autoimmune lymphoproliferative syndrome (ALPS)**

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**Introduction:** Primary immunodeficiency (PID) with massive lymphoproliferation comprise a significant cohort of PIDs. Differential diagnosis includes X-linked lymphoproliferative syndrome (XLP1), ALPS, and other disorders of immune dysregulation. Quick methods of differential diagnosis are valuable in clinical practice. One of the most reliable diagnostic criteria for XLP1 is SAP protein deficiency detected by flow cytometry. SAP is well studied as modulating molecule in the process of activation of cytotoxic lymphocytes. SAP expression is also known to be increased in activated and intensively proliferating cells. Patients with ALPS have a defect in Fas-mediated apoptotic pathway resulting in abnormal survival and proliferation of activated double-negative T lymphocytes (TCRab+CD4-CD8-). We hypothesized that SAP protein expression in this double-negative population could be increased due to its activation status.
**Methods:** Five patients with ALPS diagnosis made according ESID diagnostic criteria and confirmed mutations in TNFRSF6 gene were studied. Peripheral blood samples were collected in EDTA tubes and stored for no more than 12 hours until analysis at the room temperature. The expression of SAP protein was analyzed by flow cytometry. We gated true double negative T lymphocytes by TCRab+CD4-CD8- phenotype and then evaluate SAP expression in this particular population in contrast with normal CD3+CD4+/CD8+ lymphocytes.

**Results:** In all patients, SAP expression had bimodal distribution: one peak of normal SAP expression corresponded to the population of ordinary T lymphocytes (CD3+CD4+/CD8+), and the second peak with increased expression was found in the population of double negative T lymphocytes (TCRab+CD4-CD8-).

**Conclusions:** The additional peak of increased SAP expression we observed in patients with ALPS with Fas defect could be helpful in differential diagnosis of PIDs with lymphoproliferation by flow cytometry. Thus, patients supposed to be XLP1 by clinical manifestations, but having normal level of SAP expression, that tends to be bimodal due to even a small increased peak, should be further evaluated for flow cytometry diagnostic features of ALPS.

**IMM-12**

**Wiskott-Aldrich syndrome (WAS) protein (WASP) expression measured by flow cytometry correlates with clinical severity in Wiskott-Aldrich syndrome patients**

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**Introduction:** WAS is a rare X-linked primary immunodeficiency caused by mutations of WAS gene, coding for WAS protein. WAS disease severity ranges dramatically from isolated thrombocytopenia to severe disease with bleeding, autoimmune disorders and malignancies. Hematopoietic stem cell transplantation (HSCT) is an effective curative method of WAS treatment. Most patients with WAS require HSCT, yet the urgency of therapy depends on the severity of the disease. With advances of early WAS diagnosis – usually during the first months of life, when disease is not fully manifested - method for prognostic assessment is critical. Flow cytometry is a rapid, accurate and reliable method for WAS diagnosis. We aimed to evaluate correlation between WAS protein Stain Index value, based on mean fluorescence intensity (MFI), and severity of the disease.

**Methods:** Fifteen patients, aged 4 months to 17 years, with various WAS mutations were included in the study. WAS protein expression was assessed in mononuclear cells (PBMCs) via flow cytometry using anti-WASP, CD45, CD14 antibodies. Stain index was calculated as “(MFI of sample - MFI of isotype control sample)/100”. Healthy subject’s specimen was used as internal control for every test. Estimation of scores to determine WAS severity was assessed using severity score suggested by Zhou et al. where 1 is mild and 5 is the most severe disease.

**Results:** in WAS patients studied stain index varied from 0.16 to 3.36 and was decreased by comparison with intralaboratory references (11.9-21.1). We revealed negative moderate correlation between severity score and stain index (r=-0.5, p<0.05).

**Conclusions:** flow cytometry can be helpful to clinicians in severity disease assessment and further prediction of disease progression in a cohort of patients with Wiskott-Aldrich syndrome. Further we are going to apply such approach (not only detection of proteins but using of calculated indexes) to analysis of another proteins (e.g. LRBA, DOCK8 etc.) and also try to find correlations with clinical course and prognosis of a range of primary immune deficiency diseases.
Flow Cytometric Study of Natural Killer Cell Function in Patients with Idic15 Syndrome

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Introduction: Natural killer (NK) cells are innate immune effectors that recognize and kill cells but also to secrete cytokines. Two main subsets of human NK cells have been defined: CD56⁶⁰ CD16¹ NK cells display strong cytotoxic activity, while CD56¹⁶⁰ CD16neg/dim NK cells produce and respond to pro-inflammatory cytokines. The Idic15 Syndrome is an Autistic Spectrum Disorder (ASD) and is caused by duplications of the 15q11-q13 region, in which some genes related to the immune response are found. An ongoing study with patients recruited among the members of the Idic15 Spain Association, has shown alterations in biomarkers of lymphocyte function, consistent with a pro-inflammatory status. Thus, we have determined by flow cytometry the expression of markers CD56 and CD16 on the surface of circulating NK cells, as biomarkers of cytotoxic and regulatory NK subsets.

Methods: The study was observational, case-control, with 28 patients diagnosed with Idic15, recruited from among the members of the Idic15 Spain Association, and 17 controls matched by age, sex and geographical area. The expression in the membrane of leukocytes of 19 protein biomarkers (CD3, CD4, CD8, CD14, CD16, CD19, CD21, CD24, CD27, CD28, CD45RA, CD56, CD57, CD197, CD279, IgD, IgM) has been determined by immunophenotypic flow cytometry (Galillos, Beckman Coulter) using three panels of monoclonal antibodies conjugated with fluorochromes (Duraclone, Beckman Coulter). Cytometric data have been correlated with the manifestation of autism, with the type of duplication, according to the aCGH of the breakpoints (BP) described in the 15q11-q13 region (BP1-BP5, BP1-BP3, BP1-BP2 and BP2-BP3), and with other biochemical and cellular markers of inflammation.

Results: Our results show that Idic15 patients have increased levels of circulating CD56¹⁶⁰ CD16neg/dim regulatory NK cells and reduced concentration of CD56⁶⁰ CD16¹⁶⁰ cytolytic NK cells, most markedly in patients with autism manifestations, but without clear correlation with BP types. Conclusions: Our data suggest a possible disbalance of NK cell function in Idic15 patients, consistent with the signs of pro-inflammatory environment previously shown by our study. Project financed by donations to the “One House One Life” Initiative promoted by Great Chance SLU.

Multiplexed Analysis of Biomarkers of Endothelial Activation and Inflammation in Patients with Idic15 Syndrome

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Introduction: The Idic15 syndrome is a rare neurological disease caused by duplications of the 15q11-q13 region. It is a rare disease, heterogeneous in size and disposition of the duplications, and weak correlation between genotype and main clinical manifestations that include hypotonia, autistic disorders, recurrent infections and epilepsy. In the search for indicators that relate these processes, our group has detected that the manifestation of autism in patients with Idic15 is associated with a cellular and biochemical profile of suggesting a subclinical proinflammatory state. Given the relationship between inflammation and endothelial activation, we have evaluated both processes by means of multiplexed bead-based flow cytometry.

Methods: We have evaluated inflammation and endothelial activation by quantifying the serum levels of the cytokines sE-Selectin, sICAM-1, sVCAM-1, Apelino, IFN-γ, IL-1b, IL-6, IL-10, MCP-1, VEGF and TNF-α, using Milliplex Map assays for Luminex with Millipore panels. The study has been conducted on 28 Spanish patients diagnosed with Idic15 (http://www.idic15.es/) and 17 paired controls by age, sex and geographic area.
Results: Our results do not show significant differences between Idic15 and controls for the levels of any of the cytokines evaluated. Neither do significant changes when the Idic15 population is divided according to the clinical manifestation of autism. On the contrary, there are significant changes in apelin levels that are associated with the manifestation of hypotonia (elevation, $p = 0.035$) or hypertonia (decrease, $p = 0.032$); and patient stratification according to the length of the duplication shows significant elevation of VEGF ($p = 0.006$), TNF-α ($p = 0.011$) and IL-1β ($p = 0.022$) and, to a lesser extent of MCP-1 and sE-Selectin in the patients BP1-BP5 compared to BP1-BP3.

Conclusions: In the analysis of the meaning of these alterations, the highest prevalence of epilepsy and autism observed in patients with BP1-BP3 duplication should be considered. Project financed by donations to the “One House One Life” Initiative promoted by Great Chance SLU.

IMM-15

Immunophenotypic characterization of peripheral blood dendritic cell subsets in patients with myelodysplastic syndromes

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Introduction: Myelodysplastic syndromes (MDS) are haematopoietic neoplasms characterized by ineffective haemopoiesis and progression to acute myeloid leukemia in a relevant proportion of patients. Although an inflammatory component to MDS has long been recognized, only recently a more direct role of chronic innate immune signaling and inflammation in the pathogenesis of the disease has been issued. In this context, the involvement of dendritic cells (DCs) in MDS has been poorly characterized, so far. DCs are professional antigen-presenting cells crucial to the initiation and shaping of immune responses. The effects of DCs on immune responses depend partly on the activation state of DCs, and partly on the functional specialization of distinct subsets, with CD141+ conventional DCs (cDC1s) being critical in anti-cancer immune responses, mainly related to their ability to efficiently cross-present antigens, and activate cytotoxic T cells. In this study we characterized DC subsets in the peripheral blood of MDS patients.

Methods: Whole blood samples obtained from 10 MDS patients and 16 healthy donors were stained with standard procedures and acquired on a FACSymphony (Becton Dickinson). The frequency of DC-lineage DCs, including cDC1s, cDC2 and plasmacytoid DCs (pDCs), and the frequency of inflammatory DCs, including monocyte-derived DCs (moDCs) and slanDCs, was analyzed in basal conditions. The phenotype of each DC subset was analyzed upon exposure of WB samples to proinflammatory and anti-inflammatory stimuli. Data were analyzed with FlowJo (FlowJo LLC).

Results: All circulating DC subsets were significantly reduced in the blood of MDS patients compared with controls, this reduction not being correlated with disease severity. cDC1s from MDS patients showed an overall subversion of their inhibitory molecule repertoire, characterized by strong upregulation of ILT2 and downregulation of TIM-3 expression. We also observed an overall activation of cDC2s, and hyporesponsiveness of pDCs and cDC2s to TLR-mediated stimulation, likely reflecting the chronic hyperactivation of TLRs occurring in MDS and the subsequent desensitization of DCs to further TLR-induced stimulation.

Conclusions: The observation that the immune checkpoint repertoire of cDC1s is subverted in MDS patients may pave the way for understanding the impact of these molecules on DC function. Further studies will be needed in order to investigate whether these cDC1 changes are also present in the bone marrow, whether they are sustained by the neoplastic process or they play a causative role in it, whether they are related to the immune dysregulation occurring in MDS, or whether they are shared with other types of cancer.
MISC-01

Immunophenotyping using t-SNE Analysis with four ClearLLab 10C Panels
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Introduction: Data from development studies of ClearLLab 10C Panels, separately from the application’s original use as a tool for trained PIs, was uploaded to R Software and t-Distributed Stochastic Neighbor Embedding* (t-SNE) was used to analyze the data. A t-SNE is a non-linear dimensionality reduction algorithm used for exploring high-dimensional data. In an unbiased manner, t-SNE can convert multi-dimensional data to low dimensionality to uncover unique population patterns. ClearLLab 10C Panels (Panels) are used in the multiparameter immunophenotyping of cell populations and analyzed using CytExpert for a trained primary investigator (PI) to determine the presence of abnormal phenotypes as part of the diagnosis. The Panels include a B, T, and two Myeloid (M1 and M2) Cell Tubes. This poster will evaluate t-SNE analysis of four abnormal samples compared to non-disease peripheral blood and bone marrow samples.

Methods: Donors classified as Hematological Abnormal/ No-Disease (classification includes normal donors) and donors classified as abnormal were compared using an unsupervised t-SNE analysis with R Software. All samples were tested with all four ClearLLab 10C Panels on a Navios Instrument and CytExpert was used for LMD analysis for PI assessment. The donors were chosen based on feedback from the PI assessment and phenotypes from the PI assessment included Mature B Cell Neoplasm, Myeloma, and Acute Leukemia. R Software was used to run the t-SNE algorithm and each panel was run on with a separate t-SNE algorithm.

Results and Conclusions: No-disease donors analyzed using t-SNE displayed similar patterns, with smaller sized CD45dim clusters compared to the Abnormal donors. Each Abnormal donor clustered in distinct populations in t-SNE analysis; three donors had abnormal populations on the t-SNE from the B or M2 Cell Tubes’ markers, while only one donor had abnormal populations on the t-SNE for the T or M1 Cell Tubes’ markers. Two of the abnormal donors had abnormal populations in t-SNEs of multiple Cell Tubes.

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MISC-02

Performance Evaluation of the BD FACSDuet integrated to BD FACSLyric™ System* using human specimens.
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Introduction: The BD FACSDuet™ (automated sample processor) integrated to the BD FACSLyric™ with BD FACSuite Clinical software system* (flow-cytometer), are physically and data integrated via the BD FACSLink™ interface for data transfer to a laboratory information system. This study evaluated the performance of the integrated automated sample preparation, transfer and analysis to the manual preparation, transfer and analysis at three clinical sites for enumeration of T-, B- and NK- cells and percentage of lymphocytes in human specimens.

Methods: Two sites enrolled remnant specimens from routine laboratory testing, one site procured specimens from a vendor. Specimens were collected in K2 or K3 EDTA delinked, de-identified, stained using the with the BD Multitest™ IMK and 6-color TBNK reagents per manufacturer’s instructions. For automated preparation, specimens and reagents were loaded in the FACSDuet system, the worklist was prepared for sample staining, transfer and analysis in FACS Lyric. For manual method, samples were stained and manually transferred to a BD FACSLyric system. Data was analyzed using Deming regression for bias analysis; method agreement calculated at 200 cells/µL clinical decision of CD4+ absolute cells, and the predicted %Bias and the bias intervals were calculated.
Results: 105 specimens were tested for 6C-TBNK and 108 for IMK from HIV-positive and HIV-negative subjects. Deming regression for 6C-TBNK absolute counts provide $R^2$ values of 0.98 for CD3, CD4, CD8; CD19, and CD16+CD56. Slope values were between 0.99 and 1.04. For IMK, $R^2$ values were between 0.98 and 0.99 and slope values between 0.99 and 1.00 for absolute counts. The overall method agreement for 6C-TBNK was 98.10% (93.32%, 99.83%, CI), and for IMK was 99.07% (94.90%, 99.48%, CI). The predicted %Bias for 6C-TBNK was -0.73 (-2.42, 0.97), for IMK was -1.18% (-2.68, 0.32).

Conclusions: This study showed the investigational automated system and the manual preparation methods are equivalent methods for enumeration of the T-, B- and NK-cell counts and percentage of lymphocytes.

*BD FACSDuet integrated to BD FACSLyric with BD FACSuite Clinical software is not for sale in USA. This product is CE Marked (IVD Directive 98/79/EC).

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MISC-04 (also presented as Poster Flash Presentation # PFP-30)

The effect of TNF-α on diabetic dendritic cells derived from bone marrow. An in vivo study

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Introduction: Tumour Necrosis Factor α (TNF-α), is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a range of responses, leading to necrosis or apoptosis. Diabetic patients show high TNFα plasma levels and a direct correlation was found by our group between TNF-α and proinsulin (PI) expression on dendritic cells (DCs). DC are involved in immune response against external pathogens, secreting TNF-α upon infection.

AIM: We evaluated the effect of TNF-α and Glucose (Gluc) stimulus on PI expression in bone marrow-derived DCs from diabetic patients, with/without treatment with the inhibitor Adalimumab (ADA), a human anti-TNF-α antibody.

Methods: Bone marrow from diabetic patients with foot lesions (n=6) were processed to obtain BMMC, that were seeded and cultured for 9 days in RPMI supplemented with 10% FBS, GM-CSF (50ng/mL), IL-4 (50ng/mL), 1% glutamine, and 1% penicillin. At day 7, cells were challenged for 24h with TNF-α 10ng/mL, and Glucose 30mM, and at day 8 cultures were supplemented with ADA 10ug/mL for 24 hours. After the treatment cells were counted and analyzed by flow cytometry to evaluate the expression of PI, CD14 , and DC markers.

Results: At day 8, both glucose and TNF-α treatments reduced cell number respect to untreated cells (80±12, glucose and 106±34, TNF-α vs 147±22, WT p<0.01). We found a significant reduction of CD14+ cells and an increased number of DC in cultured in standard medium respect to T0 (7.7±2.7 vs 0.4±0.7, 4.6±2.3 vs 24.8±8.9, respectively, both p<0.001). In glucose treated cells we found an increase of proinsulin and DC markers expression respect to untreated cells (0.16±.09 vs 0.74±0.46 and 0.03±0.03 vs 0.43±0.35 respectively, p<0.001), whilst no changes were induced by TNF-α treatment. Following TNF-α and ADA treatments, PI expression (Gluc:0.74±0.46; GlucTNF-α:30 0.21±0.18; GlucTNF-α+ADA:0.22±0.19 p<0.01) and PI coexpression with DC markers (Gluc:0.43±0.35; GlucTNF-α: 0.065±0.04; GlucTNF-α+ADA:0.0235±0.03 p<0.05) were reduced versus Glucose stimulus alone.

Conclusion: We found an increase of PI and reduced DC under glucose treatment, similarly to what we had yet showed. TNF-α and ADA treatments do not affect the expression of PI in DC, and protect the cells from glucose-mediated apoptosis. Further studies are necessary to establish the real biological significance of PI in innate immune system.
MISC-05

Validation of CD34+ stem cell enumeration with the BD SCE kit on two BD FACSLyric Analyzers
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Introduction: Accurate quantification of CD34+ cells is a critical step in the bone marrow transplantation process. The most reliable methods for the enumeration of stem cells in clinical use are well-established commercial prepared kits based on the single platform ISHAGE protocol. The aim of this study was to validate the suitability of the BD FACSLyric system for the routine enumeration of viable CD34+ cells with the BD SCETM kit in samples obtained during leucopheresis.

Materials and Methods: This validation study was conducted with two BD FACSLyric analyzers at the Central Laboratory of the University Hospital Halle, Germany, based on the CLSI EP09-A3 and EP15-A3 guidelines. Estimations of precision and accuracy were carried out with commercial quality control material (BD Stem Cell Control) at two concentration levels over 6 days with 6 measurements each day. A method comparison was performed by analyzing peripheral blood (n=18) and apheresis samples (n=20), measured on a FACSLyric and a FACSCanter analyzer in duplicate each. The coefficients of variation (CV) and bias were calculated for all analyzed cell populations (CD45+, CD34+, %CD34+ of CD45+) and the comparison was conducted via Passing-Bablok regression and Bland-Altman plot.

Results: The two FACSLyric analyzers did not differ from each other concerning their CVs and accuracy. Repeatability CVs ranged from 1.4% up to 10.3%, total laboratory CVs from 3.8% to 12.3%. The repeatability CVs obtained from the duplicate measurements of peripheral blood and apheresis samples ranged from 1.3% to 7.2%. As such, all obtained CVs met the approval criteria of <10% for repeatability CV and <15% for total laboratory CV. Mean bias (±SD) over all cell populations was -5.9% ± 3.7%. Passing-Bablok regressions for the comparison of FACSLyric and FACSCanter analyzers for peripheral blood and apheresis samples gave R2 values ranging from 0.994 to 1.000. The estimated slopes of the regression analysis ranged from 0.98 and 1.06. The mean difference between FACSLyric and FACSCanter for viability CD34+ cell measurements in peripheral blood was -2.4 % (95% LoA = -13.2 to 8.4%) and in apheresis samples -0.03 % (95% LoA = -4.1 to 4.1%).

Conclusions: The performance evaluation shows that the BD FACSLyric system can provide accurate measurements for the quantification of CD34+ cells and is acceptable for routine analysis of samples obtained during bone marrow transplantation process.

MISC-06 (also presented as Poster Flash Presentation # PFP-14)

Proficiency testing of Paroxysmal Nocturnal Haemoglobinuria (PNH) using electronic data files.
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Introduction: PNH, is a rare acquired stem cell disorder, characterised by GPI-linked surface antigen loss. Flow cytometry is recognised as the method of choice to detect/quantify PNH red blood cells (RBC) and white blood cells (WBC) enabling accurate diagnosis, effective treatment and monitoring. The UK NEQAS LI proficiency testing (PT) programme has identified interlaboratory variation in detection and quantification of PNH clones. To identify the proportion of variation arising from sample testing versus that from subsequent data analysis, a series of electronic PT cases have been developed. This study looks at the analysis of anonymised data files from actual PNH cases and highlights the findings of the first electronic PNH PT distribution.

Methods: UK NEQAS LI issued 4 FCS data files to all participants (n=191) in the PNH PT programme. Patient files were obtained with differing clinical scenarios and anonymised prior to issue. Samples were stained using the 2018 ICCS/ESCCA consensus guidelines 5 colour protocol. 150,000 events were acquired on a FACSCanto II analyser for each sample giving a potential limit of detection of 0.02%. Centres were asked to analyse all samples and return results to UK NEQAS LI.

Results: 78/191 (40.8%) returned results for data file analysis. 78.6% of laboratories stated that they followed the 2018 guidelines; however, analysis found that most (62% RBC, 83% WBC analyses) were missing essential time plots. Inter-laboratory agreement with respect to clone presence/absence was 91% for RBCs, 96.8% for monocytes and 98.9% for neutrophils, comparable to levels seen when using stabilised blood samples in the existing PT programme.
Comparison of PNH clone sizes from the electronic exercise to matched stabilised samples issued in the existing PT programme found average variation of clone size, as measured by inter-quartile range, was almost ten times higher in the wet samples (average IQR 1.64%) than the electronic analysis (average IQR 0.17%).

**Conclusions:** The results highlighted that data analysis is not the main cause of variation when performing PNH testing, but issues remain related to data analysis and guideline adherence. The lower variation in clone size measurement seen in the electronic exercises suggests that testing methodology and not analysis is the main cause of variation in PNH testing, although further exercises are required to prove this. This exercise also suggests that whilst electronic exercises are a useful PT tool, they must be used in conjunction with PT programmes using wet samples to facilitate full process monitoring.

**MISC-07**

**IVDR: a new era for in-vitro diagnostic medical devices**

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**Introduction:** In-Vitro Diagnostic Device Regulation (IVDR) is a new European regulation for placing in-vitro diagnostic medical devices (IVDMD) on the European market. A fundamental revision of the current Directive 98/79/EC was done to establish a robust, transparent, predictable and sustainable regulatory framework for IVDMD which ensures high level of safety and health whilst supporting innovation. This regulation sets high standards of quality and safety for IVDMD by ensuring, among other things, that data generated in performance studies is reliable and robust.

**Method:** Beckman Coulter (BEC) has put in place an intensive worldwide program across all Business Units (BU) in order to meet the quality and safety standards requirements in a timely manner. This Global IVDR program aims to identify and remediate impacts for all current BEC IVD products to comply with IVDR.

**Results:** The Global IVDR team is comprised BU representatives. Each BU has created IVDR local teams, which are in charge of deploying local activities accordingly. BEC Marseille IVDR team is responsible for bringing more than 230 IVD CE marked flow cytometry products into compliance with the IVDR. As a first step, Marseille IVDR team has performed a detailed gap assessment of each product to identify any additional performance studies required. In addition to the gap assessment, activities targeting new state-of-the-art Shipping Stability studies and analytical performances (Accuracy, Precision, Limit-of-Detection and Staining stability) studies, Post Market Surveillance and clinical evaluation are ongoing. International Guidelines published by organizations such as Clinical Laboratory Standards Institute (CLSI) have been used as inputs for experiment design. The Technical file structure and Quality System improvement were developed following a quality master plan to fulfill Notified Body assessment.

**Conclusions:** New IVDMD classification demands a demonstration of strong clinical evidence, which resides in Analytical Performance, Scientific Validity and Clinical Performance. BEC Marseille IVDR team, on the frame work of the IVDR Global program, has established and deployed a robust project plan to fulfill IVDR Requirements of more than 230 IVD CE marked products in a given timeframe. One key of success is strong and open relationship between different stakeholders such as Quality/Regulatory Affairs, R&D, Manufacturing, Engineering and Marketing. In spite of the huge workload that IVDR represents for BEC as IVDMD manufacturer, BEC supports the continuous improvement of product quality and safety, for a better diagnosis of patients.
**MISC-08 (also presented as Poster Flash Presentation # PFP-10)**

**Identical clinical performance of the Fetal Cell Count™ kit version IV and III for quantification of Fetomaternal Hemorrhage**  
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**Introduction:** In Fetomaternal Hemorrhage (FMH), fetal red blood cells (fRBCs) enter the maternal circulation, which may result in severe fetal anemia or alloimmunization against fetal blood groups. Quantification of the amount of FMH is important in obstetrical management of pregnant women. The Fetal Cell Count™ (FCC) kit (IQ Products, Groningen, The Netherlands) is a flow cytometric assay for fast, sensitive, and specific quantification of FMH, using antibodies against fetal hemoglobin (HbF) and maternal carbonic anhydrase-II (CA-II). Currently, the FCC kit is improved by the substitution of a polyclonal anti-CA-II antibody (FCC-III) with a monoclonal anti-CA-II antibody (FCC-IV). The aim of this study is to evaluate the clinical performance of the FCC-IV.

**Methods:** Peripheral blood samples of 184 pregnant women suspected of FMH and spiked samples containing 0% – 5% cord blood cells were included in this study at two different medical centers. Quantification of fRBCs was performed simultaneously with both the FCC-III and FCC-IV according to the manufacturer’s instructions. Erythrocytes were gated based on logarithmic amplification of the forward scatter and side scatter properties. Next, positively and negatively stained erythrocytes were quantified by quadrant dot-plot analysis, determined by the use of fluorescence minus one controls. Within the erythrocyte population, fRBCs were identified as HbF-CA⁺, and adult RBCs were identified as HbF-CA⁻. To assess the agreement between FCC-III and FCC-IV Passing-Bablok and Bland-Altman analyses were performed.

**Results:** The results demonstrated good linearity and precision of both kits for spiked samples (correlation of 0.9921 at location A and 0.9995 at location B). Additional Bland-Altman analysis of the HbF-CA⁺ and HbF-CA⁻ populations in spiked samples demonstrated no significant deviation between both FCC versions. Analysis of 184 patient samples exhibited equal clinical performance of the FCC-III and FCC-IV with a non-significant bias of 0.998 (CI95%: 0.991-1.005). Furthermore, analysis of eight FMH positive patients by both versions of the FCC kits revealed an outstanding agreement and no significant differences between FCC-III and FCC-IV (bias of 1.005 (CI95%: 0.904 – 1.106)).

**Conclusions:** This clinical performance evaluation study demonstrates identical results between the FCC-IV and FCC-III. The substitution of the polyclonal anti-CAII antibody with a monoclonal anti-CAII antibody did not change the performance specifications of the FCC kit. Therefore, the Fetal Cell Count™ IV kit can be used for the detection and accurate quantification of fRBCs in maternal samples, providing gynecologists and obstetricians with reliable results in cases of FMH.

**MISC-09 (also presented as Poster Flash Presentation # PFP-13)**

**Comparison of cell counting methods: Standard Hematology Analyzer versus Volumetric Cell Counting Flow Cytometry**  
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**Introduction:** Cell counting is crucial for the hematology laboratory and many methods are available nowadays. Major neutrophils, lymphocytes and its subsets enumeration is very important for diagnosis and monitoring of a variety of conditions affecting the immune and hematopoietic system. Hematology analyzers use impedance and flow cytometry for cell number and percentage determination, as SYMEX XE 2100 lines. New flow cytometry technologies also use beads volumetric counting for single platform enumeration showing precision and reproducibility. In this study we demonstrated the comparison of volumetric cell counting and the standardized hematology analyzer one in peripheral blood (PB) and bone marrow (BM) samples.

**Methods:** 14 BM and 17 PB samples were processed at the SYMEX XE 2100 analyzer (one time) and at Beckman Coulter CytoFLEX flow cytometer (three times). The samples were antibodies free, and the results were compared using Paired t-test and Pearson’s correlation.
**Results:** 14 BM samples had media count of 32571 (2450-108930) / μL cells in the SYSMEX XE 2100 and 32146 (2596-107008) / μL cells in the CytoFLEX (X² = 1 p< 0.001 / paired t-Test p = 0.128). The 17 PB samples had media count of 8545 (3860-23230) / μL cells in the SYSMEX XE 2100 and 8713 (3697-23509) / μL cells in the CytoFLEX (X² = 0.996 p< 0.001 / paired t-Test p = 0.172). PB neutrophil absolute counting: media count of 4178 (900-13250) / μL cells in the SYSMEX XE 2100 and 4125 (950-13150) / μL cells in the CytoFLEX (X² = 0.994 p< 0.001 / paired t-Test p = 0.217). PB Lymphocyte counting: media count of 3357 (540-6100) / μL cells in the SYSMEX XE 2100 and 2536 (529-7399) / μL cells in the CytoFLEX (X² = 0.979 p< 0.001 / paired t-Test p = 0.454). PB monocyte counting: media count of 672 (113-3706) / μL cells in the SYSMEX XE 2100 and 798 (80-1620) / μL cells in the CytoFLEX (X² = 0.961 p< 0.001 / paired t-Test p = 0.04).

**Conclusions:** These results show a good correlation between the two methods allowing the use of beadless volumetric cell counting for hematopoietic cell determination. Based on this we are intending to perform lymphocyte subset (CD4, CD8, CD19, CD20, CD16, CD56) enumeration and compare this new methodology with the standardized beads based one, looking forward to have less costly and time saving workflow.

**MISC-10**

**6-(Methylsulfonyl) hexyl isothiocyanate induce autophagy in leukemia cell lines**

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²University of Valencia, Valencia, Spain

**Introduction:** Autophagy is a fundamental catabolic process of cellular survival through which the cell degrades and recycles cellular components. However, the alteration of its normal mechanism may be responsible for the development of many diseases, such as cancer. The role of autophagy in cancer is extremely complex; on the one hand, in the early stages of neoplastic transformation it can act as a tumor suppressor avoiding the accumulation of proteins, damaged organelles and reactive oxygen species; on the other hand, during the advanced stage of cancer, autophagy is exploited by cancer cells to survive under starved. Numerous substances of natural origin have shown to have chemopreventive activity and in particular, isothiocyanates are of great interest, between these, more recently the 6-(Methylsulfonyl) hexyl isothiocyanate (6-MITC) attracted the attention of researchers. Studies conducted in our laboratories and recently published demonstrating that 6-MITC is able to exhibit cytotoxic, cytostatic, and cytodifferentiation effects on different leukemic cell lines. These encouraging results stimulate us to continue research investigating other possible potentials. In this context it is extremely interesting to evaluate the modulation of the autophagic process by 6-MITC and the related pathways, with particular attention to ROS-autophagy-cancer interconnection.

**Materials and Methods:** Flow cytometric analysis of Jurkat and HL-60 cells treated with 6-MITC 0-8 µM and 0-16µM respectively for 20h allowed to evaluate the autophagy mechanism. Subsequently, to better characterize the interconnection between autophagy and oxidative stress the level of DCF, MitoPY, HE, MitoSOX, DHR123, MBC were also evaluated. At the end, in order to extended us previous experience, the data has been confirmed through InCell Analyzer 2000.

**Results:** 6-MITC induced autophagy in Jurkat and HL-60 cells at the highest concentration tested. The levels of intracellular hydrogen peroxide (DCF) increase in a dose dependent manner in both cells line while the levels of mitochondrial hydrogen peroxide (MitoPY) only in HL-60. For the other markers no statistically significant increase was observed.

**Conclusion:** Our data suggest that 6-MITC might be considered an interesting chemopreventive agent. The isothiocyanate is able to induce autophagy through ROS generation and in particular through the production of hydrogen peroxide. These first results encourage the conduct of further studies to learn more about the interconnection oxidative stress, autophagy and cancer.
Flow Cytometry Functional Assays in the Sarasota Dolphin Health Assessment Project
Mar Felipo-Benavent¹, Consuelo Rubio-Guerri², Alicia Martínez-Romero¹, Jose-Enrique O’Connor³
¹Centro de Investigación Príncipe Felipe, Valencia, Spain
²The Oceanographic Foundation, Valencia, Spain
³The University of Valencia, Valencia, Spain

Introduction: In the context of oceans increasingly contaminated by humans, there is a growing interest in understanding the effects of pollutants in the marine environment to raise awareness about the danger of their massive discharges. With this objective, our group has participated in the project Sarasota Dolphin Health Assessment in order to compare some essential immune and haemostatic functions between dolphins under human care and wild dolphins, which are more exposed to environmental stressors or toxins.

Methods: Sarasota Dolphin Health Assessment is an annual project carried out during the last 36 years in Sarasota Bay (Florida, USA). The staff involved in this project throughout the year, identify the dolphins and monitor the group that lives in the bay. Once a year, veterinarians and researchers from around the world participate in the health assessment of the animals, capturing for a brief period of time the individuals of interest (less than 1.5 hours). They perform veterinary tests and take samples or measurements for different research projects. Specifically we obtained heparinized and citrated blood samples and studied the phagocytic capacity and the platelet activation of the animals, using the same protocols applied to the control population of healthy dolphins of the Oceanogràfic Aquarium of Valencia (as previously defined by us in ESCCA 2018 and ESCCA 2017). After sampling, the animals were released to the sea and they returned to their group. The boats went back to the harbour and we started with the laboratory work.

Results: After 10 days of work in the boat, 16 wild dolphins have been checked. We have evaluated the phagocytic capacity and the platelet activation assessing the rise of cytosolic calcium and microparticles production after in vitro stimulation. These data are now under analysis.

Conclusions: The collaboration between different institutions makes possible the development of marine environmental field studies in which cytometry can have a great importance, as in immunotoxicology studies in marine animals. The project was financed mainly by Dolphin Quest, Fundación Oceanogràfic, Harbor Branch Oceanographic Institution/FAU and NOAA Prescott and organized by Chicago Zoological Society. To carry out our project, it was essential the collaboration with Beckman Coulter, assigning us a Cytoflex S during these 2 weeks and with the University of South Florida Sarasota-Manatee, allowing us to use their laboratory.

Comparison of an image-based fluorescence cell counter for CD34+ cells quantification with flow cytometry single platform assay
Lejla Kuric, Antonija Babic, Drago Batinic
University Hospital Centre Zagreb, Zagreb, Croatia

Introduction: Quantification of hematopoietic stem CD34+ cells in peripheral blood samples after mobilization is an important part of the process in which stem cells are harvested for hematopoietic stem cell transplantation (H SCT). The standard method used for CD34+ cells enumeration is flow cytometry, according to the ISHAGE protocol. Alternative technology currently being validated is based on an image (slide) based fluorescence cell counter, such as ADAM II (NanoEnTek, Korea). It has 4-channel light sources and performs assays for CD34+ cells and cell viability simultaneously. The obtained images (maximum 136) are processed by the image analysis software. All procedures are automatic once the stained sample has been dropped to the disposable chip.

Methods: We used a total of 23 mobilized peripheral blood and 2 leukapheresis samples for analyzing CD34+ count with ADAM II (NanoEnTek) in parallel with flow cytometry using single platform assay on FACS Calibur (BD Biosciences). Whole blood samples were collected and stained according to the ISHAGE single platform protocol. In parallel, samples were analyzed on ADAM II cell counter. Briefly, samples were mixed with the reagent solution containing fluorescence-labeled antibodies in a single test tube, lysed and loaded onto an assay slide. After inserting the slide into ADAM II cell counter, a manual focusing step is required for adjustment of the focus in the bright channel for discriminating cells from the debris, as well as in PerCP channel for enumerating CD45+ cells. Digital image analysis algorithms are used to determine total and fluorescent cell counts.
**Results:** We compared the two methods (ADAM II vs FACS Calibur) using Passing-Bablok regression to determine the relations between the results. Regression analysis revealed a strong relationship between the CD34+ cell counts analyzed on ADAM II and FACS Calibur. Spearman’s rho for CD34%/CD45 and CD34 cells/μL between ADAM II and FASC Calibur were 0.992 and 0.991 (P<0.0001).

**Conclusion:** ADAM II is relatively easy to run, except for the focusing step; namely, it was difficult to obtain adequate BR (bright) and CD45PerCP focus with some samples. In comparison to flow cytometric single-platform assay for absolute CD34+ count, ADAM II is more convenient and more practical since it does not require flow cytometry expertise. ADAM II demonstrated very good performance for use as a routine clinical assay and it could be used as a point-of-care device.

**MISC-13**

**Lethal and sub-lethal effects induced by Campylobacter jejuni lysates on CaCo-2 cells**

Mariele Montanari1, Erica Cesarini1, Raffaella Campana3, Maria Gemma Nasoni1, Francesca Luchetti1, Wally Baffone1, Stefano Papa1, Barbara Canonico1, F. Sola2

1University of Urbino Carlo Bo, Urbino, Italy, Urbino, Italy
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**Introduction:** Campylobacter jejuni is a Gram-negative spiral-shaped bacterium that is the most prevalent cause of bacterial diarrhea in humans and that might be responsible for 500 million cases of gastroenteritis world over each year. An important C. jejuni virulence factor is the Cytolethal Distending Toxin (CDT) that is thought to be associated with C. jejuni-induced local acute inflammation involved in enterocolitis. Our research evaluates the sequence of lethal and sub-lethal events in CaCo-2 cells, as a model of intestinal epithelium, exposed to lysates of three distinct strains, C. jejuni ATCC 33291 and C. jejuni ISS 1, compared to C. jejuni 11168H cdtA mutant.

**Methods:** Cell lysates of C. jejuni ATCC 33291, C. jejuni ISS 1 and C. jejuni 11168H cdtA mutant strains were added to CaCo-2 cell monolayers and, after 24, 48 and 72 hours, cells were analysed by means of flow cytometry and confocal microscopy, to detect DNA content, death features, mitochondrial-lysosomal network and autophagic pathway.

**Results:** C. jejuni lysates induced cell cycle arrest in G2/M phase, as highlighted by propidium iodide staining, and induction of apoptosis, revealed by 7-AAD. The C. jejuni ISS 1 wild-type strain mainly induced lysosomal alterations and the most relevant mitochondrial modifications. Instead, the C. jejuni ATCC 33291 strain causes changes in autphagic pathway as indicated by the results from monodansylcadaverine (MDC) staining, a specific autophagolysosomal marker, suggesting that CaCo-2 cells respond to stimuli by activating the autophagic machinery, largely involved in bacterial infections.

**Conclusions:** Our results suggest that C. jejuni lysates-treated CaCo-2 cells displayed different features, depending on the particular strain. In fact, lysates are able to induce lethal and sub-lethal effects differently targeting mitochondrial, lysosomal and autophagosomal compartments. These data are linked to the potentially complex interactions that underlay the etiopathogenesis of enteric infections.
PRESENTING AUTHORS INDEX

For a quick reference to find the session or poster number, the authors index listed on the next pages includes the invited speakers, oral abstract presenters, poster flash presenters and poster presenters*.

Please note that only the presenting authors are included.

Explanation of the codes used in the index:

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* The presentations of the Parallel sessions will be made available online to registered participants and ESCCA members after the Conference. An emailing will be sent out when the presentations are available.
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