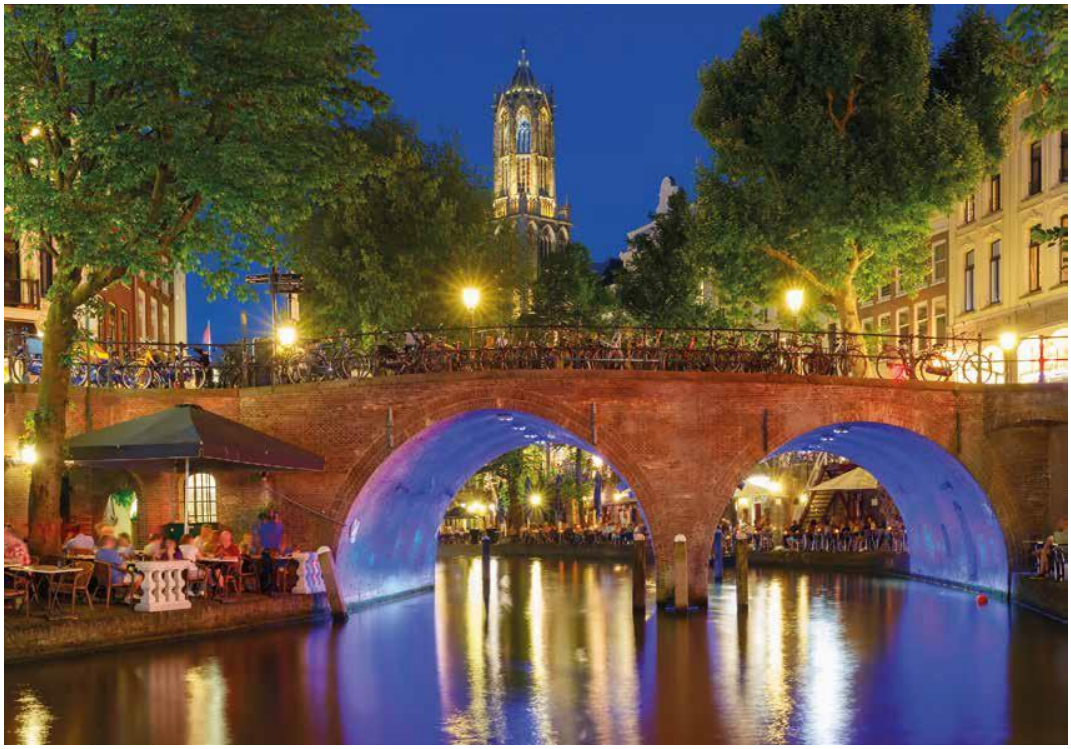
ESCCA 2023

Bridging the (cytometry) flows



UTRECHT, THE NETHERLANDS

27 - 30 SEPTEMBER 2023

ABSTRACTS

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PLENARY PRESENTATIONS

PLE01-01

MONITORING REGULATORY T CELL (TREG) THERAPY IN SOLID ORGAN TRANSPLANT (SOT) PATIENTS

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Chronic immune-related diseases triggered and nourished by pathogenic immune reactions out of natural endogenous control (autoimmunity, hyperinflammation, infection-induced tissue damage by immunoreactivity) represent an increasing burden for patients (> 10% of chronic diseases) and public society (>100 billion €/a in Europe). In addition, control of strong undesired immune reactions to solid organ and hematopoietic stem cell transplantation (SOT/HSCT) as well as to gene and cell therapy approaches is a further challenge. Current pharmacological strategies focus on chronic inhibition of immune reactivity by more or less unspecific immunosuppressive drugs, like steroids, calcineurin inhibitors, cytokine antagonists, T-cell targeting antibodies that express several limitations regarding efficacy, safety and sustainability. The adoptive therapy with regulatory T cells (Treg) is a promising new option for sustainable reshaping of the immune balance. To improve the quality of Treg cell products and to monitor therapy response regarding safety, PK, PD, mode-of-action and surrogate markers, a meaningful and robust immune assessment is necessary.

Our center has developed a full pipeline of 1st and 2nd generation Treg products based on a unique and very robust manufacturing process (about 50ml blood as starting material). We also established a qualified/validated immune assessment program for the *in depth* product characterization and therapy response monitoring.

The first-in-human study (ONE Study) of our 1st-gen Treg product in LD-kidney transplant patients revealed safety and signs of efficacy within the follow-up period of >5 yrs. The cell product showed high purity / low impurity and good functionality. For therapy response studies, >250 parameters were monitored by multiparameter flow cytometry, multiplex-ligand assays, NGS for TCR tracking, and IFNg-Elispot for antigenspecific T-cell response. We generated useful information regarding safety, therapy response failure, and mode-of-action (Roemhild et al. BMJ 2020).

To enhance the Treg engraftment and functionality under an ongoing tacrolimus therapy (necessary to control in particular initially very strong alloresponse), we generated Tac-resistant Treg cells by vector-free CRISPR/Cas-based gene editing. This resulted in new challenges for the product characterization and therapy response monitoring. We also implemented newer technologies as multiomics-single cell analyses. The updated immune assessment program is qualified/validated and the first-in-human clinical study with this first-in-class cell product is almost ready to start.

The talk will give a general overview on challenges and solutions and highlight some case studies.

PLE02-01

USING CLSI H62 TO BRING QUALITY TO THE CLINICAL FLOW CYTOMETRY LABORATORY

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The Clinical and Laboratory Standards Institute (CLSI) published the guidance document, H62 - validation of assays performed by flow cytometry in October 2021. This comprehensive document addresses all aspects of conducting flow cytometry measurements in regulated as well as non-regulated settings and brings together information regarding best laboratory practices. It follows the path of workflow: Pre-examination phase activities (sample requirements, reagent evaluation, instrument qualification and standardization, assay optimization and validation); .Examination phase activities (instrument monitoring and quality controls); and Post-examination activities (data review, reporting, storage, and retention are described).

The objective of this presentation is to provide guidance on how to distill the vast amount of information contained in this 234-page document into practice in the laboratory. Real-life examples from assays for rare events (CD34+ hematopoietic stem cells, paroxysmal nocturnal hemoglobinuria (PNH), and measurable residual disease (MRD) in blood cancers) will illustrate how to use this document to develop validation strategies and minimum requirements. A significant amount of time will be used to answer participant's questions.

PLE02-02

EDUCATION LEADS TO STANDARDIZATION

Ahmad Al-Attar

UofL Health, Louisville, United States

In this talk, I briefly discuss standardization as a concept in clinical laboratories, and how other laboratory sections have implemented it. I will try to clarify the differences between standardization and harmonization, and explain the merits and limitations of each.

I then explore the available agencies, centers, and organizations who could become partners to clinical laboratories in planning, designing and implementing protocols for standardization in flow cytometry.

The next section will cover standardization specific to flow cytometry – where we are, and where we need to be.

Afterwards, I take the audience on a trip through memory lane, in the company of Rob Sutherland and co. This will provide valuable lessons demonstrating that standardization goals are achievable in flow cytometry. The story highlights the importance of education at every level to ensure that the process is done correctly.

Finally, I talk about the importance of continued education to ensure that standards do not slip, and to safeguard the quality of standardized/harmonized flow cytometric assays, and the clinical laboratory as a whole.

PLE02-03

STANDARDIZATION IN MRD AML

Wolfgang Kern

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Assessment and quantification of measurable residual disease (MRD) in AML by flow cytometry has been subject of scientific evaluation for more than a quarter of a century but rather recently has gained attention in the context of both clinical care of patients with AML and clinical trails on AML. As any laboratory assay a flow cytometric MRD assay has to be validated and has to adhere to national and international standards. While the international standards ISO 15189 and ISO 17025 contain more general provisions applicable to a bright variety of assays including quantitative ones CAP Flow Cytometry Checklist and FDA Guidance on the use of MRD provide details on parameters to be addressed during validation, e.g. assessment of sensitivity, specificity and accuracy, as well as how these parameters should be addressed. Importantly, the consensus documents of the European LeukemiaNet MRD Working Party from 2018 and 2021 provide further specific details on the application of MRD considering also available moleculargenetic techniques and cinical aspects. Examples are the recommendation of distinct cutoff values and the preference of certain MRD assays in dependance of the specific findings in an individual patient. This presentation will provide the audience with an overview on the above mentioned sources and their implications when setting up a flow cytometric MRD assay for AML.

PLE03-01

THE ROLE OF MRD IN CURRENT TREATMENT STRATEGIES FOR CLL

Andy Rawstron

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Measurable residual disease (MRD) plays a crucial role in assessing response in chronic lymphocytic leukemia (CLL), with undetectable MRD (uMRD) being associated with improved progression-free survival (PFS) and overall survival (OS) outcomes following time-limited treatment. Due to the prolonged (>5yr) PFS with first-line treatment for most patients, MRD is frequently a primary endpoint in clinical trials for CLL and can be used as an intermediate endpoint for drug approval in the EU. MRD is a continuous variable, and it is well recognised that different MRD thresholds have different implications. Persistence of MRD at a high (>1%) level is typically associated with a poor outcome equivalent to a partial clinical response, while prolonged remission requires depletion below <0.01% (uMRD4) and achieving a functional cure likely requires depletion of disease below 1 CLL cell per million (uMRD6) in both peripheral blood and bone marrow. However, the optimal target and compartment for MRD assessment are still under investigation and recommendations from the European Research Initiative on CLL (ERIC) will be discussed. Different treatment approaches include chemotherapy, immunotherapy, B-cell receptor pathway inhibition (BCRi), or BCL2-pathway inhibition (BCL2i) and several different combinations are under evaluation. BCRi monotherapy typically requires indefinite treatment and prolonged remission can be achieved with persistently high MRD, however there are still applications for MRD assessment in identifying the evolution of treatment resistance and stratifying patients for intermittent treatment strategies. BCL2i approaches typically result in very rapid depletion initially but sustained uMRD is only attained in a proportion of cases. In combination approaches, the

MRD response kinetics vary considerably between the different components, highlighting the importance of optimizing treatment schedules to maximise depletion accordingly. The results of current clinical trials and MRD assessment strategies will be reviewed with the aim of considering how MRD may be brought into routine clinical practice.

PLE03-02

ROLE OF MINIMAL RESIDUAL DISEASE IN MULTIPLE MYELOMA: DIFFERENT TECHNIQUES FOR A CRUCIAL BIOMARKER

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Multiple myeloma is a neoplastic proliferation of plasma cells producing and secreting a monoclonal protein generally detectable in serum and/or urine. This M-protein is considered a reflection of tumor burden and, as such, it has been traditionally used to assess treatment response. The different categories of response as defined by the International Myeloma Working Group (and specially the achievement of complete response) have been related for many years to patient's outcome. However, despite achieving complete response, theoretically the highest degree of treatment response, patients continue to relapse due to "occult" disease that remains undetectable with the conventional methods used to define complete response. In this context, new methods to detect persistent disease have been introduced and broadly tested in patients with myeloma under different circumstances: transplant eligible, transplant ineligible, at diagnosis, at relapse, etc. These methods include Next Generation Flow and Next Generation Sequencing that assess the bone marrow of the patient and imaging methods, mainly PET-CT, to assess the potential presence of extramedullary disease. Overall, all these methods have shown a very solid prognostic value beyond complete response that will be reviewed in detail in the presentation. Due to the limitations associated with the use of bone marrow samples, the use of peripheral blood to assess the presence of residual disease is currently being actively investigated with very promising results. Peripheral blood is more accessible, can be obtained with a minimally invasive procedure and is potentially more reflective of the overall tumor burden; in contrast, the amount of circulating disease is generally lower as compared to the bone marrow and therefore ultra-high sensitive methods of assessment such as mass spectrometry or flow cytometry applied on enriched samples need to be used to obtain results with clinical relevance.

PLE04-01

ULTRA-SENSITIVE MOLECULAR MRD ON FLOW CYTOMETER USING SUPERRCA MUTATION ASSAY

Lei Chen [3], Shirin Khaliliazar [1], Carolin Lindholm [2], Anna Eriksson [3], Cavelier Lucia [2], Magnus Tobiasson [2], Eva Hellström Lindbe [2], Linus Boseus [1]

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Background: Rare mutations in patient samples serve as excellent markers to monitor the course of malignant disease and responses to therapy in clinical routine, and improved assay techniques are needed for broad adoption. superRCA assays provides rapid and highly specific detection of DNA sequence variants present at very low frequencies in DNA samples. Using a standard flow cytometer, precise and ultra-sensitive detection of single-nucleotide mutant sequences from malignant cells against a 100,000-fold excess of DNA, to follow the course of patients treated for acute myeloid leukemia (AML) both in bone marrow and peripheral blood.

Methods: Sequence of interest are first enriched by amplicons and converted to DNA circles which are subjected to rolling-circle amplification (RCA). Padlock probes specific for mutant or wild-type sequences are used to probe the repeated sequences of the RCA products with exquisite specificity through major voting mechanism that suppress background noise.Followed by RCA of the circularized probes, the large DNA clusters that result from each starting DNA circle are referred to as superRCA products. In each sample reaction starting from 330ng DNA, around one million superRCA particles are enumerated in the flow cytometer to gain the statistical power for low frequency detection.

Results: The low detection limit and high precision of superRCA are consequences of the highly selective genotyping of the repeated target sequences in combination with the large numbers of products that may be conveniently analyzed by flow cytometry. With spike-in dilution series, we demonstrated the superRCA assay can detect single point mutations as low as 1 in 100,000 with the flow cytometer readout.

In longitudinal AML patient sample analysis, SuperRCA assay detected remaining mutations after initial treatment and clearly revealed the remaining malignant clone, subsequently leading to a relapse. The superRCA assay also demonstrated the feasibility of detecting corresponding leukemia mutations in the PBMCs when such mutations were present in bone marrow. This would eventually enable a more frequent MRD strategy and reduce the patient burden for bone marrow sampling.

Conclusions: The superRCA assay procedure is suitable for routine use for its high sensitivity and simplicity. The single vial protocol contains seven solution additions, separated by incubations within a total time span of 3 hr, before the superRCA products are analysed using a standard flow cytometer. With ultra-high sensitivity, it's even possible to monitoring the status of AML patient with blood samples with equal utility comparing to the bone marrow samples.

PLE04-02

ANALYTICAL ASSAY VALIDATION FOR ACUTE MYELOID LEUKEMIA MEASURABLE RESIDUAL DISEASE ASSESSMENT BY MULTI-PARAMETER FLOW CYTOMETRY

Jesse Tettero [1], Naveen Dakappagari [2], Maaike Heidinga [1], Yvonne Oussoren-Brockhoff [1], Diana Hanekamp [3], Anil Pahuja [2], Kerri Burns [2], Pavinder Kaur [2], Zeni Alfonso [2], Vincent Velden [3], Jeroen Marvelde [3], Willemijn Hobo [4], Jennichjen Slomp [5], Costa Bachas [1], Angele Kelder [1], Kevin Nguyen [2], Jacqueline Cloos [1]

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Background: The assessment of measurable residual disease (MRD) in acute myeloid leukemia (AML) patients using multiparametric flow cytometry (MFC) has gained importance in clinical decision-making. However, compliance with the recent *In Vitro* Diagnostic Regulations (IVDR) in Europe and regulatory demands in the United States and Europe necessitates rigorous validation prior to the application of MFC-MRD assays in investigational clinical trials and diagnostics. Validating AML MRD-MFC assays poses challenges due to the unique underlying disease biology and limited availability of patient specimens. In this study, we conducted an extensive MFC MRD assay validation in accordance with the CLSI H62 guidelines to meet regulatory expectations.

Methods: Our analytical validation focused on our four tube, eight color MRD assay, which was previously clinically validated in HOVON clinical trials. We assessed several quality standards, including accuracy, analytical specificity, analytical and functional sensitivity (limit of blank (LoB), lower limit of detection (LLoD) and quantification (LLoQ)), precision (inter-operator, inter-gating, inter-instrument, intra-assay and inter-laboratory), linearity, sample/reagent stability and established the assay background frequencies.

Results: To evaluate the accuracy of the assay, we compared read-outs from two different MFC methods, which showed a highly significant equivalence with a correlation coefficient (r) of 0.99 for %blasts and r=0.93 for %leukemia associated immuno-phenotype (LAIP). The analytical specificity of the LAIPs (referred to as the internal positive population) was assessed by evaluating marker expression that could be differentiated from negative control cells. This differentiation was achieved by considering the mean fluorescence intensity (MFI), with a threshold set at an MFI value greater than two. The assay demonstrated a LoB of 0.03%, LLoD of 0.04%, and LLoQ of 0.1%. Precision experiments yielded highly reproducible results (Coefficient of Variation <20%). The assay demonstrated strong linearity (r=0.99, p<0.001), indicating a proportional relationship between the concentration of the aberrant subpopulation and the assay readout. The largest deviation was observed at a 0.4% dilution, with a standard deviation of 0.107%. Stability experiments demonstrated reliable measurement of both diagnostic and follow-up samples up to 96 hours after collection. Furthermore, the reference range of LAIP frequencies in non-AML patients was below 0.1%, ranging from 0.0% to 0.04%.

Conclusions: This study presents the extensive validation of an AML MFC-MRD assay using BM and PB patient specimens while adhering to best practices. Our approach may assist other laboratories in expediting their validation activities to meet current health authority guidelines.

PLE04-03

DETECTION OF MRD IN AML WITH A SEMI-SUPERVISED FLOWSOM-KALUZA INTEGRATED SYSTEM WITH A BACKGATING EVALUATION CAPABILITY

George Oudatzis [1], Christina Karela [2], Nikolaos Tsagarakis [3], Maroula Milaiou [2], Sofia Nikolaou [2], Vasiliki Zina [4], Paraskevi Vasiliou [5], Elpiniki Kritikou-Griva [6], Theodoros Marinakis [4], Francis Lacombe [7], George Paterakis [2]

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Introduction: MRD detection in AML in several time points has been shown to have significant prognostic value. Multiparameter flow cytometry (MFC) with complex gating strategy (GS) demands extensive experience and is operator dependent. Recently, FlowSOM, an unsupervised approach has been evaluated in AML MRD detection. The aim of this study was to evaluate an in-house AML MRD protocol by a semi-supervised FlowSOM-KALUZA integrated system taking advantage of its back gating capability.

Methods: An in house MFC protocol including 14 antibodies (CD66, CD14, CD45, CD33, CD34, CD117, CD123, CD10, CD16, CD38, CD36, CD41, CD19, CD64) in 10 color NAVIOS (Beckman-Coulter) analyzer was transformed to an integrated FlowSOM Kaluza@ version. Normalized merged 15 LMD files from normal patients (NMB) were compared to normalized diagnosis and normalized follow-up MRD detection points (FU). FlowSOM-Kaluza semi-supervised analysis produces nodes of interest pattern, and the user selects the nodes with twice as more percentage of MRD compared to NMB. MRD has been measured as (%). In our study, 98 FU points were evaluated (30 FU1 post induction, 27 FU2, 17 FU3, 13 FU4, 11 FU5). We performed a semi-supervised FlowSOM-KALUZA integrated analysis taking advantage of its back gating capability censoring each node by criteria of phenotype and FlowSOM discrimination. Pearson correlation was applied to correlate GS-induced MRD with FlowSOM-induced MRD.

Results: 30 cases of AML showed post-induction FU1 $r=0.8893$ ($p<0.0001$), FU2 $r=0.596$ ($p=0.0007$), FU3 $r=0.9785$ ($p<0.0001$), FU4 $r=0.9945$ ($p<0.0001$) and FU5 $r=0.0277$ ($p=0.46$). Specifically examined five cases with AML NPM1m showed $r=0.997$ ($p<0.0001$) considering all FU. Discrepant measurements were reduced by node censoring by KALUZA backgating revealing false positive populations like erythroblasts, mature cells, plasma cells or debris.

Conclusions: The FlowSOM-KALUZA transformation of an in house MFC AML MRD protocol was feasible and was applied and compared to GS. A semi-supervised approach is proposed by KALUZA backgating revealing false positive populations with statistically significantly correlation with GS.

PLE04-04

AUTOMATED EUROFLOW APPROACH FOR STANDARDIZED IN DEPTH DISSECTION OF HUMAN CIRCULATING B-CELLS AND PLASMA CELLS

Alejandro Hernandez-Delgado [1], Rafael Fluxa [2], Martin Perez-Andres [3], Annieck M. Diks , Jacqueline A.M. Gaans-van den Brink [4], Alex-Mikael Barkoff [5], Elena Blanco [3], Alba Torres [3], Magdalena A. Berkowska [6], Georgiana Grigore [2], Jacques J.M. Dongen [3], Alberto Orfao [3]

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Background: Multiparameter flow cytometry (FC) immunophenotyping is a key tool for detailed identification and characterization of human blood leucocytes, including B- lymphocytes and plasma cells (PC). However, currently used conventional data analysis strategies require extensive expertise, they are time consuming, and show a limited reproducibility. Here, we designed, constructed and validated an automated database-guided gating and identification (AGI) approach for fast and standardized in depth dissection of B-lymphocyte and PC populations in human blood.

Methods: For this purpose, a total of 183 FC standards (FCS) datafiles corresponding to newborn cord blood and blood samples from healthy volunteers, stained with the 14-color 18-antibody EuroFlow BIgH IMM panel and the EuroFlow standard operating procedures measured at four different laboratories, were used.

Results: The BIgH IMM antibody panel allowed identification of a total of 117 different B-lymphocyte and PC subsets. To build the EuroFlow BIgH IMM database 14/36 pre-selected FCS datafiles that fulfilled strict inclusion criteria were (manually) analysed by an expert flow cytometrist. Data contained in the analysed datafiles was then merged into a reference database that was uploaded in the Infinicyt software (Cytognos, Salamanca, Spain). Subsequently, we compared the results of manual gating with the performance of two classification algorithms -hierarchical algorithm vs two-step algorithm- implemented in Infinicyt for automated gating and identification (AGI) of the cell populations present in 5 randomly selected FCS datafiles. The AGI tool based on a hierarchical algorithm showed higher correlation values vs conventional manual gating (MG) (r^2 of 0.94 vs 0.88 for the two-step AGI algorithm) and it was further validated in a set of 147 FCS datafiles against conventional expert-based manual gating. For virtually all identifiable cell populations a highly significant correlation was observed between the two approaches (r^2>0.81 for 79% of all B-cell populations identified), with a significantly lower median time of analysis per sample (6 vs 40 min, p=0.001) for the AGI tool vs MG, respectively and both intra-sample (median CV of 1.7% vs 10.4% by MG, p<0.001) and inter-expert (median CV of 3.9% vs 17.3% by MG by 2 experts, p<0.001) variability.

Conclusion: Our results show that compared to conventional FC data analysis strategies, the AGI tool here proposed is a faster, more robust, reproducible and standardized approach for in depth analysis of B-lymphocytes and PC subsets circulating in human blood.

PLE05-01

B-CELL DEVELOPMENT IN A NEW CONTEXT: NEW INSIGHTS BASED ON INBORN ERRORS OF IMMUNITY

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B-cell precursors (BCP) arise from hematopoietic stem cells in bone marrow (BM). Identification and characterization of the different BCP subsets has contributed to the understanding of normal B-cell development, which is in fact a process guided by an intricate network of transcription factors as well as chemokine and cytokine signals. Regulatory mechanisms of B lymphopoiesis are similar, but sometimes different in humans and mice. The continuous discovery of monogenic defects that impact early B cell development in humans substantiates the similarities and differences with B cell development in mice. The first monogenic defects in early B-cell development were all restricted to the pre B-cell receptor (BCR) and preBCR signaling. Only during the last couple of years genetic defects have been identified in early transcription factors such as Ikaros, E2A, PAX5 and Aiolos. Cellular and

molecular analysis of patients carrying genetic defects in these key transcription factors provided a wealth of information and gave new insights into human B lymphopoiesis and into the spectrum of clinical phenotypes. Analysis of bone marrow samples of patients with IL7RA-deficient SCID unveiled a thus-far unknown role for IL-7 signaling in promoting the B-lymphoid fate and expanding early human B-cell progenitors, which defined the important differences between mice and humans that IL7R signaling completely abrogated T and B lymphopoiesis in mice, which IL7RA-deficient SCID patients still generate peripheral blood B cells. Altogether, cellular and molecular analysis of patients with Inborn Errors of Immunity affecting B lymphopoiesis provides important insights which contributes to better understanding of leukemogenesis of BCP, but will also have implications for hematopoietic stem cell transplantation.

PLE06-01 Interactive case presentation - no abstract

PLE07-01

PATIENTS WITH ACUTE STROKE SUSPICION WITHIN 24-HOURS OF SYMPTOM ONSET: MONOCYTE SUBSET PROFILES IN LARGE-VESSEL-OCCLUSION STROKE COMPARED TO OTHER DIAGNOSIS

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Introduction: Stroke is one of the upcoming epidemics of the century: it is one of the leading causes of disability and mortality. Sorting patients with acute ischemic stroke due to large vessel occlusion (LVO) is crucial to transfer them to the right center providing mechanical thrombectomy. Therefore, innovative tools are needed to screen patients. Peripheral monocyte subsets, *i.e.*, classical (Mon1), intermediate (Mon2), non-classical (Mon3) and corresponding activation marker expression could be cell marker candidates. We aimed at characterizing monocyte subset profiles and their activation marker expression in patients with stroke suspicion admitted to emergency stroke centers.

Methods: BOOST study ("Biomarkers algOrithm for strOke diagnoSis and Treatment resistance prediction", NCT04726839) is a non-interventional prospective multicenter exploratory study. Adult patients with symptoms suggesting acute stroke within the last 24 hours were included following informed consent. Four mL of blood were collected on EDTA diK+ upon admission to emergency unit before brain imaging. Flow cytometry analysis was performed on fresh blood using the six-color Becton-Dickinson FCM system/FlowJo-software. We developed a new strategy of monocyte subset gating based on CD45, CD14, CD16, CD91 (pan-monocytic), HLA-DR as well as CD62L, CD11b, CD86, CCR2, ICAM-1, CX3CR1 and tissue-factor.

Results: We included 330 patients; 297 had monocyte subsets analysis (mean-aged 62 ± 20 years, females 42.8%): 58 (19.6%) had LVO stroke, 67 (22.6%) small vessel occlusion ischemic stroke, 18 intracranial hemorrhage, two cerebral venous thrombosis, and 152 "mimics" (*i.e.*, non-LVO patients: n=239). The mean NIHSS upon admission was 12.2 *vs* 5.6 in LVO *vs* non-LVO patients. The median Mon1

count was higher in LVO *vs* non-LVO patients: 0.67 *vs* 0.56 G/L (p=0.0040); the median Mon3 count was slightly lower in LO vs non-LVO patients (p=0.0258). Using a linear mixed effect model, we evidenced a significant interaction between the expression of HLA-DR (p=0.0101) and ICAM-1 (p=0.0041) and the different Mon1, Mon2 and Mon3 subsets in the LVO diagnosis. CD86 MFI was 12% lower in LVO *vs* non-LVO patients (p=0.0094) in the three monocyte subsets.

Conclusions: In LVO *vs* non-LVO patients, the higher count of Mon1 highlights the early systemic inflammatory response associated with LVO stroke; the lower count of Mon3 could suggest an early recruitment of these monocytes at the site of injury, confirming their role as patrolling cells in case of endothelial injury and vascular inflammation. These results need to be confirmed in a validation cohort.

PLE07-02

PIDGEON: BRINGING A COMPUTATIONAL DIAGNOSTICS PIPELINE FOR PRIMARY IMMUNODEFICIENCIES CLOSER TO THE CLINIC

Annelies Emmaneel [1], Jana Neirinck [2], Sofie Van Gassen , Mattias Hofmans , Alba Valle , Martin Perez-Andres , Bart Lambrecht , Tessa Kerre , Jan Phillipé , Filomeen Haerynck , Mirjam Van der Burg, Tomas Kalina , Carolien Bonroy , Yvan Saeys

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Introduction: Patients with primary immunodeficiencies (PID) have an impairment in one or more intrinsic elements of the immune system and show a wide spectrum of clinical manifestations. This heterogeneity often complicates a fast diagnosis and timely treatment.

Methods: Our pipeline is built on multiparameter flow cytometry data, analyzing the peripheral blood within cohort of 509 patients with various PID subtypes, non-PID diseased controls and healthy controls. The cohort was diagnosed according to the IUIS and ESID criteria and the samples were immunophenotypically characterized using the PID Orientation Tube, developed by the EuroFlow consortium. Our computational pipeline "PIDgeon" starts with a preprocessing step, using the PeacoQC package and a normalization step with CytoNorm. Next, a FlowSOM model is built based on healthy controls of different ages to identify different lymphocyte populations. Subsequently, cell population counts are extracted by mapping all samples onto this model, enabling comparison between patients. These counts were then used to build a three-step classification model with extreme gradient boosting where first the most urgent IUIS I patients are classified. This is followed by the diagnosis of the diseased controls in step two and a separation of the different B cell defects in a final step. In the end, we also included a SHAP analysis, as an explainable framework, that gives more insight in the black-box classification models. Two validation cohorts were analyzed to test the models' accuracy. The first cohort consisted out of 211 patients, including a similar patient distribution as the original cohort. The second one consisted out of 26 patients considered as IUIS I.

Results: In both cohorts, our first classification model obtained a balanced accuracy of 95% where it succeeded in diagnosing all IUIS I patients. The second diagnostic model obtained a balanced accuracy of 84% when distinguishing diseased controls from PID patients and the third model received a balanced accuracy of 60%. When analyzing a new patient, PIDgeon also provides an overview report that includes the number of counts per immune cell populations, the patient's FlowSOM tree, a proposed diagnosis and a SHAP analysis, explaining that diagnosis.

Conclusion: In the end, we can conclude that PIDgeon is an explainable pipeline that is highly accurate in diagnosing IUIS I patients resulting in a faster diagnosis and treatment. We hope that PIDgeon can function as a first step toward the implementation of such a computational diagnostic tool in the clinic.

PLE07-03

PD1/TIGIT AXIS IN IDIOPATHIC PULMONARY FIBROSIS AND POST-COVID19 PULMONARY FIBROSIS

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Introduction: idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrosing interstitial pneumonia of unknown cause, limited to the lungs and occurring in older adults. A variety of intrinsic and extrinsic causes can activate the pro-fibrotic cascade. Similar to IPF, the COVID-19 activate factors involved in the fibrotic process. The longer the insult acts on lung tissue, the lower is the probability of a complete resolution of the damage. An emerging clinical entity post-COVID is pulmonary fibrosis (PCPF), that share many pathological, clinical and immune features with IPF. Due to these reasons, the aim of the present study was study PD1/TIGIT axis in patient affected of IPF and PCPF compared with healthy control (HC).

Methods: Patients affected by IPF and PCPF followed at at referral center for rare lung diseases of Siena University were enrolled. The diagnosis were confirmed by radiological data. Peripheral blood samples were stained with monoclonal antibodies CD4, CD8, PD-1 and TIGIT and they were detected through flow cytometry.

Results: Forty-eight patients affected by IPF (mean±standard deviation, 65±12; 70% males), fifty-five by PCPF (mean±standard deviation 74±14 ,61% male) and ten healthy controls (mean±standard deviation 55± 8, 50%male) were enrolled. The expression of $CD4^+$ cells is higher in PCPF than IPF patients (p=0.0002), while $CD8^+$ cells were abundant in IPF than PCPF patients (p<0.001). $PD1^+CD4^+$ and $PD1^+CD8^+$ cells are higher expressed in PCPF than HC (p=0.004, p=0.001), and also $PD1^+CD8^+$ is more abundant in IPF than HC (p=0.001). The expression of $TIGIT^+CD4^+$ is higher in IPF than PCPF and HC (p=<0.001, p=0.005). While the $TIGIT^+CD8^+$ is more abundant in PCPF than HC (p=0.029), as well as $TIGIT^+CD8^+$ is higher in IPF than HC (p=<0.001). PD1+CD4+ and TIGIT+CD4+ cells were directly correlated in PCPF patients (r=0,6 p<0.0001), as well as PD1+CD8+ and TIGIT+CD8+ cells were directly correlated in PCPF patients (r=0,5 p<0.0001).

Conclusions: The functional T-cell phenotype in PCPF patients is abnormal, similar to IPF patients. These results confirmed an unbalanced immune profile in both diseases accompanied by an increased PD-1 and TIGIT expression on exhausted cells with synergically function. The dual blockade PD-1/TIGIT axis could be considered a potential target therapy helpful to restore the T-cells in IPF and PCPF patients.

PLE07-04

DIAMETERS AND FLUORESCENCE CALIBRATION FOR PERIPHERAL BLOOD EXTRACELLULAR VESICLE ANALYSES BY FLOW CYTOMETRY

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Introduction: Extracellular vesicles (EVs) are released during different pathophysiological processes, play crucial roles in intercellular crosstalk and are increasingly thought to be new potential biomarkers in many diseases. EV analysis still represents one of the major issues to be solved in order to translate the use of EV detection in clinical settings. Even if flow cytometry has been largely applied for EV studies, the lack of consensus on protocols for flow cytometry detection of EVs generated controversy. Standard flow cytometry procedures, based on scatter measurements, only allow the detection of the "tip-of-the-iceberg" of all EVs. We applied an alternative flow cytometry approach based on the use of a trigger threshold on a fluorescence channel, with the final aim to optimize flow cytometry sensitivity for EV detection.

Methods: Here we have applied an optimized and patented procedure (Pat. nr: EP3546948A1) to identify, classify, enumerate, and separate from whole peripheral blood samples circulating EVs from different cell origins. This protocol takes advantage of a lipophilic cationic dye (LCD) able to probe EVs, used in combination with fluorescein-isothiocyanate-(FITC)-conjugated phalloidin to identify damaged EV membranes and a mix of antibodies to subtype EVs (Leukocyte-derived EVs-CD45+; Endothelial-derived EVs-CD45-/CD41-/CD31+; Platelet-derived EVs-CD41+/CD31+). Different flow cytometry platforms (BD FACSCanto II, BD FACSVerse, BC CytoFlex) were used to acquire EV samples. The same protocol was used to separate EVs (FACSAria III). All ISEV requirements were met (i.e. size, morphology, protein expression).

Results: Total EV numbers obtained by the application of the fluorescence triggering resulted significantly higher in respect to them obtained from the same samples acquired by placing the threshold on the side scatter (SSC) channel (p=0.0005). EV concentrations and percentages were analyzed from 22 healthy donor blood samples indicating that, among the detected phenotypes, the most abundant circulating EV population stems from platelets (mean = 1898.31 ± 1182.74 events/µL). Leukocyte- (mean = 621.06 ± 742.61 events/µL) and endothelial-derived EVs (mean = 346.63 ± 601.56 events/µL) were also detectable. Proteomic results demonstrated that the proteins conveyed by EVs from healthy donors were classified as having binding functions (45.50%), convey proteins with regulatory functions (25%), and catalytic activity (20.50%).

Conclusions: A protocol, using whole blood and avoiding any pre-analytical manipulation step, based on the use of a pan-EV marker, allowing to apply a florescent threshold, was optimized. This protocol allowed to obtain highly reproducible results. These results may open new routes for Flow cytometry EV studies and their clinical translation.

PLE08-01

DISSECTING MM: FROM FLOW-CYTOMETRY TO SINGLE-CELL SEQUENCING

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Flow cytometry is a powerful technique to monitor tumor and immune cells in the bone marrow and peripheral blood of patients with multiple myeloma. In addition, the patient-specific phenotypic features of tumor and immune cells can be leveraged for their isolation using FACS. These highly purified cell types can be subsequently studied using bulk sequencing approaches in large series, and/or single-cell methods in relatively smaller series. These combined immunogenomic datasets are powerful tools to advance our understanding of myeloma pathogenesis, risk stratification and precision medicine. During my presentation, I will cover the use of these techniques in the study of:

1) The impact of aging in normal and tumor-precursor cells, and the interaction between these events and myelomagenesis.

2) The biology and the role of circulating tumor cells in disease progression.

3) Phenotypic classification of unique patient subgroups.

4) Identification of key immune cell types associated with response and resistance to specific immunotherapies.

5) Clinical and biological significance of measurable residual disease in bone marrow and peripheral blood.

PLE09-01

BREAST CANCER WITH THE PRESENCE OF ATYPICAL CELLS IN PERIPHERAL BLOOD MIMICKING ACUTE MYELOID LEUKEMIA: A CASE REPORT

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A 75-year-old woman with a history of breast lobular adenocarcinoma treated with mastectomy and radiotherapy in 2021 and taking hormone therapy, presented with asthenia and tremor. Laboratory tests showed leucocytosis (23.4x10^3/ml), anemia (Hb 72 g/l), platelet count 138x10^3/ml, calcium level 13 mg/dl, LDH 600 UI/l, indirect bilirubin 5 mg/dl, haptoglobin <0.1 g/l and reticulocytes 11%. Direct Coombs test was negative and renal function was normal. Peripheral blood smear showed marked red cell anisocytosis, a remarkable proportion of schistocytes (15% of erythrocytes), erythroblasts (3% of nucleated cells) and immature granulocytes (promyelocytes, myelocytes and metamyelocytes). Furthermore, a significant number of large mononuclear cells of unclear lineage (15% of nucleated cells) was also detectable. Therefore, in the hypothesis of a macroangiopathic hemolytic anemia related to cancer recurrence, total body CT scan and ^{18}F-FDG PET/CT were planned. The former examination resulted negative, whereas the latter showed only a slight FDG uptake in the spine, ascribed to increased bone marrow activity due to macroangiopathic hemolytic anemia (MAHA). Then, to exclude the diagnosis of acute leukemia due to the presence of circulating suspected abnormal cells, bone marrow aspirate and trephine biopsy were performed, along with a cellimmunophenotyping. The

first myeloid flow cytometric panel evidenced a CD45-, CD34-, CD117+ population (20%), with high FSC and SSC All myeloid lineage markers were negative. A more extensive panel was performed, including plasma cell and erythroid markers. Interestingly, this population resulted positive for CD138 and CD71 and negative for CD38. A recent study reported that in addition to CD45 negativity, non-hematological neoplasms frequently express CD56, CD117 or CD138. Therefore, a further panel for non-hematological markers including EpCAM (epithelial cell adhesion molecule) was carried out. This population resulted CD9+ and EpCAM+, a marker typically expressed on breast neoplastic cells The flow cytometric analysis allowed to characterize the non-hematological nature of this population, circulating at unusually high level in the peripheral blood. Aspirate smears revealed the presence of the same cells, and immunohistochemistry on bone marrow confirmed the massive infiltration of breast cancer cells, allowing to diagnose bone marrow metastases.

PAR01-01 & PAR-02

NEW CLASSIFICATIONS FOR DIAGNOSIS OF HEMATOLOGICAL MALIGNANCIES: IMPACT ON IMMUNOPHENOTYPING

Kees Meijer & Maximiliano Ramia De Cap

In 2001, the 3rd edition of the World Health Organization (WHO) Classification of Haematopoietic and Lymphoid Tumours became the single prevailing system for classifying leukemia and lymphoma. This classification was developed by the WHO in collaboration with the Society for Hematopathology (SH), and the European Association for Haematopathology (EAHP). The subsequent 4th edition, and revised 4th edition, of the WHO Classification of Haematopoietic and Lymphoid Tumours (WHO-HAEM4) remained the prevailing system for classifying leukaemia and lymphoma until 2022.

In 2022, the 5th edition of the WHO classification (WHO-HAEM5) was released to address significant advances in the field of haematolymphoid disease. This classification was developed by an editorial board composed of standing members nominated by major pathology societies around the world, however, it was not endorsed by the SH and EAHP. Instead, the SH and EAHP endorsed a parallel classification system; the International Consensus Classification (ICC), which was developed using clinical advisory committees composed of pathologists, haematologists, oncologists, geneticists, and bioinformaticians from around the world. These updated classifications have, with varying divergence from the antecedent WHO-HAEM4, changed the way in which haematolymphoid neoplasms are diagnosed and prognosticated. In turn, this has changed the way diagnosticians evaluate diagnostic specimens and interpret diagnostic results.

In this session a hematopathologist and a cytometrist will use real cases to discuss some of the major changes and differences in the new classifications. The session will focus on entities for which diagnostic role of flow cytometry has been impacted by the current WHO and ICC classifications. Topics covered will include: “where is B-PLL?” and “how to approach challenging entities,” such as blastic plasmacytoid dendritic cell neoplasm (BPDCN) and monocytic leukemias.

PAR02-01

FUNCTIONAL ASSAYS OF PLATELETS IN WHOLE BLOOD

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The study of platelet phenotype and function is still considered as a complicated aspect in clinical cytometry. However, most difficulties in platelet cytometry may be overcome by appropriate sample preparation and by careful flow cytometric setup and assay optimization. Platelet surface immunophenotyping has been classically used as a convenient tool for diagnosis of inherited deficiencies of platelet glycoproteins or acquired alterations of hemostasis and vascular dynamics, but the state of the art in platelet flow cytometry has made its way far beyond the description of platelet-

related disorders. Indeed, most CD markers on platelet surface are receptors involved in signal transduction pathways central to the control of hemostasia and other physiological functions of platelets. Such pathways involve a multiplicity of biological parameters suitable of being studied by flow cytometry. Indeed, flow cytometric assay of platelet functional responses allows characterizing effector and regulatory pathways, identifying genetic variability in biomarkers of disease and risk factors, as well as validating and monitoring therapeutic targets in hemostatic disorders and cardiovascular conditions. This presentation illustrates the advantages and relevance of functional flow cytometry for the clinical analysis of platelet and platelet-derived microvesicles, including recommendations for sample preparation, assay optimization and standardization. J-EO is a member of Spanish Network of Inflammatory Diseases of the Institute of Health Carlos III (ISCIII), Spanish Government, Madrid, Spain (REI-RICORS , RD21/0002/0032)

PAR02-02

PHOSPHO-ASSAYS FOR RESEARCH AND DIAGNOSTICS OF IEI

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Inborn errors of immunity (IEI) are rare disorders caused by damaging genetic variants in genes affecting the immune system. Currently, over 450 genes have been associated with IEI. The diagnosis for IEI is based on clinical and immunologic features and genetic testing for known variants in the IEI genes. However, quite often variants of uncertain significance (VUS) are found. These VUS requires functional validation before a definite diagnosis can be made. Functional validation of these VUS can be difficult, time-consuming, and is often done in a research setting. In this presentation, I will discuss the possibilities of using flow cytometry for measuring phosphorylated proteins for the functional validation of VUS in patients with IEI.

PAR03-01

DEFINING THE NORMAL: NEW APPROACHES FOR HARMONIZED SUBSET DEFINITIONS AND GATING PROCEDURES

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Analysis of composition of leukocyte subpopulations by flow cytometry is essential in haematological and immunological diagnostics. Compositions and function of the immune system is very dynamic during ontogenesis and is influenced by interactions between host and surrounding environment. Age matched controls are needed for set up of the reference ranges. Availability of the healthy volunteers is rather limited especially in very young children. Alternatively, residual blood samples for blood count analysis i.e taken before surgery in otherwise healthy child after appropriate informed consent can be used. Reference range is typical set between 5^{th} and 95^{th} percentile in respective age category. Another factors potentially influencing composition of subpopulations is ethnicity and gender.

Subsets are defined by immunophenophenotypic pattern. There is great variability in the composition of monoclonal antibody panels between laboratories. Harmonization is the process of minimizing redundant or conflicting standards which may have evolved independently. Currently, an increasing

number of parameters are being assessed at the single-cell level and there is need to integrate computional methods enabling multivariate analyses in diagnostic process. Analysis based on computional methods might overcome subjectivity of manual gating. Automated tools may contain following functions: pre-processing, automated sequential gating, Boolean combination gates, clustering, dimensionality reduction and trajectory/pathway inference. Increasing number of parameters also enables identification of innate myeloid cells and unconventional T cells simultaneously in limited amount of tubes.

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PAR03-02

MINIMIZE TECHNICAL VARIATION TO SEE MORE BIOLOGICAL SIGNAL – AN OPTIMIZED BLOOD SAMPLING METHODS IN SYSTEMS IMMUNOLOGY

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Introduction: There is no doubt that blood sampling plays a crucial role in immunology, serving as a valuable tool for studying and understanding the complexities of the human immune system or for diagnostic purposes. A blood sample can serve as a source of immune cells and molecules in the bloodstream. However, golden standard methods, such as gradient centrifugation isolation of PBMC, require larger volumes of blood and are laborious as well as selective with respect to cell populations. As a standard, five to twenty milliliters of blood are collected for multiple analyses in diagnostic or research laboratories due to inefficiencies on multiple levels in blood sample processing. The delay between blood drawing and processing increases technical variation, representing another challenge. To address these limitations, we developed reagents and simplified sampling pipelines that make deep immune profiling from as little as one hundred microliters of blood samples possible.

Methods: We developed multiple reagents or pipelines using commercially available options, and we employed multiple cytometric, proteomic, or genomic methods throughout multiple studies, demonstrating the power of optimized blood sampling for high-dimensional analyses of blood samples.

Results: To minimize the hands-on time for every sample collection, we developed protocols and reagents that allowed us to simplify sample handling at the point of blood collection to the liquid handling step followed by incubation (variable for each type of sample) and freezing, postponing the sample processing for months or even years and minimizing the time between blood drawing and sample storing. Some of the protocols were developed to improve cell isolation efficiency. As a result of the continuous development, we enabled a collection of a large number of samples within a short period of time or multidimensional analyses from minute volumes of blood.

Conclusions: Simplified blood sampling that enables sample collection directly at busy clinical sites such as delivery wards, ICU units, or in the field leads to large-scale reproducible sampling and allows the generation of reliable multidimensional data sets, representing a valuable resource for systems immunology. In addition, recently developed methods for painless blood sampling in combination with

simplified and efficient blood sample processing will allow large-scale analyses from minute blood samples, essential for the future of research, laboratory-based testing, and point-of-care.

PAR04-01

TARGETING CD123 FROM IMMUNOTHERAPY TO CART: BPDCN AND BEYOND

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CD123 is the interleukin-3 receptor alpha chain (IL-3Rα), a cell-surface protein that plays a crucial role in the growth and differentiation of hematopoietic cells. Its expression can be abnormal in certain blood disorders, including acute myeloid leukemia (AML), a subset of acute lymphoblastic leukemia (ALL), and blastic plasmocytoid dendritic cell neoplasm (BPDCN). It is believed to contribute to the growth advantage of leukemic cells. Notably, CD123 is highly expressed in malignant stem cells that can initiate and maintain disease but is minimally expressed in normal hematopoietic stem cells (HSCs). Therefore, targeting CD123 could be an effective way to eliminate malignant stem cells without harming normal HSCs. Currently, there are ongoing clinical trials using different approaches to target CD123 and also approved treatments aimed at targeting CD123 for BPDCN, highlighting the importance of this protein as a therapeutic target.

PAR04-02

CAR T CELL TREATMENT FOR CD19 POSITIVE MALIGNANCIES

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Chimeric antigen receptor T (CAR T) cells targeted against CD19 have revolutionised the treatment of B-cell malignancies with cure rates of 50-60% in relapsed B-acute lymphoblastic leukaemia and 40% in relapsed diffuse large B-cell lymphoma. CD19 CAR T cell treatment has now become standard of care in many countries and is gradually replacing conventional chemotherapy and being introduced earlier in the disease course. For those patients where CAR T cell treatment fails to achieve a cure there is increasing understanding of the mechanisms underlying treatment failure such as target antigen downregulation, lack of CAR persistence, impaired T-cell fitness, immunogenicity and the effects of the immunosuppressive tumour microenvironment. Strategies to overcome these mechanisms are being actively explored and include dual antigen targeting, developing CARs with improved co-stimulation, fully humanised CARs, improved CAR manufacturing processes, use of allogeneic CARs and combination treatment with immunomodulatory agents. Furthermore CAR T cells are now being used successfully to treat non CD19 expressing malignancies including multiple myeloma (target BCMA), T-ALL (target CD7) as well as solid tumours such as mesothelioma (target mesothelin) and neuroblastoma (target GD2). This talk will summarise the clinical data of the landmark CAR T cell trials and discuss some of the exciting new developments in this rapidly evolving field.

PAR05-01

IVDR AND FLOW CYTOMETRY: IMPACT ON ROUTINE DIAGNOSTICS AND CLINICAL TRIALS

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IVDR – or Regulation (EU) 2017/746 on in vitro diagnostic medical devices – really has become a buzzword in the medical laboratory, sometimes creating confusion and anxiety over the continued availability of in vitro diagnostic tests for laboratory professionals and hence, for patients. The field of clinical flow cytometry (FCM) is certainly one area where IVDR could prove to be disruptive. Indeed, all clinical FCM assays are now in scope of IVDR, and medical laboratories fear that they will no longer be free to deploy their own clinical expertise to build, validate and implement their FCM panels of choice. IVDR already impacts laboratory-developed or laboratory-modified FCM assays today, and more regulatory requirements are set to become applicable. Moreover, many manufacturers of commercial FCM reagents and kits are revising their claims to ensure compliance of their products with IVDR. Hence, medical laboratories must carefully explore how the revised intended purpose of these CE-marked products may impact their continued use. Whether FCM assays are used as part of a routine diagnostic work-up or as part of the laboratory testing in clinical trials, it is likely that many laboratories will shake up their FCM test menu in the coming years.

PAR05-02

ASSESSMENT OF IMMATURE PNH RBCS (IRBCS) BY FLOW CYTOMETRY: ASSAY VALIDATION FOLLOWING THE EU 2017/746-IVDR FOR IN-HOUSE DEVICES

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The assessment of iRBCs enables better separation of PNH RBCs type III and II, reflects the real size of the PNH RBCs clone and provides better correlation with values for PNH neutrophils and monocytes. The EU IVDR 2017/746 clearly defines the conditions for devices in-house to qualify for exemption of regulation rules (Article 5.5) including compliance with QMS and ISO 15189. We present our data for EU IVDR 2017/746 compatible iRBCs assay validation, based on current ISO 15189 and CLSI H62 recommendations, which could serve as a practical approach for qualitative and semi quantitative LDTs validation.

PAR06-01

BROADER EPSTEIN–BARR VIRUS–SPECIFIC T-CELL RECEPTOR REPERTOIRE IN PATIENTS WITH MULTIPLE SCLEROSIS

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Epstein–Barr virus (EBV) infection precedes multiple sclerosis (MS) pathology and cross-reactive antibodies might link EBV infection to CNS autoimmunity. As an altered anti-EBV T-cell reaction was suggested in MS, we queried peripheral blood T-cell receptor β chain (TCRβ) repertoires of 1,395 MS

patients, 887 controls, and 35 monozygotic, MS-discordant twin pairs for multimer-confirmed, viral antigen–specific TCRβ sequences. We detected more MHC-I–restricted EBV-specific TCRβ sequences in MS patients compared to controls, while no differences could be found for CMV-, influenza A- or SARS-Cov-2-specific TCRβ sequences. Differences in genetics or upbringing could be excluded by validation in monozygotic twin pairs discordant for MS. Anti–VLA-4 (natalizumab) treatment, which sequesters leukocytes in peripheral blood, led to a specific increase of EBV-specific sequences. In healthy individuals, EBV-specific CD8+ T cells were of an effector-memory phenotype in peripheral blood and cerebrospinal fluid. In MS patients, cerebrospinal fluid also contained EBV-specific central-memory CD8+ T-cells, suggesting recent priming. Therefore, MS is not only preceded by EBV infection, but also associated with broader EBV-specific TCR repertoires, consistent with an ongoing anti-EBV immune reaction in MS.

PAR06-02

DEFINING T CELLS POISED TO INFILTRATE THE BRAIN AND TRIGGER MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is the most common cause of non-traumatic neurological disability among young adults. Although the etiology is still unknown, $CD4^+$ T cells are considered one of the first immune cells that trigger MS by interacting with and crossing the blood-brain barrier (BBB). The aim of this part of our work is to pinpoint the functional program used by $CD4^+$ T cells to invade and trigger inflammation in the brain of people with MS. To achieve this, we not only use state-of-the-art technology including in-depth, T cell-focused (spectral) cytometry, single cell RNA-seq and *in vitro* BBB transmigration assays, but also compare people with MS who do or do not respond to immunomodulatory treatment and analyze paired human body compartments including blood, cerebrospinal fluid and postmortem brain tissues. Thus far, we found that a $CD4^+$ T-cell subset termed Th17.1 ($CCR6^+CXCR3^+CCR4^{-/dim}$; IL-$17^{low}$IFN-$\gamma^{high}$GM-$CSF^{high}$) is selectively associated with the early MS disease process based on the following observations: 1) the proportion of blood Th17.1 cells is reduced in people who rapidly develop an MS attack, 2) Th17.1 cells accumulate in the blood of people with MS who clinically respond to natalizumab (anti-VLA-4 Ab; which prevents entry into the brain), 3) Th17.1 cells predominate the cerebrospinal fluid of people with MS and not neurological controls, 4) brain-infiltrating Th17.1 cells display glucocorticoid-resistant features, which can be targeted *in vitro* by the use of vitamin D and female hormones, and 5) Th17.1 is the only $CD4^+$ T-cell subset that can spontaneously cross the BBB *in vitro*, likely because of its distinctive expression of effector molecules.

PAR07-01

TRANSLATING COMPUTATIONAL FLOW CYTOMETRY TO THE CLINIC

Sarah Bonte

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Machine learning models based on cytometry data can be of use in various clinical settings, such as automated diagnosis, prediction of therapy response and survival analysis. In this talk, several algorithms developed by our team will be discussed, and how we translate our computational research

to a clinical setting. It is important to note that machine learning models only work well with good quality input data. Therefore, our team has developed several algorithms for cytometry data preprocessing: PeacoQC evaluates the stability of each sample during the acquisition and removes low quality events, while CytoNorm is able to align samples across multiple batches, thereby correcting batch effects. In addition, we automatically remove margin events, debris and doublets in our preprocessing pipeline. As the amount of data collected and the number of markers measured in one panel keep increasing, computational analysis will provide an alternative for time-consuming and subjective manual analysis of cytometry data. We have developed FlowSOM, a clustering algorithm able to return high-quality clustering similar to manual gating. In collaboration with clinicians, we applied our computational preprocessing pipeline and downstream FlowSOM analysis already to aid in the diagnosis of myelodysplastic syndromes, automated diagnosis of primary immunodeficiencies and analysis of acute myeloid leukemia.

PAR07-02

MACHINE LEARNING FOR AUTOMATED FLOW-MRD ASSESSMENT

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Introduction: Acute leukemia is the most frequent cancer in children and adolescents. Despite continued progress, disease relapse remains the number one cause of treatment failure. Assessment of the therapy response via flow-cytometric (FCM) based quantification of minimal residual disease (MRD) is a fundamental diagnostic tool to tailor therapy for personalized treatment in individual patients in acute lymphoblastic (ALL) as well as in acute myeloid leukemia (AML). In particular, children with AML still face a need for highly toxic therapies due to a high relapse-propensity. Hence, there is a need for better risk assessment using quality assured MRD quantification. While sample preparation can be harmonized, data analysis and interpretation strongly rely on individual operator skills and experience. Hence, we aimed to develop an objective and automated analysis strategy based on machine learning with the expectation to be integrated into routine clinical practice.

Methods: We examine the transformer model originally developed for fully automated MRD detection in ALL flow cytometry samples and demonstrate its extension to emulate the manual FCM-MRD gating procedure. By generating interpretable predictions in form of automatically generated gates, this extended model brings us closer to its practical use in routine clinical practice. Our transformer models are lightweight with about 30000 trainable parameters which are trained using manually gated data derived from the clinical routine. Considering the constraints posed by the limited availability of training data and the diverse phenotypic variations in AML, we will also provide an overview of our ongoing research on enhancing data efficiency using graph representations.

Results: We address the challenge of data scarcity and explore strategies to optimize the model's performance for application in AML-MRD. A quantitative comparative analysis in terms of F1-scores shows that the enhancement with feature embedding by means of input graph representations improves classification performance on small data sets. The combination of newly introduced methodologies enables a 95% reduction in training dataset size while maintaining a competitive F1-score of 0.818 in ALL patients.

Conclusion: Our findings highlight the potential of automated MRD quantification in acute leukemia. While a quantitative study specifically focused on AML is still ongoing, the findings from our research with ALL data provide a valuable research direction for automated MRD detection in AML. Given the lower incidence of AML compared to ALL, the emphasis on label-efficiency becomes particularly significant in optimizing the use of available data.

PAR08-01

HARMONIZATION EFFORTS IN AML MRD

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Measurable residual disease (MRD) together with baseline genetics are the most important independent prognostic factors in patients with acute myeloid leukemia. The deployment of MRD testing is expanding in clinical practice to guide treatment choices including stem cell transplantation and subsequent interventions. Of the various technologies in use, multi-parameter flow cytometry (MFC) is arguably the most accessible and can be used to detect MRD by identification of abnormal leukemic immunophenotypes at a single cell level in over 90% of patients to a sensitivity of 10^{-3} to 10^{-4}. MFC was the MRD methodology applied in 6115 of the 11,151 patients in remission analysed by the recently published meta-analysis of AML MRD studies. Although MFC-MRD assays with their rapid real-time results have been remarkably successful in generating a wealth of MRD data for prognostic validation (clinical evidence), this benefit is offset by several technical, biological and analytical variables that may blunt both prognostic / predictive accuracy of the result and its intra- and inter laboratory comparability. MFC-MRD is currently classified as a semi-quantitative assay (in the absence of calibration using standard reference material). Harmonisation efforts to facilitate validation of LDTs for AML MFC-MRD include consideration of marker combinations, LOD/LOQ, and EQA. These will be discussed in this talk with recent developments.

PAR08-02

DEVELOPMENT OF FULL SPECTRUM FLOW CYTOMETRY ASSAYS FOR THE DETECTION OF MEASURABLE RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

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Multiparameter flow cytometry (MFC) has emerged as a standard method for quantifying measurable residual disease (MRD) in acute myeloid leukemia (AML). However, the limited number of available channels on conventional flow cytometers requires the division of a diagnostic sample into several tubes, restricting the number of cells and the complexity of immunophenotypes that can be analyzed. Full spectrum flow cytometers overcome this limitation by enabling the simultaneous use of up to 40 fluorescent markers. Consequently, several spectral panels for AML Flow-MRD have been proposed recently. However, the panels developed to date have too many colors and were mostly developed on and for non-IVD certified research instruments. With this in mind, we aimed to merge the multi-tube

MFC-MRD assay most widely used throughout Europe -including the HOVON (Netherlands), SAKK (Switzerland) and the German-Austrian AML study groups (AMLSG) into an 19-color single-tube MRD assay that complies with recommendations of the EuropeanLeukemiaNet MRD Working Party (ELN-DAVID). We based our assay on clinically-validated antibody clones and evaluated its performance on an IVD-certified full spectrum flow cytometer. We measured MRD and normal bone marrow samples and compared the MRD data to a widely used reference MRD-MFC panel generating highly concordant results. Using our newly developed single-tube panel, we established reference values in healthy bone marrow for 28 consensus leukemia-associated immunophenotypes and introduced a semi-automated dimensionality-reduction, clustering and cell type identification approach that aids the unbiased detection of aberrant cells. In summary, we provide a comprehensive full spectrum MRD-MFC workflow with the potential for rapid implementation for routine diagnostics due to reduced cell requirements, ease of data analysis and increased reproducibility in comparison to conventional Flow-MRD routines.

PAR09-01

NEUTROPHIL OXIDATIVE BURST CAPACITY AND NK CELL FUNCTION ASSAYS IN CELLULAR DIAGNOSTICS

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Defects in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex consisting of five proteins lead to chronic granulomatous disease (CGD). The dihydrorhodamine (DHR) 123 flow cytometry assay can be used as initial diagnostic test to screen for superoxide production. This test makes it possible to detect a defective or decreased respiratory burst in neutrophils. Abnormal results should be confirmed by mutation analysis of the CGD-associated genes. The most frequently (~70%) occurring deficiency in CGD is the X-linked form with mutations in the *CYBB* gene encoding for NOX2 (gp91-phox), mostly resulting in absence of the respiratory burst. The other autosomal recessive forms of CGD are caused by pathogenic variants in the genes *CYBA*, *NCF1, NCF2* and *NCF4*. Several samples have been used to validate the DHR 123 test. To interpret the results, it is important to know the performance of the functional assay used.

Other important functional assays are the NK cell assays testing the capacity of killing and degranulation (measuring CD107a expression) upon stimulation with target cells in the context of diagnosing hemophagocytic lymphohistiocytosis (HLH). Additionally, intracellular expression of perforin in NK cells and effector $CD8^+$ T cells and surface expression of SAP and XIAP on $CD8^+$ T cells are measured by flow cytometry. Because HLH is an acute and life-threatening syndrome of hyperinflammation, there is an urgent need of rapid testing. In HLH, NK cells and/or cytotoxic lymphocytes fail to eliminate activated macrophages. Defects in the granule-mediated cytotoxicity pathway should be confirmed by genetic analysis. Different results of NK cell function obtained in the last fifteen years will be presented.

PAR09-02

FUNCTIONAL STUDIES ON INBORN ERRORS OF INNATE IMMUNITY

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Inborn errors of immunity (IEI) are rare diseases caused by single gene defects that lead to a broad range of immunological manifestations, including severe infections, immune dysregulation, and autoinflammation. These IEI can be considered experiments of nature, as these genetic defects can aid in understanding the roles of specific genes in the immune system. The application of next-generation sequencing has revolutionized the discovery of IEI, although the majority of patients remain unsolved. In some cases, strong genetic candidate variants are identified that could be causative of the patient's immunological phenotype, but their functional effect remains inconclusive. Functional validation of these variants of unknown significance is therefore required to prove pathogenicity, as this can guide diagnosis and clinical decision making. We will discuss several IEI affecting innate immune cell function and their laboratory evaluation, including immunophenotypic and functional assays.

PAR10-01

PHENOTYPIC MYELOID ALTERATIONS IN MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS) AND IN DE NOVO MULTIPLE MYELOMA (MM) PATIENTS

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Introduction: Multiple myeloma is an incurable hematopoietic malignancy caused by uncontrolled proliferation of clonal plasma cells (PC) in the bone marrow (BM). It is always preceded by an asymptomatic premalignant Stage termed Monoclonal Gammopathy of Undetermined Significance. The progression from MGUS to Myeloma requires the establishment of a permissive bone marrow (BM) microenvironment. Maia et al. showed that a risk of developing myelodysplastic syndrome (MDS) is significantly increased in both MM and MGUS.

Methods: We used multidimensional flow cytometry (MFC) to prospectively study the presence of MDS-associated phenotypic alterations (MDS-PA) in the bone marrow of 105 patients with MM treated according protocols of the Intergroupe Francophone du Myélome study and of 113 patients with MGUS.

Results: At diagnosis, 15 (14,7%) of 105 myeloma cases displayed MDS-PA. 9/15 cases presented MDS-PA before the presence of morphological dysplasia. All these patients had an expanded Precursors CD34+CD117+ compartment, with the expression of phenotypic aberrations. All 15 patients had two or more MDS-PA in one or two myeloid cell lineages.

After myeloma treatment, 81/90 non MDS-PA patients had no alterations and 9/90 became MDS-PA detectable. 87% of non MDS PA patients are alive after four years of follow-up. MDS-PA persisted in 12/15 MDS-PA patients and became undetectable in three. 33,3% of MDS-PA patients are alive after 60 months. Xx evolved to AML. Regarding MGUS patients, **14/113** presented MDS-PA at the moment of

the first study to discard a MM. Of 113 cases, 95 could be followed for several years, including 5 with CD34+ compartment expansion and the 14 patients with MDS-PA. 14/19 evolved to overt myeloma.

Conclusions: Our study shows that the MDS-PA at diagnosis is associated with higher risk of myeloma evolution in MGUS and shorter survival. Identifying myeloid dysplasia at the time of myeloma diagnosis may be important for early detection of patients with shorter survival. The presence of the phenotypic alterations in MGUS can be a relevant marker to identify patients at higher risk of developing myeloma.

References

Maia et al. Biological and clinical significance of dysplastic hematopoiesis in patients with newly diagnosed multiple myeloma.
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PAR10-02

INTEGRATED FLOW CYTOMETRY AND SEQUENCING TO RECONSTRUCT EVOLUTIONARY PATTERNS FROM DYSPLASIA TO ACUTE MYELOID LEUKEMIA

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AML is the most common acute leukemia in adults and its prevalence significantly increases in the elderly. The 5-year survival rate for adults younger than 60 is around 40% and decreases to 10% in patients above this age. The study of the genetic landscape of residual mature dysplastic cells offers valuable insights into the evolutionary process from dysplasia to AML. However, dissecting leukemic transformation at the onset of AML is challenging without single-cell sequencing because of the dominant blast population, and most clinical laboratories do not have the infrastructure to perform single-cell studies routinely. Moreover, the assessment of MRD could be clinically relevant in elderly AML patients. The genomic characterization of treatment-resistant cells and phenotypically normal progenitors helps to understand the mechanisms of resistance. To address these hypotheses, flow cytometry was integrated with NGS and RNAseq data from isolated cell types.

Targeted sequencing of dysplastic cells and blasts uncovered three evolutionary patterns of leukemogenesis: stable transition, branching, and clonal evolution. The high throughput of WES detected mutations only present in dysplastic cells, mutations shared between these cells and blasts of diagnosis (absent in MRD), and only with the resistant cells (absent in diagnosis blasts). Clinically, MRD status in patients achieving CR had independent prognostic factors for CIR. The phenotypically normal progenitors of CR_{MRD-} patients had mutations and copy-number abnormalities consistent with genetic MRD. While elderly AML patients have similarly dire survival regardless of achieving PR or CR with persistent MRD, the former appears to be characterized by primary resistance, whereas CR with persistent MRD is associated with the emergence of molecular traits of acquired resistance. These findings uncover that genetic alterations that are critical in leukemic transformation and chemoresistance, may not overlap. The possibility of identifying the genetic drivers in AML pathobiology could be clinically meaningful to develop tailored treatment strategies aiming at the eradication of genetically diverse leukemic clones.

PAR11-01

STANDARDIZED FLOWCYTOMETRIC MRD ANALYSIS IN BCP-ALL PATIENTS

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Presence of minimal residual disease (MRD) in patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is of strong prognostic significance and used to adjust therapy. One of the methods to measure MRD is flowcytometric immunophenotyping, based on the fact that leukemic cells show an aberrant immunophenotype that allows distinguishing them from normal B-cells.

A diagnostic flowcytometric MRD assay should be sufficiently specific, sensitive (at least 0,001%) and reproducible. Within EuroFlow, a standardized antibody panel was designed, allowing separation of normal B-cells and BCP-ALL cells in >98% of cases. In addition, a bulk-lyse method was adapted to allow acquisition of at least 4 million cells, required to reach a Lower Limit of Quantitation of at least 0,001%. This standardized assay was mainly validated on patients receiving chemotherapy; treatment of BCP-ALL patients with more novel targeted therapies (e.g. targeting CD19) may however impact the applicability and/or reliability of the assay. Therefore, alternative gating strategies, independent of CD19, were successfully designed for patients treated with these targeted therapies. In addition, distinguishing normal and malignant B-cells can be challenging and more automated approaches may provide more objective data. Using the Automated Gating and Identification tool and a database consisting of normal bone marrow samples stained and processed according to the EuroFlow protocols, semi-automated analysis of te data files can be performed. This approach resulted in appropriate MRD results in both chemotherapy and targeted therapy treated patients. Finally, the 8-color EuroFlow panels are being adapted to 12-color tubes to allow inclusion of additional markers, in order to improve MRD analysis in BCP-ALL patients treated with targeted therapies.

Standardization of flowcytometric MRD assays should facilitate the analysis of MRD in BCP-ALL patients and future efforts should focus on further automated processing of samples and automated analysis of the data.

PAR11-02

CHALLENGES IN MRD ANALYSIS IN T-ALL BY FLOW CYTOMETRY

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Flow cytometry (FC) has become a well-established technique of measurable residual disease (MRD) detection in acute lymphoblastic leukemia (ALL) patients. MRD is defined as low numbers of leukemic cells remaining after treatment, detectable in peripheral blood (PB) or bone marrow (BM) of patients. The level of MRD reflects patient response to treatment and potential risk of leukemia relapse. Information on actual level of MRD at defined time points is used to make key clinical decisions including treatment stratification with modification of intensity and possibly duration of chemotherapy. Importantly, currently employed treatment regimens require MRD to be evaluated with at least 10^{-4} or higher sensitivity levels, which at least in part of the samples can be technically challenging.

In minority of ALL cases (10-15%), leukemic cells originate from T-lineage committed lymphoid progenitor cell, resulting in T-ALL. Usually the malignant transformation takes place in the thymus and

leukemic cells are secondarily disseminated into BM and PB. Typically, blasts in T-ALL can be recognized by the expression of immaturity markers such as CD34, terminal deoxynucleotidyl transferase, in combination with cytoplasmic CD3, usually without or partial expression of this molecule on the surface membrane, CD10, CD99 and/or CD1a. Other typical T-cell markers, such as CD2, CD3, CD4, CD5, CD7, CD8 and CD45 are expressed on both immature and mature T cells. Even though T-ALL blasts can differ in expression level of these markers at diagnosis, as compared to mature T-cells, the stability of these markers during treatment is variable, making them insufficient for MRD monitoring. This is why alternative markers of T-ALL blasts are highly desirable in the field and seeking of them became one of the most important goals for many scientific groups. One of such extended multicenter studies was performed within the EuroFlow Consortium and included more than ten new markers in 12-color setting. Based on current EuroFlow experience, still, it might be difficult to design a single 12-color tube that might be applicable for MRD detection in all T-ALL patients.

MRD monitoring in T-ALL can also be challenging because of the need of intracellular staining. This can bring some troubles in data interpretation and produce both false positive and false negative results. It is also important to choose appropriate clones of monoclonal antibodies for intracellular (e.g., CD3) staining, assuring proper epitope recognition.

PAR12-01

CYTOMETRIC PROFILING OF AUTOANTIGEN-SPECIFIC T CELLS IN HUMAN AUTOIMMUNE DISEASE

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Introduction: Antigen-specific CD4+ T helper (Th) cells orchestrate adaptive immunity including protective responses against infections or tumors but also pathogenic responses driving chronic inflammatory diseases such as allergies or autoimmune diseases. Therefore, their direct identification and quantitative and qualitative characterization is essential to better understand the cellular basis of immune protection and pathology and to develop specific diagnostic and therapeutic targeting strategies. However, antigen-specific CD4+ T cells are rare, ranging from about 1% e.g. in acute viral infections to as little as 1-10/10e6 CD4 T cells in autoimmunity. Thus, their detection and functional characterization requires highly specific and sensitive technologies.

Methods: We employed antigen-reactive T cell enrichment (ARTE) for their in-depth characterization. ARTE relies on detection of CD154 and CD137 upregulation by conventional (Tcon) and Foxp3+ regulatory T cells (Tregs), respectively, upon short-term (7h) *in vitro* stimulation with antigen. Subsequent magnetic enrichment allows detection of rare reactive T cells and their in-depth characterization using multiparameter flow cytometry, single cell “omics” or functional assays. Here we utilized ARTE for the direct ex vivo characterization of autoreactive T cells in patients with neuromyelitis optica spectrum disorder (NMOSD), a prototypic autoimmune disease targeting aquaporin-4 (AQP4).

Results: *Ex vivo* single cell phenotypic, transcriptional, and functional characterization identified low frequencies (10e-5-10e-4) of clonally expanded, high avidity AQP4-specific effector memory-type T cells targeting immunodominant peptides. Unexpectedly, proliferation as well as pro-inflammatory T cell cytokines and effector T cell lineage defining transcription factors were shut off or downregulated, while co-inhibitory receptors and an exhaustion-associated transcriptional program were increased. AQP4-specific effector T cells expressing the canonical Treg transcription factor Foxp3 marked a pronounced exhausted state along a developmental trajectory. Blocked proliferation and cytokine

production was partially relieved by checkpoint inhibition, unmasking potent autoantigen-specific and CD154/IL-21-dependent B cell helper activity.

Conclusion: Thus, the direct ex vivo characterization of autoreactive T cells identified a novel molecular exhausted state of autoreactive effector CD4+ T cells in human autoimmune disease. Exhausted cells preserved basic functionality under conditions of chronic antigen stimulation reminiscent of cancer or chronic infection. This suggests common mechanisms of human CD4+ T cell adaptation to chronic (self-)stimulation, which will significantly impact on strategies for therapeutic targeting of autoreactive T cells in human autoimmunity and beyond.

PAR12-02

DIRECT ISOLATION OF DISEASE-DRIVING T CELLS IN CELIAC DISEASE WITHOUT HLA MULTIMERS

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Characterization of disease-specific T cells and their cognate antigens may enable better diagnosis and more targeted treatments in autoimmune disorders. Peptide-major histocompatibility complex (pMHC) multimers and flow cytometry have been the gold standard for direct detection of antigen-specific CD8+ and CD4+ T cells. We have showed that celiac disease, a chronic enteropathy with a strong HLA-class II association and autoimmune components driven by dietary gluten, can be diagnosed with high sensitivity and specificity though detection of gluten-specific CD4+ T cells in blood using gluten-HLA-class II multimers.

By combining MHC-class II multimers with cytometry by time of flight (CyTOF), which allows far more comprehensive profiling of the gluten-specific CD4+ T-cell response in blood and gut tissue, we found that such cells have a distinct phenotype associated with multiple functions, including T-cell help to B cells. The cell phenotype rapidly shifted with gluten withdrawal and re-introduction in the diet. We confirmed the findings with RNA sequencing.

CD4+ T cells with highly similar phenotype are increased across multiple autoimmune conditions without knowledge about the disease-driving T-cell antigens. Thus, we asked whether gluten-specific CD4+ T cells may be isolated without HLA multimers, i.e. solely based on the particular cell phenotype. Indeed, we demonstrated that disease-driving CD4+ T cells in celiac disease can be isolated in high frequencies from inflamed tissue using a simple 11-color flow panel. We based the sorting panel on our previous CyTOF results and we sorted the cells without using antigen-stimulation or HLA multimers. Our findings on gluten-specific CD4+ T cells are highly relevant to the many efforts to detect disease-driving T cells and antigens in celiac disease and other autoimmune conditions. Furthermore, detection of glute-specific T cells in celiac disease is proof of concept for T-cell based diagnosis in autoimmune disease.

PAR13-01

DEVELOPMENTS IN DIAGNOSTICS TO MONITOR PNH CLONES AND COMPLEMENT ACTIVATION

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, life threatening disease characterized by thrombosis and bone marrow failure. Furthermore, patients suffer from complement mediated intravascular hemolysis due to the partial or absolute absence of GPI-anchored proteins such as the complement-defense antigens Decay Accelerating Factor (DAF, recognized by CD55) and the Membrane Inhibitor of Reactive Lysis or Protectin (MIRL, recognized by CD59) on erythrocytes. As a result, the terminal complement cascade from factor C5 to C9 is not blocked and erythrocytes can be eliminated.

Therapeutic treatment of PNH is mainly limited to an anti-C5 antibody, Eculizumab, resulting in effective reduction of intravascular hemolysis. However, 25 – 50 % of patients remains transfusion dependent due to complement C3-mediated extravascular hemolysis by the proximal complement cascade starting with C3b. Spliced products of C3b, the C3d fragments, can bind on erythrocytes in PNH patients that are resistant for terminal complement cascade inhibitor treatment. This binding of erythrocytes results in recognition by complement receptors on phagocytes leading to phagocytosis of these erythrocytes in the liver and spleen. Recently, clinical trials are initiated to investigate the improvements by new proximal complement inhibitors with or without the terminal inhibitors.

In these trials determination of PNH-clones in erythrocytes and white blood cells can be efficiently performed by multi-parameter flow cytometry using an advanced gating protocol and panels containing MoAbs to select monocytes (CD45 and CD64), granulocytes (CD45 and CD15) and erythrocytes (CD235), combined with MoAbs to determine the absence of GPI-antigens on the PNH targets within these populations (resp. CD14/FLAER, CD24/FLAER or CD157/FLAER and CD59). Furthermore, a method was developed to determine C3d on erythrocytes using a panel of an anti-C3d MoAb in combination with CD235 to select mature erythrocytes and CD59 to recognize the PNH clone, to predict the chance of extravascular hemolysis.

By using these flow cytometry assays, the reduction of PNH erythrocytes by intravascular (terminal complement cascade) or extravascular (proximal complement cascade) hemolysis can be efficiently monitored.

PAR13-02

MODERN TREATMENT APPROACHES TO PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) TREATMENT

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disease characterized by complement mediated intravascular hemolysis (IVH), thrombosis, and associated bone marrow failures. It is due to a somatic mutation of PIG-A gene in hematopoietic stem cells, impairing the production of glycosyl-phosphatidyl-inositol (GPI)-anchor molecule, and resulting in the loss of the natural complement inhibitory molecules CD55 and CD59 at the cell surface. Complement inhibition with the anti-C5 monoclonal antibody eculizumab revolutionized PNH treatment, reducing the most feared thrombotic risk and

improving (nearly normalizing) survival. Several drawbacks of eculizumab included the route and schedule of administration (i.e. intravenous every 14 days), the risk of hemolytic exacerbations before the next dose (pharmacokinetic breakthrough hemolysis, PK-BTH), and residual anemia in about 2/3 of patients with transfusion need in some of them. The first two unmet needs were partly resolved by ravulizumab, a long-half-life analogue of eculizumab, administered every 8 weeks and able to exert a stable control of hemolysis abolishing PK-BTH. Additionally, the anti-C5 crovalimab, administered subcutaneously every 4 weeks, improved patient convenience and is active on C5 polymorphism not responding to eculizumab. As regards residual anemia, a role of iatrogenic extravascular hemolysis (EVH), due to C3 accumulation on erythrocytes after C5-blockade, has been demonstrated. C3-coated PNH-erythrocytes are in fact phagocyted in the reticuloendothelial system with consequent chronic anemia, unconjugated bilirubin increase, and direct Coombs test positive for C3. The development of "proximal" complement inhibitors was aimed at resolving EVH in suboptimal responders to anti-C5 and, further, to control IVH and prevent EVH in naïve PNH patients. The first to be studied is the anti-C3 pegcetacoplan, which is administered subcutaneously twice a week. In the phase 3 trial the drug was able to improve Hb and abolish transfusion need in a large proportion of suboptimal responders to eculizumab. Pegcetacoplan is now approved for all PNH patients in USA and for suboptimal responders after 3-month anti-C5 therapy in Europe. Similarly, oral small molecules inhibiting factor-B (iptacopan) and factor-D (danicopan) of the alternative complement pathway showed impressive results in early trials, and phase 3 trials are ongoing. The short half-life of these drugs, with the need of frequent self-administration, poses several questions regarding patient compliance. Additionally, the risk of "pharmacodynamic" (PD)-BTH due to over-activation of complement during infections, traumas, surgery, etc., warnings close monitoring and need of common management and prevention strategies. Individualizing treatment on disease/patient features will be the next step for PNH "modern" management.

PAR14-01

NEW FLOW-CYTOMETRY APPROACHES FOR DIAGNOSIS (OF CLONALITY), CLASSIFICATION AND MONITORING OF T-CELL CLPD

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Diagnosis of T-cell chronic lymphoproliferative disorders (T-CLPD) is often challenging, due to the lack of fast and reproducible routine diagnostic assays for T-cell clonality. Recently, a single antibody against one of the T-cell receptor β-chain constant domains (TRBC1) has been claimed as a useful marker to assess Tαβ-cell clonality by flow-cytometry (FCM). We aimed to optimize the FCM method for routine use of anti-TRBC1 to assess T-cell clonality in a fast and robust manner, and to validate it in a large series of normal/pathological samples. We showed that TRBC1 labeling significantly improved in the presence of CD3, the best resolution to accurately identify TRBC1+ cells being achieved by adding the CD3 antibody either simultaneously or after TRBC1. We also provide $TRBC1^{+}/TRBC1^{-}$ ratios within the different Tαβ-cell subsets, as reference for polyclonal cells, among which a bimodal pattern of TRBC1-expression profile was found for all TCRVβ families. Surprisingly, highly variable and more disperse $TRBC1^{+}/TRBC1^{-}$ ratios were observed in more mature (effector memory, early effector and terminal effector cells) vs. Tαβ-cell subsets at earlier maturation stages.

Then we validated the optimized approach in 117 samples from both healthy/reactive conditions and patients with T-CLPD and detected monoclonal Tαβ-cell populations with high specificity (112/117;

96%). Further, we showed a high analytical sensitivity/level of detection (≤10^{-4}) when clonal T-cells exhibited immunophenotypic aberrancies. Additionally, we also validated the TRBC1-FCM assay for the diagnosis of T-cell clonality of T-large granular lymphocyte leukemia (T-LGLL), taken into consideration that they derive from effector cells, and showed that it is also valid for detecting T-cell clonality in T-LGLL.

In summary, our results support implementation of the optimized TRBC1-FCM approach as a fast, simple, cost-effective and accurate method for assessing T-cell clonality, showing a high specificity and sensitivity for detection of monoclonal Tαβ-cells in patients suspicious of T-CLPD, including those with T-LGLL. Therefore, appropriate integration of the TRBC1/CD3 reagents into comprehensive lymphocyte screening panels for the diagnostic work-up of patients presenting with lymphocytosis is strongly recommended, as well as into the current T-CLPD classification and measurable/minimal residual disease (MRD) monitoring panels.

PAR14-02

B-LPD, DIAGNOSIS BEYOND TYPICAL IMMUNOPHENOTYPIC FINGERPRINT ENTITIES

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The immunophenotype is a key element to classify B-cell Lymphoproliferative Diseases (B-LPD). However, in B-cell non-Hodgkin's Lymphomas (B-NHL), although the use of multiparametric flow cytometry (FC) can bring several advantages, the gold standard for diagnosis remains immunohistochemistry. Few FC laboratories can rely on a long-standing practical experience on tissue cell suspension, and the literature in support is still limited; as a result, the use of FC is generally restricted to the analysis of B-LPD with bone marrow or peripheral blood involvement.

Previously, we demonstrated that Artificial Intelligence (AI), applied to a wide series of mature B-LPD, allows us to define homogeneous groups of neoplasms characterized by the expression of surface markers.

Using additional intracellular markers on a series of 615 tissue samples from B-NHL patients, whose diagnosis, grouped in 8 main categories, were confirmed by histological analysis, and applying a more articulated system of AI analysis, we investigated whether we could obtain an AI based classification and estimate which markers, among the 30 used for immunophenotyping, are more specific to differentiate lymphoma groups.

Leveraging the Predictive Power Score, we evaluated the performance of each individual marker in predicting lymphoma categories, identifying 10 markers significantly correlated with the diagnosis. These markers were further validated by combining them in a classification tree. Here, each marker is analyzed in combination with all the others leading to a structural relationship tree that separates the entire database in quasi-homogeneous groups of lymphomas. Finally, using the Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction technique, we observed that the 8 lymphoma categories were substantially grouped and separated in clusters. Ten or less markers seem to be sufficient to achieve an adequate classification capability. Nevertheless, the use of all markers increases the ability of UMAP to separate different entities. In conclusion, it is conceivable that the implementation of AI applied to multiparametric FC, combining well-established diagnostic markers (CD5,CD10,CD23,CD20) with intracellular (MIB1,Bcl-6,IRF4) and unconventional markers

(CD6,CD31,CD44,CD81,CD305), could contribute significantly to an optimal diagnostic process in B-NHL.

Finally, we also demonstrated that, in Follicular Lymphoma (FL), three markers correlate with time to post-therapy relapse: the proliferative index MIB1, the cell surface adhesion receptor CD44, that interacting with the extracellular matrix promotes metastatic process, and CD72, a positive regulator of B lymphocyte functions in autoimmune diseases, whose overexpression in FL could likely sustain increased proliferation and confer resistance to immunochemotherapy agents.

PAR15-01

IMMUNOLOGICAL DIFFERENCES BETWEEN HEART AND KIDNEY-TRANSPLANTED CHILDREN: A CROSS-SECTIONAL STUDY.

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Introduction: Our institution faced an increased number of post-transplant lymphoproliferative lymphoma (PTLD) among heart transplanted children. As the immune system has a crucial influence on the susceptibility to develop lymphoma, we decided to investigate the immune system in heart transplanted children.

Methods: A prospective case control, cross section study for one year was performed. 36 children who had underwent heart transplantation were compared to 34 age- and sex-matched kidney transplanted children and 33 healthy age and sex-matched subjects. Subpopulations of lymphocytes in blood were determined by flow cytometry. The analysis included T-cells, and subtypes, B-cells, monocytes, recent thymic emigrants and T-cell receptor excision circles (TREC).

Results: Heart transplanted children had lower levels of TREC, T-cells, all subpopulations of naive T-cells, recent thymic emigrants, mononuclear cells, and B-cells than the healthy control group ($p < 0.05$) and kidney transplanted children ($p < 0.05$). The immunosuppressive regimen at kidney transplantation in children comprise the same drugs but at slightly lower doses compared to heart transplantation. However, kidney transplanted children had higher levels of T-cells, subpopulations of T-cells, naive cytotoxic T-cells, lymphocytes, mononuclear cells and B-cells compared to the healthy control group ($p < 0.05$). Within the T-cell population heart transplanted children had lower proportions of naive T-cells, and recent thymic emigrants but higher proportions of T-follicular helper cells and effector T-cells compared to the healthy control group and kidney transplanted children ($p < 0.05$)

Conclusion: Heart transplanted children had significantly lower T-cells, B-cells and TREC than the healthy control group and kidney transplanted children. In contrary, kidney transplanted children had higher levels of T-cells despite the immune suppression. Therefore, it is plausible that the immune dysfunction after heart transplantation has other explanations than the immune suppressive treatment.

PAR15-02

DIRECT DETECTION OF SINGLE EXTRACELLULAR VESICLES IN KIDNEY TRANSPLANTATION

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Introduction: Extracellular Vesicles (EVs) represent stable, tissue specific submicron-sized particles that reflect the conditional state of their tissue of origin. To identify, quantitate and characterize single EVs ≤400-nm in diameter *without* prior isolation, we developed a calibrated imaging flow cytometry (IFCM)-based assay. To assess the applicability of this assay in clinically relevant, molecularly complex samples, we measure allograft-derived EVs both before (released during Normothermic Machine Perfusion (NMP)), and after kidney transplantation (in recipient plasma samples).

Materials: Perfusate samples of discarded kidneys (n=8) were stained with general EV markers (tetraspanins CD9/CD63/CD81). EDTA blood samples from kidney transplant donors (HLA-A2+, n=21) and recipients (HLA-A2-, n=33) were collected before transplantation as well as after transplantation (both during stable allograft function as well as allograft dysfunction). Platelet-poor plasma was stained with a donor-specific HLA antibody (HLA-A2) in combination with the common EV marker CD9. All samples were measured using calibrated IFCM.

Results: EV release during NMP occurred largely during the first 3 hours (~740 fold increase compared to baseline perfusion fluid). Different EV subsets were detected based on their tetraspanin profiling, with the majority (>75%) of released EVs being CD81+. Analysis of EV subset concentrations showed correlations with well-established clinical parameters indicative for kidney quality before transplantation such as cold ischemia time and donor age. Based on HLA phenotype differences, donor-derived EVs (dd-EVs) can be detected directly in recipients' circulation if their fraction exceeds ~1% above the recipient-specific background. After transplantation, dd-EVs were present at higher concentrations in recipients with stable allograft function than in recipients with allograft dysfunction.

Conclusion: Our calibrated IFCM-based assay facilitates the direct detection of single EVs and identification of EV subsets in molecularly complex samples as demonstrated in 1) perfusion fluids (containing EVs originating from multiple cell types/single organ), and 2) human plasma (containing EVs from multiple cell types/organs). This assay allows researchers to study EVs in diverse biofluids, and expand on the usage of EVs as non-invasive biomarkers in a plethora of clinical settings.

POSTERS

Posters have been clustered according to the topic:

- Hematology-Oncology (HEM)
- Immunology (IMM)
- Novel applications in cytometry (NOV)
- Other (OTH)

HEMATOLOGY-ONCOLOGY (HEM)

HEM-01

PREDICTION OF VENETOCLAX RESISTANCE BEFORE THE THERAPY BY FLOW CYTOMETRY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Venetoclax, a Bcl-2 inhibitor, has a high efficacy in the treatment of chronic lymphocytic leukemia (CLL), however in certain situations, venetoclax resistance develops. It is well known that the microenvironment plays a significant role in the development of drug resistance. In this investigation, we compared the immunophenotype of venetoclax sensitive and resistant cases, focusing on markers that interact with the microenvironment, to determine which surface protein pattern may indicate the resistance.

Methods: We analysed the immunophenotype of CLL cells collected from peripheral blood of venetoclax resistance (VR)(n=6) and sensitive (VS)(n=13) patients. The levels of surface proteins were assessed at the start of therapy and at the time of resistance. We determined the level of adhesion molecules (CD49d, CD44), activation markers (CD27, CD69, CD20, CD38), co-stimulator molecules (CD40, CD86) and chemokine and Wnt5a receptors (CXCR4, CXCR5, ROR1), which are critical in the pathogenesis of CLL, by multicolor flow cytometry.

Results: Comparing the immunophenotype of VS and VR cases prior to therapy, we found that expression of CD44, CD27, CD69, CD20, CD40 and CD86 were significantly higher, whereas CXCR4, CXCR5 levels were lower. By examining the immunophenotype of VR cases before the treatment and at the time of resistance, we detected increased expression of CD38, CD69, CD40 and CD86, moreover decreased expression of CXCR4. The proportion of the proliferative fraction ($CD5^{bright}$, $CXCR4^{low}$, $CD86^{pos}$) among CLL cells did not vary at the start of the therapy between VR and VS cases but at the onset of resistance, this fraction increased.

Conclusions: Our findings indicate that phenotype of CLL cases, which develop venetoclax resistance is more activated and aggressive compared to sensitive cases. Based on these findings, we can predict at the start of the therapy which case may develop venetoclax resistance. We hypothesise that CLL cells with immunophenotype showed by resistant cases may migrate more often to lymphoid tissues, where the protective microenvironment may mitigate the effect of venetoclax monotherapy.

HEM-02

FLOW CYTOMETRIC CHARACTERIZATION OF B-CELL SUBSETS ENABLES THE DIAGNOSIS OF B-CELL LYMPHOMAS IN BONE MARROW AND PERIPHERAL BLOOD SAMPLES

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Introduction: B-cell subpopulations can be defined by immunophenotypic analysis according to the differentiation and maturation of B-cells. Most published studies are focusing on analysing B-cell subpopulations in peripheral blood, mostly their role in autoimmune diseases. Only limited studies describing B-cell subpopulations in bone marrow samples and very few include cases of lymphomas. The aim of the study was to define immunophenotypic features of B-cell subpopulations according to their differentiation in reactive lymphocytic proliferations (RLP) and B-cell lymphoma (BCL).

Methods: We analysed 28 bone marrow aspirations and 3 peripheral blood samples. To describe the subsets of B-cells in this study we used classification method based on IgD/CD27 determination by 6-color flow cytometry. Comparison of B-cell subpopulations in RLP and BCL was done. IgD index was calculated as the ratio of mean channel fluorescence in CD19 positive to that of CD19 negative cells. The study was approved by The National Medical Ethics Committee of the Republic of Slovenia (109/02/14).

Results: Among 31 of our samples, in 11 (35%) final diagnosis was BCL (2 diffuse large B-cell lymphomas, 3 chronic lymphocytic leukemias, 2 mantle cell lymphomas, 1 marginal zone lymphomas (MZL), and 3 B-cell lymphomas not otherwise specified) and 20 (65%) RLP. Five different mature CD19 positive B-cell subpopulations were found in RLP samples. BCL samples differed from RLP in four characteristics: a) at least one of the B-cell subpopulations observed in RLP was lost, b) expression of the analysed antigens was different than in RLP, c) lymphoma cells had immunophenothypic features of one or two of B-cell subpopulation found in RLP, d) the IgD index in RLP was significantly higher than in BCL.

Conclusions: Presented study showed that immunophenotypic features of B-cell subpopulations in BCL and RLP are different which enables more reliable BCL diagnosis based on flow cytometric IgD/CD27 classification epically in equivocal cases.

HEM-03

EXPRESSION OF IMMUNE CHECKPOINT ON TUMOR AND IMMUNE CELLS IN DIFFUSE LARGE B CELL LYMPHOMA

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Introduction: Diffuse large B-cell lymphoma (DLBCL) is an aggressive malignancy and is the most common type of malignant lymphoid neoplasm. Its heterogeneity leads to a situation where approximately 35% of DLBCL cases do not respond to the conventional treatment these patients experience relapsed or refractory disease. Therefore, it is critical to identify additional biomarkers and new therapeutic targets for DLBCL. The programmed cell death 1 (PD-1)/programmed cell death ligand 1(PD-L1) pathway is an immune checkpoint pathway that plays an important role in the maintenance of self-tolerance and the control of excessive immune responses.

Methods: We include 37 patients with DLBCL diagnosed from May 2019 to August 2022. We use samples of fine needle aspiration and lymph node excision. From cytological sample we also prepare a cell block. In all cases flow cytometry (FC) and immunohistochemical (IHC) staining (on excision and cell block) was done to determine PD-1 and PD-L1 positive tumor and immune cells.

Results: Comparison of results FC and IHC showed good concordance. We observed that ABC subtype showed higher PD-1 and PD-L1 expression on tumor cells. On immune cells was higher expression PD-1 and PD-L1 on GCB subtype.

Conclusions: Determining PD-1 and PD-L1 with FC provides comparable results to IHC. This can make the process of obtaining samples for determining PD-1 and PD-L1 easier and can help us identify candidates for immune therapy.

HEM-04

SURPRISING DISCRIMINATING POWER OF INTRACELLULAR MARKERS DETECTED BY FLOW CYTOMETRY IN LYMPHOMA DIAGNOSIS UNVEILED BY ARTIFICIAL INTELLIGENCE TECHNIQUES

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Introduction: In a previous study, we demonstrated that Artificial Intelligence (AI), applied to a wide case series of mature B-cells Leukemia and Lymphomas (B-NHL), allows us to define homogeneous groups of neoplasms characterized by the expression of one or more surface markers. Using additional intracellular markers on a series of tissue samples from B-NHL patients, and applying a more articulated system of analysis, we investigated whether we could obtain an AI based classification and estimate which markers are more specific to differentiate Lymphoma groups.

Methods: We collected phenotypic analysis of 615 biopsy samples, whose diagnoses, all confirmed by histological analysis, were grouped in 8 major categories of B-NHL. Leveraging the Predictive Power Score, we evaluated the predictive performance of each individual marker against all lymphoma categories. Among all, we identified 10 markers strongly correlated with the diagnosis. We further validated the role of these markers by combining them in a classification tree. Here, each marker is analyzed in combination with all the others leading to a structural relationship tree that separates the entire database in quasi-homogeneous groups of lymphomas. Finally, using the Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction technique, we observed that the 8 lymphoma categories were substantially grouped and separated in clusters.

Results: The results obtained demonstrate how the use of surface and intracellular markers allows us to define the major categories of B-NHL with a high degree of accuracy. Ten or less markers seem to be sufficient to achieve an adequate classification capability. Nevertheless, a greater number of markers, combining intracellular with unconventional markers (CD305, CD81), increases the ability of UMAP to separate different entities.

Conclusions: It is conceivable that the implementation of AI applied to multiparametric flow cytometry (MFC) could contribute significantly to an optimal diagnostic process in B-NHL, where histopathological examination remains the gold standard. It is still being investigated whether the use of these methods with a large number of markers can also be predictive of categories of neoplasms carrying molecular

or genetic alterations, which would be useful for a better classification even for therapeutic purposes of B-NHL.

HEM-05

CD371 EXPRESSION IN B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA AND ITS CORRELATION WITH THE GENETIC ABNORMALITIES

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Introduction: CD371 (CLL-1) is a cell surface antigen expressed by myeloid cells and is currently being targeted for immunotherapy. The overexpression of CD371 has been shown to be associated with DUX4 rearranged B-acute lymphoblastic leukemia (B-ALL) among patients with B-ALL not otherwise specified (B-ALL-NOS). However, data on the frequency of its expression in all B-ALL and its expression pattern in patients with disease-defining recurrent genetic abnormalities is limited. With the advent of upcoming antiCD371-immunotherapy, we aimed to assess the expression of CD371 in patients of B-ALL and its correlation with underlying genetic abnormalities.

Methods: Expression of CD371 (fluorochrome-PE, clone- 50C1) was evaluated in consecutive B-ALL patients from January 2020 to December 2021 using 12-color MFC immunophenotyping on DxFLEX flow-cytometer. Data was analysed using Kaluza-softwareV2.1. CD371-expression was correlated with demographic details, cytogenetics using FISH, and molecular genetics data using RNA sequencing including DUX4 rearrangement.

Results: CD371 was assessed in 690 B-ALL patients (412 paediatric, age 0-14 years and 278 adult-B-ALL, age more than 14 years). CD371 was positive in 64 patients (9.28%) of B-ALL. Incidence of CD371 expressing B-ALL was higher in paediatric patients (59.3%, n-38). Median-age of CD371 positive B-ALL was 11.5 years (range: 0.4-61 years) which was slightly higher compared to the CD371 negative patients (median age- 10 years, range: 0.5-78 years). Cytogenetic and RNA-sequencing data was available in 673/690 and 484/690 patients respectively. Of 484 patients with RNA-sequencing data, DUX4 rearrangement was detected in 14/484 (2.9%) patients. CD371 was positive in 12 of these 14 patients (85.7%, p-value <0.0001). CD371 was also detected in patients with other genetic abnormalities including 3/20 (15%) B-ALL with KMT2A-rearrangement (p-value 0.63), 7/99 (7.1%) B-ALL with BCR::ABL1 (p-value 0.61) and 1/180 (0.5%) patients of B-ALL with hyperdiploidy. CD371 was also positive in 19.5% patients of B-ALL-NOS, where no disease-defining genetic abnormality could be detected. We did not study DUX4-gene expression. Co-expression of CD371 and CD2 was noted in 5/14 patients (35%) with DUX4-rearrangement (p-value <0.0001).

Conclusions: Aberrant expression of CD371 was seen in 9.28% B-ALL patients with slightly higher incidence in paediatric B-ALL. It was significantly associated with DUX4 rearranged B-ALL. However, it was also positive in other genetic abnormalities including KMT2A rearrangement and BCR::ABL1. Thus, in the absence of routinely studied recurrent genetic abnormalities such as KMT2A rearrangement and BCR::ABL1, CD371 expression strongly suggests DUX4-rearrangement.

HEM-06

FLOW CYTOMETRIC STANDARDIZATION OF MUTANT NPM1 AND ITS UTILITY IN RAPID DETECTION OF NPM1 MUTATED AML AND MRD ASSESSMENT

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Introduction: Nucleophosmin1 (*NPM1)* mutated AML is a distinct subtype of AML with favourable prognosis contributing upto 35% of adult AML and upto 60% of AML with normal karyotype. Its rapid detection provides valuable clinical information. As multicolour flow cytometry (MFC) provides rapid results, we standardized mutant NPM1-protein expression (mNPM1) by MFC. Further, MRD study in NPM1+AML is challenging by conventional MFC-based minimal residual disease (cMFC-MRD) assay. Hence, we also evaluated its utility in the MRD assessment.

Methods: Anti-NPM1 mutant antibody (polyclonal, Novus) staining was standardized using OCI-AML3 cell-line and known-cases of AML-samples using 13-color MFC. We compared two permeabilization reagents: FACS Lyse (BD) and Fix-&-Perm (Invitrogen). Results of mNPM1 expression were correlated with the *NPM1*-gene mutation using molecular techniques. MRD was studied using MFC-mNPM1, 16-color cMFC-MRD and targeted NGS-based MRD (NGS-MRD).

Results: Based on MFI & S/N ratio of anti-NPM1-ab expression**,** FACS Lyse was found to a better permeabilization reagent. mNPM1 expression was studied in 37 AML cases. Isotype control was taken as a negative control with a median MFI of 0.47 (range 0.19-2.66). Median (range) of normalized-MFI and percentage of mutant NPM1 in AML with NPM1 mutation were 3.44 (1.14-21.15) and 39.98%(3.08-80.75). 20/37 AML were positive for NPM1-mutation by molecular methods, among these MFC-based mNPM1 expression was detected in 18 (90%) cases and was undetectable in 16/17 (one case showed mNPM1 expression). Thus, sensitivity & specificity for MFC-based mNPM1 detection were 90.00% and 94.1% respectively.

In dilution and spiking assay (with 5%, 1%, 0.1% and 0.05%), we could reliably detect mNPM1 population upto 0.05%. MFC-based mNPM1 MRD (MFC-mNPM1) was studied in 13 AMLMRD samples and was detected in 7/13 samples (53.8%). cMFC-MRD was positive in 6/7 of these samples. Thus, one case was false-negative for cMFC-MRD (as it was also positive by NGS-MRD). NGS-MRD results were available in 11/13 samples. Among these 7 cases, NGS-MRD was available in 5 samples and also positive in all 5 samples. Of 6 MRD negative cases (by cMFC-MRD and NGS-MRD), one was positive by MFC-mNPM1 MRD. Thus, the sensitivity & specificity for MFC-mNPM1 MRD were 83.3% and 100% respectively.

Conclusions: We successfully standardized flow cytometry evaluation of mutant NPM1 expression in the AML patients. FACS Lyse was the best permeabilization reagent. Mutant NPM1 expression by flow cytometry is highly specific and sensitive to detect AML with NPM1 mutation and MFC-based mNPM1 MRD monitoring in combination with other immune markers.

HEM-07

MONITORING CAR-T CELLS USING FLOW CYTOMETRY-A SINGLE CENTRE EXPERIENCE

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Introduction: Adoptive immunotherapy using chimeric antigen receptor (CAR)-T cells leads to impressive remission rates in patients with refractory B-cell leukemia and B-cell lymphomas. In order to estimate response and severe side effects of CAR-T cell therapies, longitudinal monitoring of CAR-T cells is desirable and sometimes critical in taking treatment decisions.

Methods: Our cohort consists of 35 patients, who have received CAR-T cell treatment since 2020, F/M: 12/23, of median age 47 (20-74). Diagnosis at presentation was B-ALL/DLBCL/PMBCL/MCL/FL in 5/16/5/5/4 patients, respectively. The administered CAR-T product was Axicabtagene ciloleucel/Brexucabtagene autoleucel/Tisagenlecleucel in 16/6/13 patients, respectively. Median follow-up was 8 (1-33) months. CAR-T monitoring using flow cytometry was available in 28/35 patients at several timepoints, mainly including day 5 and upon immune-mediated toxicity [cytokine release syndrome (CRS) or immune effector cell-associated neurotoxic syndrome (ICANS)]. A three-color flow cytometry panel was designed using a commercial CD19 CAR Detection Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). The reagent consists of a biotinylated CD19 antigen that specifically binds to CD19-targeted CARs. In a second incubation step the biotin-labeled CAR-T cells are then stained with a fluorochrome-conjugated anti-biotin antibody.

Results: Thirty/35 and 13/35 patients experienced CRS grade 1/2/3/4: 12/13/3/2 and 13/35 patients experienced ICANS grade 1/2/3: 11/1/1 with a median onset time of 2 (0-12) and 8 (3-12) days, respectively. Disease assessment was available in 33/35 patients. Best response was CMR/CR/PR/SD/PD in 10/8/11/1/3 patients, respectively. Fourteen patients (40%) eventually relapsed - 5 of them having achieved CR/CMR at interim evaluation. Five/35 (14%) patients died due to progressive disease: 4, /TRM(CRS): 1. Peak expansion levels of CAR-T cells were >100/μl (group1) /1-100/μl (group 2) /<1/μl (group 3) in 7/15/6 patients, respectively. The occurrence of CRS/ICANS were 85%/71% for group 1, 73%/33% for group 2 and 66%/17% for group 3. Peak expansion levels of CAR-T cells in the peripheral blood of the patients were not shown to be higher in responding patients than in non-responding patients. Furthermore, the duration of detectable CAR-T cell levels has not been found to correlate with progression free survival.

Conclusions: Flow cytometric CAR-T cell detection is a reliable method to monitor CAR-T cells. In our analysis, higher expansion levels correlate with higher frequency of CRS or ICANS. No correlation with treatment outcomes was noted, perhaps due to the limited patient number. However, CAR-T cell levels at certain timepoints post infusion provide guidance to therapeutic decisions such as lenalidomide administration to boost T cell expansion.

HEM-08

FLOW CYTOMETRY IN CLINICAL TRIALS. AN UPDATE ON MULTI-CENTER RESEARCH STUDIES

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Introduction: A clinical trial is a complex setup that requires intensive management of logistical details and constant control, from patient recruitment and running biological analyses to data analysis and reporting. Flow cytometry has significantly contributed to the understanding of physiology and pathology in the context of immunology and hematological disorders. As the number of clinical trials is on the rise, especially since the discovery of checkpoint inhibitors, the use of flow cytometry in clinical trials has also increased.

Methods: We describe here the outcomes of clinical trials where flow cytometry has been used. Biological samples from patients were tested using a combination of methods aimed at improving the comparability of the data and, ultimately, the reproducibility of similar results in multicenter research studies. These methods include the control of reagent variability and improved processes for measurement and data analysis.

Results: There are multiple ways to reduce the variability of flow cytometry experiments. Reducing steps and human intervention is one of the most important. Flow cytometry panels can include >10 different fluorescence-conjugated antibodies, and manual cocktailing may introduce high variability due to the low reagent volumes required. The utilization of dry reagents has significantly improved the workflow of hematology laboratories. As clinical research often seeks novel biomarkers, tailored dry reagent solutions show similar technical benefits. The inclusion of appropriate controls and standardized analysis also shows significant benefits for clinical research studies.

Conclusions: Flow cytometry is a fairly accurate technology to assess, one by one, hundreds of thousands of cells in biological samples by using fluorescence-conjugated antibodies targeting the biomarkers of interest. As described, improved standards to increase the robustness of data exist. Not only do they offer an advantage in terms of the quality of information generated, but they also offer the possibility to include flow cytometry in clinical trials by streamlining workflows and reducing the burden on clinical scientists throughout the studies.

HEM-09

SINGLE-CENTRE UK EXPERIENCE: THE USE OF TRBC1 FOR THE DIAGNOSIS AND MONITORING OF T CELL LYMPHOPROLIFERATIVE DISEASE

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Introduction: Current immunophenotyping methods for T cell lymphoproliferative disease (TLPD) aim to identify neoplastic T cells by their aberrant antigen expression. This can be challenging as benign conditions, such as infection, can also result in aberrant antigen expression. Conversely, some T cell neoplasms have subtle changes in antigen expression that are not easily distinguishable from normal cells. An anti-TRBC1 (T cell receptor beta constant 1) monoclonal antibody allows for incorporation of

clonality testing into the immunophenotyping method, providing simultaneous evidence of clonality in an immunophenotypically aberrant T cell population. This study describes a single-centre study of the clinical validation of a new TRBC1 flow cytometry panel.

Methods: Excess peripheral blood and bone marrow aspirate samples (n = 41) were sourced from the Immunology Laboratory (part of the Haematological Malignancies Diagnostic Link Service) at the University Hospitals of Leicester NHS Trust. A nine-colour flow cytometry panel containing anti-TRBC1 was designed, optimised, setup and technically validated on the BD FACS Lyric. Data was analysed for the presence and quantification of an aberrant T cell clone, and compared to results from other diagnostic investigations.

Results: Of the 41 samples tested, 20 had a phenotypically aberrant T cell population that was monotypic for TRBC1 expression (clonal). Fourteen of these samples were tested for T cell receptor (TCR) molecular gene rearrangement and were confirmed to contain clonal T cell populations (6 samples were not tested). All 20 samples that had aberrant T cell populations were in consensus with clinical features and the final clinical diagnosis. This included two samples which had small T cell clones of uncertain significance ('T-CUS'), as described elsewhere, in patients that were subsequently confirmed to not have T cell lymphomas. A further 9 samples had a phenotypically aberrant T cell population, where TRBC1 analysis was not suitable due to the lack of cell surface expression of TCR-alpha:beta. The remaining 12 samples with no clear TRBC1-monotypic T cell population were in consensus with TCR gene rearrangement studies in two samples and with the clinical conclusion of an absence of T cell lymphoma in all samples.

Conclusions: This study confirms the ease, rapidity and clinical utility of a flow cytometry panel for T cell lymphoproliferative disease that contains anti-TRBC1 to simultaneously assess T cell clonality. There was 100% concordance with TCR gene rearrangement studies, where performed, and with clinical investigations in patients with a range of T cell lymphomas or healthy/reactive conditions.

HEM-10 Abstract has been cancelled

HEM-11

CD44 EXPRESSION IN THE CD34+CD38- COMPARTMENT OF AML

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Introduction: Acute myeloid leukemia (AML) is a heterogeneous neoplastic disease characterized by an aberrant proliferation and differentiation of hematopoietic stem cells (HSC). Leukemic stem cells (LSC) are therapy-resistant cells responsible for refractory disease and relapse. Several cell-surface markers have recently been identified as LSCs biomarkers, like CD44, a cell membrane glycoprotein implicated in cell-cell and cell-extracellular matrix interactions via the binding to hyaluronate. CD44 is

involved in homing, migration, and lymphocyte activation. It is expressed on HSCs and all types of mature blood cells, varying in expression level. This study aims to characterize the expression of CD44 in the immature myeloid compartment (CD34+CD38-) of AML.

Methods: Twenty-five adult patients diagnosed with AML were included in this study, and five healthy bone marrow (BM) samples were also used as controls. Seven-color multiparameter flow cytometry was performed using Omnicyt (Cytognos) with the following antibodies: CD38-FITC, CD45RA-PE, CD34-PerCP-Cy5.5, CD123-PE-Cy7, CD33-APC, CD44-PB and CD45-OC515. From 1.000.000 to 2.000.000 viable events were acquired. Samples were analyzed using the Infinicyt software 2.0.6 (Cytognos). The CD34+CD38- compartment from AML patients was characterized within the blast population (45dim) after discriminating debris and doublets. Regarding the controls, immature myeloid precursors were defined as CD34+CD38-SSCdim once debris and doublets were eliminated, and dendritic cells (CD123+), lymphocytes (CD45+), and granulocytes (CD45dimSSChigh) were defined. Positivity thresholds were established as follows: negative (from 1-10^3), dim positivity (from 10^3-10^4), and strong positivity (10^4-10^6).

Results: All patients expressed CD44 within the most immature compartment. Specifically, in 21/25 cases, CD44 was expressed with a strong positivity (84%) and 4/25 with a dim positivity (16%). Two patients with a dim CD44 expression did not express CD123 (8%), whereas all the others expressed CD123: 7/25 with a strong positivity (64%), and 16/25 were dim (28%). CD44 was also found in healthy BMs, in 4/5 with a strong positivity (80%) and 1/5 was dim (20%). Interestingly, AML patients had a higher mean fluorescence intensity (MFI) of CD44 than healthy donors (36202.46 and 9318.38, respectively). A negative subpopulation was also found in each healthy donor's CD34+CD38- compartment, which was nearly undetectable in AML patients (0,00028-0,17532%, mean value 0,0243). The CD34+CD38- compartment in AML patients was 0.013-23.534% (mean value 1.722), whereas, in the healthy donors, this population ranged from 0.0037-0.0310% (mean value 0.0123).

Conclusion: CD44 was constantly expressed in the CD34+CD38- compartment in AML samples, and its MFI was higher than in healthy BMs.

HEM-12

THE ROLE OF THE PROXIMAL COMPLEMENT FACTOR C3D IN EXTRAVASCULAR HEMOLYSIS IN PNH PATIENTS TREATED WITH TERMINAL COMPLEMENT INHIBITOR ECULIZUMAB

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Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, life threatening disease characterized by complement mediated intravascular hemolysis due to absence of GPI-anchored proteins on blood cells, thrombophilia and bone marrow failure. Treatment of PNH is mainly limited to a monoclonal antibody (MoAb) directed against complement factor C5, Eculizumab resulting in effective reduction of the intravascular hemolysis and, as such, normalization of hemoglobin in one third of cases. However, 25 – 50 % of patients remains transfusion dependent as a result of remaining C3b-mediated extravascular hemolysis. C3d complement fragments, splicing products of the C3b, can bind to erythrocytes that are recognized by complement receptors on phagocytes. This leads to phagocytosis of the erythrocytes in the liver and spleen. Therefore determination of C3d on erythrocytes is essential to predict the chance at extravascular hemolysis. C3d can be determined by multiparameter flow cytometer (FCM) using an anti-C3d MoAb. The aim of our study was to follow the

extravascular hemolysis by determination of C3d bound to erythrocytes in treatment-resistant PNH patients.

Methods: Twenty patients, consisting of 15 severe PNH patients, all treated with Eculizumab, and 5 non-treated patients with only small PNH clones as control, were screened for C3d expression on their erythrocytes. All peripheral blood samples were analyzed with a 4-color MoAb panel (CD235a-FITC, C3d-PE, CD71-PeCy7 and CD59-APC) using FCM. GPI-deficient erythrocytes were gated to determine C3d expression. Furthermore, the absolute reticulocyte counts was determined as a value for hemolysis. An estimation of extravascular hemolysis was done by a Hematologist. Subsequently, linear regression analyses between C3d% and absolute reticulocyte count was performed.

Results: The expression of C3d on erythrocytes of patients with severe PNH was in 9 samples (60%) >30% C3d positive, 3 samples (20%) >14% C3d positive and 3 samples (20%) <10% C3d positive. The 12 samples with a C3d expression of >14% showed absolute reticulocyte count of <180^10*9/l (normal value: 20.0-80.0^10*9/l). The patients with samples of >30% C3d expression showed a moderate to severe extravascular hemolysis. The regression analyses between the C3d% and the absolute reticulocyte count showed a R^2 of 0.56.

Conclusion: The expression of C3d on GPI-deficient erythrocytes matched with the severity of PNH and was higher in the treatment-resistant PNH patients with extravascular hemolysis. The patients with moderate to severe extravascular hemolysis showed a high C3d% of more than 30% C3d positive GPI-deficient erythrocytes, in contrast to PNH patients with no to less extravascular hemolysis.

HEM-13

FLOW CYTOMETRIC EVALUATION OF CD38 EXPRESSION IN T-CELL NON-HODGKIN LYMPHOMA (T-NHL) AND ITS IMPLICATION FOR ANTI-CD38 TARGETED THERAPY

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Introduction: T-NHL is an aggressive and heterogeneous group of lymphomas with poor prognosis. No therapy provides long-lasting response and there is a need for new targeted therapies. Anti-CD38 monoclonal antibody (antiCD38-Mab) therapy has become a promising targeted therapy in many hematological malignancies. Its anti-tumor efficiency depends on level of CD38 expression on abnormal cells. Data on CD38-expression levels on T-NHL is scarce. Hence, we evaluated CD38 expression levels in tumor cells from T-NHL patients.

Methods: Expression pattern (percentage and mean fluorescent intensity (MFI)) of CD38 on atypical lymphocytes (ATLs) was studied in 50 T-NHL patients in samples for diagnosis or staging using Multicolor Flow Cytometry (MFC) between January 2021 to June 2023. MFC was performed on 10-13 color Cytoflex (Beckman Coulter, USA) and data was analyzed using Kaluza-v2.1 software. Normalized MFI (nMFI) was determined using positive control (PC) and negative control (NC) with the formulae

nMFI = (Tumor MFI - NC MFI)*10/(PC MFI - NC MFI)

Results: Out of 50 T-NHL (median age- 49 years; range- 1-80 years), 23(46%) were Angioimmunoblastic T-cell Lymphoma (AITL), 6(12%) Anaplastic Large Cell Lymphoma (ALCL), 6(12%) Gamma-Delta T-cell Lymphoma (GD-TCL), 2(4%) T-Lymphoblastic Lymphoma (T-LBL), 2(4%) T-cell Large Granular Lymphocytic leukemia (T-LGLL), 2(4%) T/NK-Non Hodgkin Lymphoma (T/NK-NHL), 2(4%) Cutaneous T-cell Lymphoma (CTCL), 1(2%) T-cell Prolymphocytic Lymphoma (T-PLL) and 6(12%) Peripheral T-cell Lymphoma–NOS (PTCL-NOS). Median age was 49(range 1-80) years. NK-cells were taken as internal positive control and granulocytes as internal negative control, the median MFI of which were 8.12(range 1.71-17.35) and 1.41(range 0.10-4.41) respectively.

The nMFI and percentage of CD38 expression in T-NHLs were 1.98(range 0-15.74) and 43.59%(range 0.54-99.19%) respectively. CD38 was homogeneously expressed in 11/23(47.8%) AITL, 3/6(50%) GD-TCL, 2/6(33%) ALCL, 2/2(100%) T-LBL, 2/2(100%) T/NK-NHL, 1/2(50%) T-LGLL, 1/1(100%) of T-PLL, 0/2(00%) CTCL and 2/6(33%) PTCL-NOS.

Conclusion: We report the expression pattern and levels of CD38 expression in patients with T-NHL. Our data showed that CD38 was homogenously expressed in 48% of all T-NHL studied. It was robustly expressed in 48% cases of AITL and 50% cases of GD-TCL making it potentially eligible for antiCD38-Mab therapy.

HEM-14

A PROPOSED NOVEL ROLE OF FLUORESCENT PROAEROLYSIN (FLAER) AS A BIOMARKER IN THE HIERARCHICAL STUDY OF NORMAL AND MALIGNANT HEMATOPOIESIS

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Introduction: The use of FLAER in the diagnosis of PNH is well established and applied in peripheral blood. In contrast a similar study in bone marrow (BM) encounters difficulties, because in non-PNH hematopoiesis there is reduced expression of GPI chain linked proteins. Only limited studies have shown differential fluorescent FLAER intensity in certain normal BM cell subpopulations [Semin Hematol. 2019 Jan;56(1):65-68]. The purpose of this study was to include FLAER in multicolor flow cytometry protocols of BM investigation in order to clarify a putative selective binding diversity in non-PNH subpopulations.

Methods: 38 BM samples were studied: PNH n=3, infections/reactive n=4, MDS or MDS/MPN n=8, CML n=2, AML in diagnosis n=9, MRD in AML n=7 and MRD in B-ALL in the end of therapy in remission n=5. Three BM samples in patients with PNH were analyzed for comparison. There was no PNH clone detected in any of the non-PNH cases. A ten-color protocol was used with FLAER/HLA-DR/CD14/CD33/CD34/CD117/CD56/CD38/CD64/CD45. Hierarchical study discriminated CD34++/CD38- as ancestral CD34+, CD34+/CD38+ stem cells, CD34-/CD117+/DR+ myeloid precursors

and CD64+/CD14-/DR+ monocyte precursors. Mature populations detected included mast cells, plasma cells, NK cells, monocytes, and neutrophils. FLAER highest intensity was defined by normal polymorphonuclear neutrophils (PMN) and minimal intensity by PNH PMN. Special care was provided to FLAER titration and compensation.

Results: A discrete FLAER binding clearly distinguishable was observed in the PNH patients with two populations of different intensity in PMN, eosinophils, monocytes, and promyelocytes. In CD34 ancestral a discrete negative FLAER clone was observed. However, in non-PNH samples a spectrum of intensity was noted. An association of intensity and maturity was observed, with the lowest intensity of all marrow cells to be detected in CD34-/CD117+/DR+ myeloid precursors. Ascending order of intensity was observed in CD34+ stem cells and monocyte precursors, intermediate intensity in promyelocytes and promonocytes and higher intensity in monocytes and PMN. Strangely mast cells, NK cells and plasma cells were found in the intermediate spectrum. Ancestral CD34+/CD38- had higher intensity than myeloid and monocyte precursors. In reactive BM, MDS and CML, the CD34-/CD117+ precursors had lower intensity, while in AML a variable deviating pattern was observed.

Conclusions: Differential FLAER binding was observed in hierarchically different populations with a higher attenuation in CD34-/CD117+/DR+ (c-kit myeloid precursors), and with lower intensity in CD34+ and CD34- myeloid and monocytic precursors than mature PMN and monocytes. We propose further study of FLAER within MRD and diagnostic protocols.

HEM-15

THE ROLE OF CD13 AS A MARKER OF LYMPHOPLASMACYTIC DIFFERENTIATION

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Introduction: Immunophenotyping by flow cytometry is a fundamental tool for Chronic B Lymphoproliferative Disease's diagnosis and subclassification. Although some histological subtypes have typical immunophenotypic profile, others particularly splenic marginal zone lymphoma and lymphoplasmacytic lymphoma, lack markers that are specific for diagnostic conclusion, requiring correlation with clinical and laboratory findings, including histological, cytogenetic, molecular and biochemical data. This work intends to evaluate the role of myeloid antigen CD13 as a surrogate marker of lymphoplasmacytic differentiation.

Methods: We retrospectively evaluated CD13 antigen expression on clonal B lymphocytes and CD13 mean fluorescence intensity (MFI) ratio between B lymphocytes and non-B lymphocytes (T and NK) by flow cytometry. We used bone marrow samples of 11 patients with histologically confirmed Lymphoplasmocytic Lymphoma / Waldenstron's Macroglobulinemia (LLP/MW) from a single center between August/2020 and May/2023. CD13 expression was also evaluated in other 44 bone marrow samples from patients with other B-lymphoproliferative diseases (Chronic Lymphocytic Leukemia (12), Follicular Lymphoma (4), Mantle Cell Lymphoma (5), Hairy Cell Leukemia (7), Marginal Zone Lymphoma (3), CD5neg B Chronic Lymphoproliferative Disease (13)), chosen randomly and carried out using the same 8-colour panel. Samples's aquisition were perfomed on FACS LYRIC flow cytometer and analyzes were done with Infinicyt software.

Results: Regarding 11 LLP/MW cases, median age was 67 years (49-85). Altered cytogenetics were found in 3/7 cases. MYD88 L265P mutation was found in the only case which the test was done. The percentage of plasmocytes in the samples ranged from 0.008% to 1.3%, all showed monoclonal Kappa restriction and the most frequently altered marker was CD20 (positive in 5/9 cases). Absence of CD19 and CD27 were seen in only 1 case. Ectopic positivity for CD56, CD117 and CD200 were seen in 0/11, 1/11 and 1/11 case respectively. The percentage of clonal B lymphocytes ranged from 1.2 to 71.5% of the total cell count. Positivity for CD5, CD10, CD23, CD200 was observed in 2/11, 0/11, 6/11, 5/11 cases respectively. CD13 was positive in 8/11 samples (72%) and MFI ratio ranged from 8.1 to 44.2 for positive cases and from 0.56 to 1.6 for CD13 negative cases. None of the 44 control cases were positive for CD13.

Conclusion: CD13 proved to be a useful marker for diagnosing chronic B neoplasms with plasmacitic differentiation. However its negativity does not exclude the diagnosis.

HEM-16

CASE REPORT OF A RARE ACUTE MYELOID LEUKEMIA WITH T(8;16)(P11;P13)

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Introduction: Acute Myeloid Leukemia (AML) is a complex, genetic and clinically heterogeneous hematological disease. Cytogenetic alterations have great prognostic impact. AML t(8;16) is a rare leukemia (<1% of AMLs), with unique clinical, morphological and cytogenetic characteristics. It can affect any age group and has a poor prognosis. It can be *de novo* AML or therapy-related AML from previous treatment of solid tumors. Morphological subtypes are usually FAB M4 or M5. It may present Disseminated Intravascular Coagulation (DIC) (39%), skin involvement (58%) and erythrophagocytosis (70%). We will describe a 28-year-old female patient, previously healthy, with a history of skin bleeding who was hospitalized and diagnosed with Acute Leukemia.

Methods: Blood count: Automated Hematology Counter using flow cytometry/impedance analysis. Morphological analysis of bone marrow aspirate stained with Leishman dye, Immunophenotyping by flow cytometry (ICF) with an 8-color panel on the Lyric Cytometer®; analysis using the Infinicyt® software. Cytogenetics by conventional method (culture without stimulating agents and with G-banding (GTW)) resolution 300-550 bands, description of abnormalities according to ISCN-2016. Molecular methods by RT-PCR and in situ hybridization by immunofluorescence. Hemostasis tests: Time of Prothrombin (TP) and Fibrinogen (FIB): Coagulometric.

Results: Blood count: Hemoglobin: 10.1g/dL, (4EOC/100 leukocytes). Leukocytes: 13000/mm^3 (Blasts: 10%, Neutrophil granulocytes: 23%, Eosinophils: 1%, Lymphocytes: 63%, Monocytes: 1%). Platelets: 69000/mm^3. Myelogram: 81.5% blasts: “Acute Monoblastic Leukemia” (FAB Classification: AML-M5a). Immunophenotyping: (+) markers: CD2+, CD15+, CD33+++, CD38, CD64++, HLA-DR and MPO+;(-) markers: CD3(m and cy), CD7, CD11b, CD13, CD14, CD19, CD34, CD56, CD61, CD71, CD79a, CD117, IREM-2, TdT. TP: 20,8sec, Activity: 51%, INR:1.62. Fibrinogen 52mg/dL (Reference values: 200 to 400mg/dL). Karyotype: 46,XX,t(8;16)(p11.2;p13.3) and in 11 metaphases it also presented trisomy 18. FLT3 and NPM1 mutations, PML-RARA, inv16 or t(14;11) and TP53gene: Negative.

Conclusion: This patient presented the typical features of this rare leukemia described in the literature. She showed a picture of DIC mimicking Acute Promyelocytic Leukemia, that was later discarted. The typical immunophenotyping of this pathology is negative for precursors CD34, CD117 and positive for myelomonocytic antigens CD15, CD33 and CD64. She was treated with D3A7 and achieved remission. After intensification, she will undergo to a bone marrow transplantation.

HEM-17

CHARACTERIZATION OF CLONAL HETEROGENEITY IN ACUTE MYELOID LEUKEMIA NPM1 TO DISENTANGLE CLONAL EVOLUTION

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Introduction: Flow cytometry (FC) enables immunophenotypic characterization of clonal hierarchy of acute myeloid leukemia (AML) *NPM1* patients. The remarkable stability of this mutation during relapse, present from the earliest stages of AML, provides an opportunity to study evolving clonal heterogeneity. The aim was to identify and characterize phenotypically and genetically the leukemic subclones of the AML *NPM1* and which mutations were responsible of the leukemic transformation.

Methods: Bone marrow samples from AML *NPM1* adult patients at diagnosis and during follow-up were identified and characterized using FC. Populations were isolated by cell sorting considering the immunophenotype of the leukemic and residual cells previously determined. NGS was performed using a gene-panel of 40 key myeloid genes (Oncomine Myeloid Research Assay v2) in the sorted leukemic cells.

Results: Analysis of 10 isolated populations at diagnosis revealed minority (0.04-0.3%) myeloid $CD34^+$ cells without immunophenotypic alterations harbouring preleukemic mutations (*DNMT3A*, *TET2*, *IDH1*) associated with clonal hematopoiesis (CH) that occurred early in AML ontogeny (VAF 9%). No founder (*NPM1* or *CEBPa*) or signal activating variants (*FLT3, NRAS*, *KRAS*) were observed. On the other hand, the main leukemic populations (inmature myeloid and/or monocytic leukemic clones) showed CH variants (e.g, *DNMT3A*, *TET2*, *IDH1)* with higher VAF (mean 50%) and acquisition of *NPM1* mutations and signalling genes (*NRAS, FLT3*). High *DNMT3A*, *TET2* and *IDH1* VAF could indicate their clonal selection leading to leukemogenic through the acquisition of a driver mutation. The presence of *KRAS* alteration exclusively in the monocytic population (with low VAF) suggests that these mutations were acquired in a minor subset of cells at a later stage in leukemogenesis. After complete remission achievement (n=2), only *DNMT3A*, *TET2*, and *IDH1* alterations were detected, supporting they could correspond to CH. A targeted analysis of molecular alterations detected at diagnosis were performed in isolated populations at relapse, showing loss of *NPM1* and *FLT3* (each in one case) and maintenance of the gene alterations acquired early in AML ontogeny. Immunophenotypically different clones from the diagnosis were isolated at relapse in 2 cases, identifying new minority mutations in *KRAS* and *TP53* (VAF 3%), not detected in the NGS performed on the bulk.

Conclusions: The integrated methodological approach combining FC, cell sorting, and NGS enabled the identification of molecular profile changes and clonal selection with greater sensitivity in the several populations present in the bone marrow during leukemia progression.

HEM-18

PROGNOSTIC IMPACT OF CLONAL COMPLEXITY AND LEUKEMIC COMPARTMENTS BY FLOW CYTOMETRY IN ACUTE MYELOID LEUKEMIA NPM1

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Introduction: *NPM1* acute myeloid leukemia (AML) is considered a unique biological entity, with a very stable mutation present from its earliest stages. It has been proposed as an optimal model for the study of clonal heterogeneity, which is also reflected in the different leukemic compartments identified by flow cytometry (FC). We analysed the prognostic value of clonal complexity and predominant population (monocytic and non-monocytic leukemic myeloid population) *NPM1* AML at diagnosis and its impact on overall survival (OS) and cumulative incidence of relapse (CIR).

Methods: A total of 89 adult patients diagnosed with AML *NPM1* at the Hospital Universitario y Politécnico La Fe, València (2016-2023) were included. Bone marrow samples at diagnosis were characterized by FC according to the Euroflow procedures.

Results: FC analysis at diagnosis revealed 2 major subtypes of myeloid leukemic populations: non-monocytic or monocytic clones, which were accompanied by other minority clones. In non-monocytic clones was not possible to distinguished between less differentiated leukemic cells and the more committed to the granulocityc/erythroid lineage. Clonal heterogeneity (>1 leukemic clone) was evidenced at diagnosis in 46% patients. Among the other 48 cases (only 1 leukemic population at diagnosis), 42 showed a myeloid non-monocytic population, whereas in 6 the population corresponded to monocytic cells. Relapse and/or resistance occurred in 22 patients (86% with a non-monocytic major population). According to the number of leukemic clones at diagnosis (1 or ≥2), there were no significant differences in the global cohort in OS and CIR (p=0.13 y p= 0.35, respectively), either in a subgroup of 53 patients treated with intensive chemotherapy (p=0.45 both). The analysis of the subtype of the major population at diagnosis did not show differences in OS and CIR (p=0.81 y p= 0.16, respectively), either in OS in patients treated with intensive chemotherapy (p=0.89). However, in the patients with intensive therapy and with non-monocytic major leukemic population, we found significant differences in CIR (p= 0.04), with more leukocytes in peripheral blood (p=0.001) and *FLT3-ITD* mutations (p=0.01).

Conclusions: Most cases of *NPM1* AML of our series had non-monocytic myeloid leukemic cells as major population. These patients showed a higher relapse rate and correlated with a higher number of leukocytes in peripheral blood and *FLT3-ITD* mutations at diagnosis. Clonal complexity does not appear to negatively impact in CIR and OS. Funding: PI18/01340, Ayudas predoctorales AECC, IISLAFE 2019-052-1

HEM-19

DON'T ALWAYS TRUST CD157 EXPRESSION: THE IMPORTANCE OF COMBINATION WITH FLAER FOR DIAGNOSIS OF PNH

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Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematopoietic stem cell disorder resulting from the somatic mutation of the X-linked phosphatidyl-inositol glycan complementation Class A (PIG-A) gene. The genetic severity of this mutation affects the glycosylphosphatidyl-inositol (GPI)-anchored proteins production. These proteins include complement-defense structures such as CD59 on Red Blood Cells (RBCs) and CD24, CD157 in White Blood Cells (WBCs). High sensitive flow cytometric detection of PNH clones is now the gold standard for diagnosis, monitoring and clinical management of patients with PNH.

Methods: 224 patient samples were analysed for PNH between September 2020 and May 2023 in our hub laboratory. Samples were acquired by Beckman Coulter DX-Flex flow cytometers with a 6-color assay following ICCS/ESCCA PNH Consensus Guidelines 2018. High sensitive analysis was able to quantify a PNH clone down to a lower limit of quantification (LLOQ) of 0.01% for RBCs, 0.1% for Neutrophils and 1% for Monocytes.

Results: The analysis of 6 patients (2.68%) out of 224, showed an atypical expression defect of the marker CD157 in neutrophils and monocytes, concomitant with a normal expression of the other GPI-linked markers in RBC (CD59), neutrophils (CD24, FLAER) and monocytes (FLAER). CD157 expression was completely absent in neutrophils and monocytes of 3 patients, partial in 2 patients, less than 1% in 1 patient. The subjects in question were equally distributed between males and females. The mean age was 60 years (range 38-79). The mean value of RBC, Neutrophils, Monocytes and Hemoglobin respectively was $4.02*10^{12}$/L, $3.89*10^{9}$/L, $0.48*10^{9}$/L, 12.01 g/dL. Four patients (67%) presented thrombosis in an atypical location, 1 (17%) was a carrier of myeloproliferative neoplasm, 1 (17%) was affected by medullary aplasia. Two patients (33%) were receiving direct-acting oral anticoagulants.

Conclusions: The isolate defect of CD157 expression in Neutrophils and Monocytes is not indicative of a PNH clone and could be related to an antigenic polymorphism not recognized by the monoclonal antibody used. As described in literature, the commonly available CD157 antibody clone SY11B5 fails to detect a diffuse polymorphic variant of CD157 (p.Arg145Gln). This polymorphism explains only a few cases; other unknown mechanisms are waiting to be clarified. Our case reports remark the importance of including GPI-reagent FLAER in the panel in order to prevent misinterpretation of results and to identify real PNH phenotypes in WBC by the loss of at least two GPI-linked structures per lineage.

HEM-20

BONE PAIN AND DIFFUSE BONE MARROW PET POSITIVITY: AN UNEXPECTED DIAGNOSIS OF ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction: A patient with diffuse bone pain and total body PET positivity: could the bone marrow (BM) biopsy analysis lead to the correct diagnosis? We report the case of a 60-year-old man with several accesses to the emergency room for diffuse bone pain, in absence of previous pathologies.

Methods: The following study was performed: a complete blood count, inflammatory and autoimmunity markers, first line coagulation tests, total body 18F-FDG PET, BM biopsy, flow cytometric analysis, FISH and cytogenetics.

Results: The laboratory results showed: hemoglobin of 129 g/L (RV: 110-138), platelet count of 343 x 10^9/L (RV: 140-400), leukocyte count of 9.14 x 10^9/L (RV: 4.00-10.00) with normal differential count without any instrumental flags (XN 9000 Sysmex). Among the inflammatory and autoimmunity markers only the following results were out of the RV: C-Reactive Protein 136.7 mg/L (RV: <5), Ferritin 1885 µg/L (RV: 30-400), Rheumatoid Factor 22.4 KU/L (RV: <20). Total body PET requested by the rheumatologist showed a diffuse BM hypercaptation, suggesting a diffuse BM pathology. The clinical picture, together with the diffuse PET positivity, induced the hematologist to perform a BM biopsy. The sample was hypercellular with numerous megakaryocytes, marked granulocytic hyperplasia and normal erythropoiesis. A more careful and fortunate observation highlighted an expansion at the promyelocytic stage. Few atypical promyelocytes with abundant purple granulations and some Auer rods were detected. Flow cytometric analysis of BM blood was then performed and showed 20% of CD45 positive cells with the following immunophenotype, suggestive for atypical promyelocytes: CD33+bright CD34-/+ CD38+medium/bright CD117+/- CD11b+dim CD13+ CD15-/+ CD64+ CD157+ MPO+; HLA-DR expression was negative. The suspicion of 'early stage' acute promyelocytic leukemia was confirmed with a cytogenetic/FISH study. FISH analysis showed 29% of 200 interphase nuclei with the standard double fusion signal. Cytogenetic analysis revealed t(15;17)(q24;q21) classic balanced translocation. Screening coagulation tests were consistent with hyperfibrinolysis: PT (INR) ratio 1.22 (RV: 0.80-1.20), aPTT ratio 0.76 (RV: 0.80-1.20), Fibrinogen 169 mg/dL (RV: 150-400) and D-Dimer > 35000 µg/L FEU.

Conclusions: Two aspects can be highlighted: a BM biopsy should be considered in patients with bone pain and diffuse BM PET positivity with no apparent cause; moreover, the clinical picture presented should alert the pathologist to investigate for an acute disease such as leukemia even if the peripheral blood is still normal and BM abnormalities could be interpreted as reactive. Flow cytometry, even if not diagnostic for APL, is useful for the prompt evaluation of the disease.

HEM-21

EXTENSIVE CHARACTERIZATION OF REACTIVE AND NEOPLASTIC PLASMACYTOID DENDRITIC CELL PHENOTYPE FOR BPDCN DIAGNOSIS AND FOLLOW-UP

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Introduction: Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare form of acute leukemia with poor outcome. At diagnosis, a BPDCN is identified by increased frequencies of blasts expressing CD123, HLA-DR, BDCA2, as well as CD45RA and CD56. However, determining BPDCN measurable residual disease (MRD) upon treatment of patients is challenging due to phenotypic similarities with healthy and/or reactive (pre-)DC. Hence, we extensively phenotyped (pre-)DC in bone marrow of healthy donors and pathological controls, as well as bone marrow of BPDCN patients.

Methods: "normal"bone marrow samples of healthy donors were obtained upon informed consent and bone marrow left-over material of pathological controls and BPDCN patients was obtained from our diagnostic laboratory. Leukocytes were prepared using bulk lysis. Subsequently, (pre-)DC were stained with an extensive panel including the following markers CD2, CD11c, CD14, CD19, CD33, CD34, CD45RA, HLA-DR, BDCA1, BDCA2, BDCA3, CX3CR1, DNGR1, XCR1 and ViaKrome808 for live/dead staining. Next, cells were measured on a Navios or Cytoflex LX flowcytometer and analyzed with Kaluza analysis software. Gating strategy focused on selection of DC by exclusion of CD3- and CD19-positive cells and expression of DC subset markers BDCA-1, -2 or -3 to respectively distinguish cDC2, pDC and cDC2.

Results: Normal bone marrow samples showed an average of 0.3% pDC within CD45 positive leukocytes. Furthermore, normal bone marrow samples enriched for mononuclear cells (BM-MNCs) showed an average of 0.5% pDC within CD45 positive leukocytes. Both cDC1 and cDC2 showed an average frequencies of <0.1% in total bone marrow and pre-DC were only lowly abundant (<0.01% in total). Next, we extensively characterized the pDC phenotype of a BPDCN patient and found various aberrancies. DC blasts showed mature pDC expressions of BDCA-2 and BDCA-4 combined with CDP marker expression of CD135 (FLT3). Furthermore we found cross lineage expression of CD19 and over-expression of CD38 by the BPDCN.

Conclusion: In order to correctly distinguish BPDCN-MRD from normal CDP to pDC differentiation, knowledge of expression patterns and frequencies is warranted. We established normal CDP and pDC frequencies and expression patterns and subsequently compared this with the phenotypic aberrancies in BPDCN bone marrow that could be exploited for MRD assessment upon treatment.

HEM-22

CD117 NEGATIVE ACUTE PROMYELOCYTIC LEUKEMIA, WITH T/MYELOID IMMUNOPHENOTYPE, SUCCESSFULLY TREATED WITH ATO-ATRA

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Introduction: Acute promyelocytic leukemia (APL) is an hematologic emergency due to the high mortality rate, requiring a rapid diagnosis and immediate initiation of therapy. Morphological and immunophenotypical criteria establish a preliminary diagnosis before molecular confirmation, thus enabling immediate, appropriate management. Classically, the leukemic promyelocytes express myeloid antigens CD117, CD33, CD13 and myeloperoxidase (MPO) whereas they do not express CD34 and HLA-DR. Aberrant expression of T cell antigen CD2 and of CD34 may be seen in the microgranular variant of APL. Here we describe a 22-year-old patient diagnosed wih a classical variant of APL (vAPL)

with CD117 negative blasts and a T/myeloid immunophenotype, successfully treated with all-trans retinoic acid (ATRA)-and arsenic trioxide (ATO)-based therapy.

Methods: The morphological evaluation was performed on May Grunwald-Giemsa stained peripheral blood (PB) smears, and hematoxylin and eosin-stained histopathological sections. Bone marrow aspirate resulted in a dry tap. The immunophenotype evaluation was performed using a Navios cytometer (Beckman Coulter) and Navios software, on EDTA whole blood samples, with a lyse-no-wash technique and a panel of directly conjugated antibodies (HLA-DRFITC-CD34PE-CD13ECD-CD33PC5.5-CD117PC7-CD14APC-CD19A700-CD10A750-CD2PB-CD45KO). The following markers were evaluated with a four fluorochrome staining: CD3, CD4, CD8, CD7, CD1a, CD56, CD11c, CD11b, CD64, CD15, CD36, CD22, CD38. For intracytoplasmic staining, TdT, cytoplasmic CD3, cytoplasmic CD79a, cytoplasmic CD22, MPO, were performed prior fixation and permeabilization. Conventional metaphase karyotyping by GTG-banding, FISH on interphase cells, and qRT-PCR for the PML-RARA mRNA (bcr1, bcr2, and bcr3 isoforms) was performed. Molecular studies also included FLT3-ITD and D385 point mutation.

Results: On PB smears blasts showed round to irregular, rarely folded nuclei, fine chromatin, and few amount cytoplasmic granules, without Auer rods. Histopathology disclosed 80% of blasts immunoreactive for CD33, partially for CD34 and CD68 (KP-1), negative for CD117, PAX-5, CD3m, CD13 and CD68R (PG-M1). PB flow cytometry showed 78% of blasts characterized by CD45+dim/SSClow, CD34+, CD33+, CD2+, MPO+bright, and partial expression of CD56 and cyCD3, suggestive for mixed phenotype acute leukemia (MPAL) T/myeloid, while CD117 and HLA-DR were negative. The t(15;17) PML/RARA translocation was identified by metaphase cytogenetics and FISH, PCR identified two atypical PML/RARA transcripts and FLT3-ITD mutation. She was diagnosed with vAPL and successfully treated with ATO-ATRA.

Conclusions: Atypical morphology and immunophenotype may rapresent a diagnostic challenge on APL. A diagnosis of MPAL requires exclusion of recurrent cytogenetic abnormaliities such as t(15;17), and the PML/RARA translocation. These diagnostic tools enable early detection of APL, thus lowering the risk of early death.

HEM-23

NOVEL IDENTIFICATION OF THE WHOLE NK-CELL POPULATION BASED ON THE EXPRESSION OF NKP80

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Introduction: NK-cell population is usually identified by flow cytometry with a combination of markers ($FSC^{lo}SSC^{lo}CD45^{hi}CD56^{+/++}CD16^{lo/+}CD3^{-}$) since a single marker for the specific identification of the whole population of NK cells is still lacking in routine (diagnostic) laboratories. However, in case of lost/downregulation of CD56, either under reactive (i.e., virus/bacterial response) or pathological (i.e., tumor NK cells) conditions, NK-cell identification is not optimal. CD314, CD335 and NKp80 are activating receptors expressed by virtually all mature NK cells, which would be good candidates as a marker for the identification of total NK cells. In this study we purposed to select a single marker for the flow-cytometry identification of total NK cells in peripheral blood (PB), and to validate its utility in

healthy donors (HD) and different pathological conditions, particularly those associated to CD56 downregulation.

Methods: PB samples from 101 HD, 29 patients with reactive processes (viral/bacterial infection), 12 chronic lymphoproliferative disorders of NK cells (NKCLPD) confirmed to be clonal, and 12 patients with NK-cell expansions carrying phenotypic aberrations but without confirmation of clonality, were analyzed in a Cytek 5L-Aurora spectral flow-cytometer, following EuroFlow SOPs. Firstly (I), CD314, CD335 and NKp80 were tested in HD to select the best candidate for the identification of total NK cells. (II) Different NKp80 reagents were tested to identify the best conjugate. (III) The best NKp80 reagent was then validated in parallel in HD and patients. Data-analysis was performed using INFINICYT^{TM} and GraphPad Prism 8 software.

Results: (I) The anti-NKp80 antibody showed a much higher median fluorescence intensity and stain index ($p<0.01$) than CD314 and CD335 in all samples tested for the selection of all NK cells based in one single marker. Moreover, (II) all NKp80 clones/conjugates tested identified all NK cells with high/very high resolution, stain index being systematically >70. (III) Overall, when comparing the NKp80 expression pattern in HD vs patients, in all cases (even when CD56 was lost in clonal NK cells) NKp80 improved NK-cell identification, with a median fluorescence intensity of 81,134 (HD) vs 57,554 (CLPD-NK) vs 57,590 (NK-cell expansions/aberrations) vs 70,774 (viral/bacterial infection), without differences in the expression between $CD56^{bright}$ and $CD56^{dim}$ NK cells.

Conclusions: NKp80 is a suitable marker for the identification of all blood NK cells, and resulted the best marker particularly for the identification of $CD56^{-}$ NK, both in reactive and clonal conditions, though it is not useful for subsetting NK cells into $CD56^{bright}$ and $CD56^{dim}$ subpopulations.

HEM-24

BIOLOGY OF ACUTE MYELOID LEUKEMIA AND RESPONSE TO THERAPY: THE ROLE OF BCL-2, CD47 AND PD-L1

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Introduction: Acute Myeloid Leukemia (AML) is still the most common type of leukemia, with patients that may eventually relapse even after initial clinical remission and response to treatment. AML cells can easily evade or suppress the immune system by the altered action of immune checkpoint pathways such as PD-1 / programmed death ligand-1 (PD-L1) and/or macrophage immune checkpoint CD47, or by resistance to apoptosis via Bcl-2. Moreover, the high relapse rate in AML is related to the persistence of a reservoir of quiescent Leukemic Stem Cells (LSCs) that are protected by the immunosupressive microenvironment of the BM niche and are difficult to identify and quantify.

Methods: Next Generation Flow-cytometry (NGF) will be used to analyze the expression of Bcl-2, CD47 and PD-L1 on AML blasts cells. Evaluation of Bcl-2 and CD47 will been espressed as Mean Fluorescent Index (MFI) comparing it to median value of MFI on lymphocytes population. PD-L1 expression on AML cells will be evaluated as positive or negative.

Results: Starting from 2021, 59 consecutive AML patients, unselected for fitness, entered the study at moment of diagnosis to evaluate firstly the expression of Bcl-2 on AML blasts: 13/59 (22%) had an high expression of Bcl-2. 25/59 patients have been evaluated for CD47 expression; 4/25 (16%) resulted

having an high expression of CD47 on AML cells. 27/59 (46%) patients have been evaluated for PD-L1; 16/27 (59%) resulted positive. Regarding post-induction response up to now we have data on 28/59 (47%) patients, treated with standard high-dose chemotherapy or hypomethylating agents; 12/28 (43%) obtained a Complete Response (CR) while 16/28 (57%) did not. In the cohort of 16 patients who did not achieved a CR, 7/16 (44%), 3/4 (75%) and 5/8 (63%) had respectively high levels of Bcl-2, CD47 and PD-L1. On the contrary, of the 12 patients obtaining CR, just 1/12 (8%) had high Bcl-2 level, no one had high levels of CD47 and 4/7 (57%) were PD-L1 positive.

Conclusions: Up until now, these preliminary data seem to corroborate the idea that both Bcl-2 and CD47 expression on AML blasts could represent a negative prognostic factor for response to therapy. Future analysis on a bigger cohort of patients are needed to confirm these hypothesis. Moreover, the role of these parameters in creating a immunosuppressive environment, that help AML blasts survival, could be better clarified by identifying and characterizing the quiescent LSCs responsabile of AML relapse.

HEM-25

CLINICAL VALIDATION OF CML LSC EVALUATION BY FLOW CYTOMETRY FOR THE DIAGNOSIS OF CHRONIC MYELOID LEUKEMIA

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Introduction: The diagnosis of chronic myeloid leukemia (CML) is based on the presence of the Ph chromosome, the rearrangement of bcr-abl1 genes and bcr-abl1 fusion protein. The goal of the present study was to evaluate the diagnostic sensitivity, specificity, positive and negative predictive values of CML leukemic stem cells (CML LSCs) detection in peripheral blood by flow cytometry.

Methods: A single tube 8-color assay: CD25 BV421 (clone BC96) /CD45 BV510 (clone 2D1)/CD15 FITC (clone MMA)/CD26 PE (clone 2A6)/CD34 PerCP-Cy5.5 (clone 581)/CD117 PE-Cy7 (clone 104D2)/CD38 APC (clone HB7)/HLA-DR APC-H7 (clone L234) was used for the detection of CML LSCs following a bulk lyse/stain/wash protocol. Acquisition was performed on a BD FACSCanto II flow cytometer, equipped with FACSDiva software (BD Biosciences) followed by data analysis with Infinicyt software (Salamanca, Spain). Detection of bcr-abl1 transcripts by multiplex RT-PCR was considered as the reference diagnostic method.

Results: A total of 213 CML patients were analyzed. The results for clinical sensitivity (TP/TP+FN), clinical specificity (TN/TN+FP), positive (TP/TP+FP) and negative (TN/TN+FN) predictive values were 100% (0 false negative results), 92.5% (7/213 false positive results), 94.5% and 100% respectively. Overall agreement between the methods was 96.7%. In 1/7 false positive results CML diagnosis was confirmed, in 2/7 false positive results atypical CML was confirmed.

Conclusion: Evaluation of CD34+CD38-CD25+CD26+ CML LSCs in peripheral blood by flow cytometry demonstrates favorable performance characteristics for rapid and reliable diagnosis of CML and could provide valuable information for the differential diagnosis of atypical (bcr-abl1 negative) CML.

HEM-26

ABSOLUTE COUNT OF CD26+ LSCS CAN PREDICT THE TKI TREATMENT RESPONSE IN CHRONIC MYELOID LEUKEMIA PATIENTS

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Background: In spite of the revolutionary therapeutic discovery of tyrosine kinase inhibitors for the treatment of CML, a good proportion of patients show disease relapse. One of the probable reasons for these relapses and poor therapy responses has indicated the presence of TKI refractory leukemic stem cells (LSCs). CD26 has been identified as a very specific biomarker for the identification, purification, and quantitation of CML LSCs in PB and BM samples of CML patients.

Methods: Peripheral blood samples from all consecutive cases of CML-CP, diagnosed at our institution were subjected to flow cytometric (FCM) analysis for LSCs. The assessment was done by a custom-made pre-titrated antibody cocktail comprising CD45-BV510/CD260-PE/CD34-PerCP-Cy5.5/CD38-APC-H7. Stem cell (SC) was identified as CD34 +/CD38 dim to negative population. While the CD26+ SC was considered as CML LSC. The response of TKI treatment was assessed by attainment of MMR (BCR-ABL1/ABL1 ratio <0.1%, on IS Scale) at 12 months. Statistical evaluation was performed to see the difference in the parameters related to CD26 positive LSCs between those who achieved MMR and those who didn't.

Results: A total of 78 CML CP patients who completed 12 months of TKI treatment and had available data on CML LSC at diagnosis as well as BCR-ABL1 transcript ratio at 12 months of TKI, were included in the analysis. The median age at diagnosis was 34 years (range 4-74 years) with a male-to-female ratio of 1.8:1. The EUTOS score was high in 23/68 (33.8%) patients and low in 45/68 (66.2%) patients. MMR at 12 months was achieved in 30/78 (38.5%) and not achieved in 48/78 (61.5%) patients.

All the cases showed the presence of CD26-positive LSCs in their PB at diagnosis. The median value of TLC, the proportion of SCs (CD34+/CD38- population) of TLC, the proportion of CD26 positivity in SC component, and an absolute number of CD26 + LSC was higher in the non-MMR achievers (1,96,000 cells/cumm vs 1,14,000 cells/cumm; *p value= 0.055*:, 0.29% vs 0.13%; *p value 0.031*:, 62.3% vs 50%; *p value 0.614*:, 278.6 cells/cumm vs 110.69 cells/cumm; *p-value 0.035*:, respectively). Using ROC curve analysis, a cut-off of <241 CML LSCs /cumm had 89.9% sensitivity and 62.5% specificity of achieving an MMR at 12 months.

Conclusions: We conclude that CD26+ LSCs are detectable in all CML-CP cases at diagnosis and their absolute numbers at diagnosis can help in predicting attainment of MMR in CML patients on TKI treatment.

HEM-27

FLOW CYTOMETRIC IMMUNOPHENOTYPING (FCI) OF CEREBROSPINAL FLUID (CSF) FOR THE DETECTION OF CNS INVOLVEMENT OF HEMATOLOGICAL NEOPLASM- THE LESSON LEARNT

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Background: CNS involvement in patients with hematological malignancies is a serious concern that adversely affects the prognosis of these patients. The microscopic examination of CSF is traditionally used to detect CNS involvement in most clinical settings. However, it lacks sensitivity owing to the small sample volume, the pauci-cellular nature of the sample, and processing issues. Flow cytometric Immunophenotyping (FCI) on the other hand is a highly sensitive technique that can objectively detect and quantify even a small number of tumor cells in a sample.

Material Method: This was a retrospective study where data were compiled for the FCI of the CSF samples received in the lab over the last two and half years. (Dec 2020 to May 2023). The CSF received in the lab (1-2 ml) was divided into two parts. One part was used for the cytomorphological examination, using an MGG-stained cytospin preparation. While the other part was subjected to the FCI. For FCI, whole sample was centrifuged and the pellet was stained with a single tube 6-10 color antibody panel, depending on the primary diagnosis. The whole of the tube was acquired and analyzed. The CSF was labeled as positive when more than 20 clustered events of diagnostic immunophenotype were found. Any genuine abnormality in less than 20 events was labeled as highly suspicious. A comparative analysis of the microscopic and FCI diagnosis was conducted.

Result: A total of 265 CSF samples from 216 patients (147 males and 69 females) suffering from different hematological neoplasms were analyzed. The median number of all viable gated cells was 1,537 (range 68-1,82,000) and the median tumor load of atypical cells in CSF on FCI was 43 % (range-0.4-98%) of the viable cells. Forty samples (15.09%) were positive for involvement and 5 cases were highly suspicious. The microscopic examination could label only 14 of these 40 FCI-positive samples as positive, while 26 were falsely labeled as negative. Four CSF samples, one each from cases of DLBCL, B ALL, Burkitts, and AML were falsely labeled positive on cytomorphology. However, on FCI, these samples consisted of CD3-positive mature T cells and a few monocytes only.

Conclusion: FCI is a robust, highly sensitive tool that can objectively detect tumor cells even in pauci-cellular CSF samples. Cytomorphology alone has a high chance of misleading results. Hence, it may be prudent to subject these precious samples directly to FCI for objective characterization of rare events.

HEM-28

CONFORMATIONAL CHANGES OF PD-L1 IN CIRCULATING MDSCS TO PREDICT ESCAPE FROM IMMUNOTHERAPY IN LUNG CANCER PATIENTS RECEIVING ANTI-PD-1/PD-L1 CHECKPOINT INHIBITORS

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Background: Most lung cancer patients are treated with anti-PD-1/PD-L1 immunotherapy (IT), and although there is an increase in survival for most patients, not all of them benefit from this strategy. The IT indication is based on PD-L1 tumor expression, with a controversial predictive value. This work focuses on the detection of conformational changes of the PD-L1 molecule from myeloid-derived suppressor cells (MDSCs), which are circulating cells that can infiltrate and proliferate in the tumor environment, inducing immunosuppression. We studied the potential value of PD-L1 conformational changes as a predictor of escape from IT in patients with non-small cell lung cancer (NSCLC) prior to receiving IT.

Methods: Peripheral blood from patients with stage III-IV NSCLC (n=37) was processed immediately, using minimal sample disturbance protocols. Briefly, blood was stimulated with phorbol esters (PMA), leading to conformational changes of PD-L1 and increased accessibility to the epitopic target. MDSCs were identified as HLA-DR$^{lo/-}$CD33^{+}CD11b^{+} and analyzed on the Attune™ NxT Flow Cytometer (Thermo Fisher Scientific). Comparative analysis of conformational dynamics was assessed using the PD-L1 index (PD-L1$_i$), obtained by calculating the difference in PD-L1 mean fluorescence intensity between stimulated PD-L1^{+}MDSC and non-stimulated MDSCs, divided by two times the standard deviation of the non-stimulated populations.

Results: PD-L1$_i$ allowed the classification of responders and non-responders at baseline, prior to IT administration. With a PD-L1$_i$ >5.88, disease progression occurred in 58.33% patients (progression free survival, PFS, median =5.73 months; 95% CI =2.67, 20.53), showing significant differences when compared to a PD-L1$_i$ ≤5.88, in which only 7.69% underwent disease progression (PFS median not reached; p-value =0.0042). Overall survival (OS) was significantly worse in the group with higher PD-L1$_i$ (41.67%; OS median =18 months; 95% CI =6.77, 25.23) vs lower PD-L1$_i$ (76.92%; OS median not reached); p-value = 0.035.

Conclusions: The conformational dynamics of PD-L1 in MDSCs has been shown to be a good biomarker for predicting NSCLC progression in patients receiving anti-PD-1/PD-L1 IT. Importantly, this approach uses a non-invasive and rapid flow cytometry assay with a promising biomarker for the evaluation of IT escape. Additionally, our results highlight the important role of MDSCs in cancer and immunotherapy resistance.

HEM-29

BLASTS IN ETP-ALL EXPRESS A HIGH LEVEL OF THE BCL2 PROTEIN COMPARED TO ITS EXPRESSION IN OTHER SUBGROUPS OF T-ALL.

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Introduction: T-cell acute lymphoblastic leukaemia (T-ALL) is a neoplasm of lymphoid cells that are committed to the T lineage. Early T-cell precursor (ETP) ALL is a distinct subgroup of T-ALL. It has a unique immunophenotypic and genomic profile. The ETP phenotype is a predictor of poor clinical

outcome and has a high risk of relapse in comparison to other subtypes of T-ALL. Recently, it has been shown that T cells are dependent on the anti-apoptotic B-cell lymphoma 2 (BCL-2) protein in the immature stage (equivalent to ETP-ALL blasts). However, this dependence switches to BCL-XL during the double-positive developmental phase and persists until the dependence switches back to BCL-2 in the mature single-positive stage. Therefore, BCL-2 may be a clinically relevant target for the therapeutic management of ETP-ALL. The aim of our study was to evaluate and compare the level of BCL-2 expression in ETP-ALL and other T-ALL subgroups of primary patients by flow cytometry.

Methods: bone marrow samples were collected from 29 patients with T-ALL followed at the National Research Centre for Hematology in Moscow, Russia, in 2022-2023. Immunophenotyping was performed at the time of diagnosis prior to treatment using the 10-colour BD FACSCanto™ cytometer BD antibody panels. All samples were recorded with the same cytometer baseline settings. The CST setup settings were applied daily before the samples were run. According to WHO 2016 and EGIL classifications, 8 ETP-ALL, 4 near ETP-ALL, 1 TI-ALL, 8 TII-ALL, 1 TII-TIII-ALL and 7 TIII-ALL patients were diagnosed. BCL2 and BCLXL protein expression was analysed in parallel in tumour cell populations, lymphocytes and granulocytes. The median MFI of each cell population was used for analysis.

Results: BCL2 MFI detected in blasts from patients with ETP was statistically significantly higher compared to other T-ALL subtypes blasts, lymphocytes and granulocytes (T-test, $p<0.05$). The BCL2 MFI of blasts is statistically significantly different between ETP blasts and other T-ALL blasts when normalised to the BCL2 MFI of granulocytes (since normal granulocytes do not express BCL2 and can be used as a control) (Mann-Whitney test, $p<0.05$). According to our data, BCLXL expression did not show statistically significant differences in different cell populations of T-ALL patients.

Conclusions: our data revealed statistically significant differences in BCL2 expression between ETP blasts and other T-ALL blasts. These findings may be useful in targeted therapy of ETP-ALL.

HEM-30

UTILITY OF FLOW CYTOMETRY IN THE DIAGNOSIS OF THE RARE AND AGGRESSIVE: MYELOID LYMPHOID NEOPLASM WITH FGFR1REARRANGEMENT

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Introduction: Myeloid/lymphoid neoplasm with FGFR1 rearrangement (MLN-FGFR1r) is a rare, aggressive hematolymphoid neoplasm with a poor prognosis. It is clinically heterogeneous and may present with either lymphadenopathy, or increased WBC counts (neutrophilia), or both and abnormal lymphoid blasts. Its diagnosis requires molecular or cytogenetics studies. However, in real-world practice, genetic studies are only done occasionally as a first-line investigation and need a high index of suspicion. Additionally, genetic studies are not available in many places. Otherwise, it can be missed easily. We evaluated the role of flow cytometry in the early diagnosis MLN-FGFR1r patients.

Methodology: We studied 7 patients with a diagnosis of MLN-FGFR1r over a period of the last 4 years. The clinical details, laboratory findings and treatment history were recorded from the electronic medical records. Flow cytometric immunophenotyping (FCI) was performed using a 5 tube 10-13 color

antibody panel and an additional antibody panel to determine clonal mast cells. Data was acquired on Cytoflex (BC) and analyzed using KaluzaV2.1 software.

Results: We diagnosed 7 patients over the past 4 years (in 10000 patients) signifying the rarity of the disease. Patients were predominantly young adults aged between 23-46 years (median 27 years) with a male predilection (M:F- 6:1). 5/7 patients presented with multiple lymphadenopathies, 3/7 with peripheral blood eosinophilia (>6%). On bone marrow examination, 4/7 patients had <10% blasts. On FCI, 6/7 showed cases blasts with mixed phenotypic lineages (B/T- 2/6, Myeloid/T- 2/6, Myeloid/B- 1/6, M/B/T- 1/6) and 1 case as T-LBL. In 4/7 cases, blasts also showed aberrant CD25 expression. Notably, clonal mast cells were identified in 5/5 (100%) samples in which mast cells were analyzed. Samples also showed immunophenotypic dyspoiesis of one more lineage.

Conclusion: Our data showed that abnormal blasts with mixed immunophenotype lineage, (usually less than 10%) and clonal mast cells are characteristic features of myeloid/lymphoid neoplasm with FGFR1 rearrangement. Thus, with typical clinical features and unique features of FCI, this rare entity can be easily identified. FCI-guided genomic testing can confirm the diagnosis of MLN-FGFR1r. Thus, FCI plays a vital role in the diagnosis of MLN-FGFR1r.

HEM-31

EXPRESSION PATTERN OF NEW MARKERS IN ACUTE MYELOID LEUKEMIA AND THEIR UTILITY FOR MEASURABLE RESIDUAL DISEASE ASSESSMENT

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Introduction: Measurable residual disease (MRD) is the most relevant biomarker for prognosis and treatment efficacy monitoring in patients with AML. Multicolor flow cytometry (MFC)-based MRD monitoring is a widely used and sensitive technique. However, its application is still limited to 85-90% of patients due to the overlap of routinely used antigen expression with normal myeloid progenitors (NMP). There is a need to evaluate additional markers to increase the applicability and accuracy of MFC-MRD. We studied expression of CD52, CD54, CD97, CD93, CD9 and CD44 in AML and their practical utility in MRD monitoring.

Method: 13-16 color MFC-MRD was performed using bulk-lysis and stain method using Cytoflex (Beckman Coulter) and LSR-Fortessa (BD). Data was analyzed using Kaluza-software. The expression intensity and pattern of markers studied in the myeloid blasts at diagnosis and MRD. Normalized mean fluorescence intensity (nMFI) and coefficient of variation of immunofluorescence (CVIF) was calculated. Expression pattern were compared with NMP.

Result: A mixed cohort of 60 adult and pediatric patients was studied. Median age of patients was 18 years (range 5-67 years). Of 60, MRD was available in 32 patients and 21/32 were MRD positive. Amongst the 6 markers studied, the highest frequency of LAIP in diagnostic samples was observed with CD54 in 35/60(58.3%) followed by CD93 in 15/60(25%), CD52 in 12/60(20%), CD97 in 12/60(20%), CD9 in 11/60(18.3%) and CD44 in 11/60(18.3%). We studied the post-therapy stability of these markers in 21 MRD-positive samples. The proportion of samples with immune shift in order of “Stable”, “Loss” and “Gain” of expression for these markers was as follow: CD54-53.8%, 46.2%, 33.3%; CD93-

zero%,100%; CD52- 82.4%, 17.6%, zero%; CD97- 40%, 60%, 6.2%; CD9- 20%, 80%, 6.2%; and CD44 40%, 60%, 12.4%. Thus, CD54 and CD52 showed highest LAIP in MRD positive samples with high stability.

Conclusion: Out of 6 markers studied for AML-MRD monitoring, CD54, and CD52 showed the highest frequency of LAIP and the best post-treatment stability in their expression. Thus, incorporating CD54, and CD52 in MFC-MRD analysis in AML patients shall further enhance the accuracy and applicability of MRD assay.

HEM-32

CD72 - A PROMISING MARKER FOR MRD MONITORING IN PATIENTS AFTER CD19-TARGETED IMMUNOTHERAPY

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Introduction: B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common hematological malignancy in children. In recent years, the immunotherapy treatment for BCP-ALL patients is increasingly used for high-risk and relapsed patients. CD19 is one of the most specific molecules determining the B-cell lineage of blast cells in BCP-ALL. Thus, it became a "default" target marker in BCP-ALL immunotherapy. However, in part of the patients with targeted treatment, blast cells lose CD19 molecule, which makes them no longer sensitive to anti-CD19 immunotherapy. On the other hand, with the loss of CD19 expression, detection of minimal residual disease (MRD) also becomes more difficult. To increase the efficacy of MRD monitoring in those specific cases, additional alternative markers should be investigated.

Methods: We have evaluated CD72 expression on blast cells in a group of 83 patients with BCP-ALL at diagnosis and at day 15 of induction treatment. After initial frontline therapy, part of the patients has undergone CD19-targeted immunotherapy. Within this group of patients, nine were MRD-positive with CD72 expression evaluated.

Results: Expression of CD72 on BCP-ALL blasts was found at diagnosis in 99% of patients with mean median of fluorescence intensity (MFI) value of 2156. Mean MFI of CD72 was lower (dim) than on mature B-cells (MFI=7748), but still clearly positive. Expression level of CD72 at day 15 of induction treatment was stable, with the mean MFI=2858. In 6 of 9 patients with positive MRD after CD19-targeted immunotherapy, loss of CD19 on blast cells was observed, whereas all 9 patients showed stable expression of CD72 on blasts, with mean MFI=1891.

Conclusions: Stable expression of the CD72 marker on blasts after CD19-targeted immunotherapy, makes it a useful marker for MRD monitoring. Moreover, CD72 could be an alternative and promising target for immunotherapy in case of CD19 loss.

HEM-33

DETECTION OF MEASURABLE RESIDUAL DISEASE IS AN IMPORTANT PREDICTOR OF RELAPSE IN CHILDHOOD AML

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Introduction: Measurable residual disease (MRD) is emerging as an important monitoring tool in Childhood Acute Myeloid Leukemia (AML). We are presenting our experience using a 16 colour single tube flow cytometric MRD assay.

Methods: All children upto the age of 15 years who were diagnosed with AML as per WHO 2016 criteria and underwent standard 3+7 Induction with Daunorubicin and Cytosine Arabinoside followed by 3 cycles of High dose Cytosine Arabinoside treatment at Tata Memorial Hospital between April 2018 to March 2021 were included in the study after informed consent. Diagnostic immunophenotyping was done using 10-13 colour multicolour flowcytometry (MFC) on DxFlex. The AML MRD was performed using Difference from Normal approach with a 16 colour tube on LSR Fortessa(BD) using Bulk Lyse stain method and minimum 1.6 million cells were acquired. Data was analyzed using Kaluza 1.3 & 2.1 analysis software. The MRD was performed at post induction and post consolidation time points.

Results: 93 Children with AML were enrolled into the study during this period. The children were aged 0.1- 15 years (median – 8 years). There were 62 males and 31 females. The baseline TLC in these patients ranged from 2.61 x 10e9/L to 266 x 10e9/L (median – 19x 10 e9/L). 60/93 (64.51%) patients were in favourable risk based on 2022 ELN genetic risk classification at initial diagnosis. The children had a median follow up of 15.17 months (Range, 1.7-42.6 months). The children with favourable risk AML had longer relapse free survival (RFS) of 40.6 months as compared to 12.76 months (Hazard ratio – 0.32 p= 0.0002) in non-favourable risk. 40.8% (38/93) patients had a median detectable MRD at end of Induction of 0.74% (Range, 0.006% - 69.3%). The end of Induction MRD was associated with increased risk of relapse ($p<0.0001$) with Hazard ratio of 3.63. The median RFS was 14.37 months for children with detectable MRD at end of Induction as compared to median survival not reached in undetectable MRD. Absence of detectable End of Induction MRD and Favourable risk were independently associated with longer RFS on Multivariate analysis. Post Consolidation MRD was available in 37/38 post induction MRD positive patients. 13/37 had detectable Post Consolidation MRD with median of 0.19% (Range 0.008% - 36%). However, it was not significant for RFS (17.3 months vs 10.2 months, $p = 0.09$)

Conclusion: End of Induction MRD is a powerful independent predictor of RFS in Childhood AML

HEM-34

A SIMPLE PROTOCOL FOR DETECTING CLONAL T CELL EXPANSIONS IN CHILDREN WITH PERSISTENT EOSINOPHILIA.

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Introduction: Diagnostic approach of persistent unexplained eosinophilia includes investigation of clonal T cell expansion, especially concerning the Th2 subset. The availability of TCR Cβ1 Antibody (JOVI.1) facilitates the study of T cell clonality. We aimed to evaluate the applicability of a single tube flow cytometry protocol in the study of the T cell compartment and its clonality in children with eosinophilia.

Methods: Blood was collected in EDTA tubes from 17 patients (median age 7.9 years) with persistent eosinophilia. Additionally, 15 more patients (median age 11.2 years) referred for other causes and with normal eosinophil counts were also tested. The following panel was used: CD183-AF 488/ TCR Cβ1 -PE/ CD45RA-ECD/ CD19-PC5.5/ CD197-PC7/ CD279-APC/ CD161-APC-AF 750/ CD194-BV421/ CD4- BV 605/ CD8-KRO. Percentage of TCR Cβ1+ cells among various memory CD4+ subsets was recorded. DxFlex (Beckman Coulter) instrument was used for data acquisition and analysis.

Results: Based on the above combination we were able to identify the following memory (NOT CD45RA+CD197+) CD4+ cell subsets: Tfh (CD279+CD185+), Th1/Th9 (NOT Tfh AND CD183+CD161-CD194-), Th2/Th22 (NOT Tfh AND CD183-CD161-CD194+), Th17 (NOT Tfh AND CD183-CD161+CD194-) and Th1/17 (NOT Tfh AND CD183+CD161+CD194-). Th2/Th22 percentages on memory CD4+ cells were 3.8%-35.5% (median 19.6%) in the non-eosinophilic controls and 7.0%-36.2% (median 17.3%) in the group of patients with eosinophilia, showing no statistically significant differences. TCR Cβ1 expression distribution on the above subsets was even in all 15 controls and in 16 out of 17 eosinophilic patients. However, in a 14-year-old patient with hypereosinophilic syndrome, not only was a significant elevation of Th2/Th22 cell percentage noticed (35.5% on memory CD4+ cells), but also an additional atypical CD183+CD194+ double positive subset was present in 37.1%. TCR Cβ1 expression on both typical Th2/Th22 and Double Positive CD183+CD194+ subsets was unevenly distributed, with 86% and 88% of them being TCR Cβ1 negative respectively.

Conclusions: Our center's experience shows that this easily applicable one-tube flow cytometry protocol was able to detect a clonal T cell expansion in an eosinophilic patient, which would not be easily noticed on total T cells. In depth T cell immune profiling may facilitate the detection of otherwise occult expansions in minor T cell subsets and provide a tool for further studies of unexplained persistent eosinophilia. The use of additional markers (as CD196) could assist further characterization of subsets with statistically significant elevations.

HEM-35

DEVELOPMENT AND VALIDATION OF FLOW CYTOMETRY ASSAYS FOR AUTOLOGOUS AND ALLOGENIC CAR T IN GLOBAL CLINICAL PROGRAMS

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Introduction: With the breakthrough in cell & gene therapies as hematological malignancy treatment, such as chimeric antigen receptor T (CAR T) cell therapy, it is critical to assess CAR T cell expansion and persistence in patients as recommended by the FDA draft guidance. Flow cytometry plays a central role in monitoring CAR T cells in clinical trials. Most current CAR T therapies use patient's own engineered T cells, i.e., autologous CAR T. Due to the reduced cost and "off-the-shelf" applicability, more and more clinical trials are performed using the next generation allogenic CAR T, which are engineered T cells from healthy donors.

Method: General challenges for validating CAR T flow cytometry assays are the lack of CAR+ validation samples prior to trial start and the lack of CAR+ control material for transparency testing between global labs. Additionally, patients are lymphodepleted before CAR T infusion so the patient samples will have low lymphocytes at early time points. These challenges must be considered during assay development and validation. Allogenic CAR T cells are designed to avoid graft vs host disease, so the panel design needs to fit specific CAR T immunophenotypic profile to appropriately identify the CAR product.

For assay validation, CAR T cells are spiked into blood from healthy donors. CAR T cells and detection antibodies (anti-idiotype) are specific to each CAR T clinical program. Validation parameters assessed are based on the context-of-use of the data. As allogenic CAR T cells may require more intense lymphodepletion than autologous CAR T, assay development for these trials should also include bulk-lysis procedure to stain more cells.

Results: Autologous CAR T cells are usually identified with T cell lineage marker and anti-idiotype antibodies (sponsor-specific), but allogenic CAR T can additionally be identified with a tag-antibody or a labeled target peptide, requiring adapted gating strategies. Some of the challenges and resolutions in validating and testing patient samples for autologous and allogenic CAR T trials will be discussed.

Conclusions: At Cerba Research, we have successfully validated and implemented flow cytometry assays for both autologous and allogenic CAR T therapies in various Cerba Research associated laboratories for global trials. Bulk lysis procedure and specific gating strategy were implemented for allogenic CAR T trials. In addition, CAR T enumeration assay was implemented globally for secondary endpoint assessment to measure absolute count of CAR T cells in patients.

HEM-36

VALIDATION OF A HARMONIZED ASSAY FOR EX VIVO MONITORING OF CD19 CAR-T CELLS

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Introduction: New strategies in immunotherapy in the last decennium have led to a breakthrough in the treatment of several cancer types. One of these new therapies involves chimeric antigen (CAR)-modified T cells. There is evidence that CAR-T cell persistence and effector/memory differentiation status of the CAR-T cell product are important for therapy response. As long as CAR-T cells are present, the chance of antigen+ relapse is minimal, but one must be alert to an antigen-negative relapse. In this context, it is desirable to monitor CAR-T cells *ex vivo* in patient samples (e.g. peripheral blood, bone

marrow, cerebrospinal fluid). Here, we describe the extensive validation of a harmonized CAR-T cell monitoring assay, according to the NVC/HOVON immune monitoring group recommendations.

Methods: In our validation we used *ex vivo* generated CART-T cells, diluted in PBMC or PBS. First, the cells were stained with indirect CD19 CAR detection reagent, followed by a second staining with the following markers, CD8-FITC, Biotine-PE, CD14-ECD, CD45RO-APC-AF750, CD4/CD20-PacB, CD45-KO, 7AAD. The cells were analyzed on a Navios flow cytometer, the acquired data were reanalyzed in Kaluza software. Multiple quality standards were evaluated including accuracy, intra-assay variation, inter-assay variation, measurement uncertainty, specificity and selectivity and detection limit. Evaluations were performed for different concentrations of CAR-T cells (in PBMC of PBS) in manifold at 1 day and/or at multiple time-points.

Results: Accuracy of the mean % CAR-T cells within CD3+ T-cells was 100% (range 96.1-103.5%). Intra-assay variation of %CAR-T cells, reflecting precision, was 4.2% (range 2.9-5.9%). Inter-assay variation (1 dilution determined by measurements on 3 separate days) was 9.3%. Measurement uncertainty, defined as 2x the intra-assay variation, was 8.5% (range 4.7-11.8%). Specificity of the %CAR-T cells was evaluated within WBC of healthy donors, showing≤0.01% CAR-T cells within WBC. Detection limit of CAR-T cell population was ≥0.01%, requiring >20 CAR-T+ T-cells were measured per 500,000 viable leucocytes.

Conclusion: Our study demonstrates the validation of a harmonized CD19 CAR-T panel for post-infusion monitoring of CAR-T cells.

HEM-37

WHEN CMML FLUSHES OUT PNH: THE FLOW CYTOMETRY WEIGHT

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Introduction: A 75-year-old male was referred for a bone marrow aspiration due to chronic anemia and thrombocytopenia, raising suspicion of myelodysplastic syndrome (MDS). The complete blood count showed aregenative anemia, thrombocytopenia, relative and absolute monocytosis. The blood smear revealed dysgranulopoiesis without abnormal cells. Bone marrow smears were rich, with numerous megakaryocytes, a significant dysmyelopoiesis and no excess blasts. LDH was increased. These findings collectively pointed towards a diagnosis of chronic myelomonocytic leukemia (CMML).

Methods: As recommended by the 2022 WHO classification, monocyte subset partitioning in peripheral blood was performed by flow cytometry to accurately quantify the three monocyte subsets (classical cMo $CD14^{++}CD16^{-}$, intermediate iMo $CD14^{++}CD16^{+}$ and non-classical ncMo $CD14^{low/-}CD16^{-}$). This analysis involves an exclusion strategy using lineage antibodies to exclude T lymphocytes, neutrophils, eosinophils, immature granulocytes (IG) and B lymphocytes. Subsequently, a CD14/CD16 bi-parametric histogram is used to eliminate residual contaminating cells $CD14^{-}CD16^{-}$ (basophiles and NK cells not previously excluded).

Results: A significant population of $CD16^{-}CD24^{-}$ neutrophils was detected (65% of neutrophils). The blood smear did not show neither IG nor eosinophils, which are two $CD16^{-}$ populations that could have corresponded to the identified $CD16^{-}$ cluster. This fortuitous discovery of combined deficiency of CD16

and CD24, two GPI-anchored proteins on neutrophils, led to suspicion of the presence of a Paroxysmal Nocturnal Hemoglobinuria (PNH) clone. The analysis of monocyte subsets repartition was uninterpretable due to significant loss of CD14 and CD16, two GPI-anchored proteins on monocytes, resulting in an underestimation of the three monocyte populations. Subsequent hemolysis assessment revealed decreased haptoglobin levels and confirmed elevated LDH. Analysis of other GPI-anchored protein expression (FLAER, CD55 on leukocytes, CD59 on red blood cells) demonstrated the expected presence of a large clone on neutrophils, monocytes and red blood cells. The diagnosis of CMML was further confirmed through cytogenetic (trisomy of chromosome 14) and molecular (mutations of *U2AF1*, *SETBP2* and *MPL* genes) data.

Conclusions: This case illustrates the previously reported association of MDS/CMML and PNH, regardless of hemolysis in affected patients. The French National Authority for Health has already recommended testing for a PNH clone in all patients with MDS, further highlighting the necessity of systematic PNH clone testing in patients with MDS/CMML. Moreover, it emphasizes the importance of careful antibody selection when designing panels and analysis protocols for the incidental diagnosis of hemopathy associations, particularly when such associations have been previously reported and are not initially suspected by clinicians.

HEM-38

A MACHINE-LEARNING–BASED APPROACH TO AUTOMATICALLY DETECT LYMPHOPROLIFERATIVE DISORDERS USING FLOW DATA COLLECTED FROM THE BD ONEFLOW™ LST PANEL

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Introduction: Automated analysis of multiparametric flow cytometry data is an emerging demand due to the growing complexity and improvements of flow cytometry capabilities. The BD OneFlow™ Lymphoid Screening Tube (LST) is an 8-color assay that identifies the lineage of lymphoid neoplastic cells. This study developed a machine-learning analysis tool for automated analysis of the BD OneFlow™ LST reagent to determine normal or abnormal cell phenotypes in peripheral blood (PB) specimens.

Methods: A subset of 176 fcs files (108 normal and 68 abnormal) from peripheral blood (PB) from a multisite OneFlow™ LST study using FACSCanto™ II and FACSLyric™ were employed to train the disease classification models using a previous training framework model and lymphocyte classification models. Five-fold cross validation was applied to evaluate performance and detection threshold of the disease classification model, which was optimized to reduce false negative rate before was deployed to the cloud platform. The time from upload fcs file to result prediction on the user-interface was measured to calculate the speed of automated analysis of the deployed model.

Results: Classification model of abnormal phenotype achieved an average AUC of 97.1%, and accuracy average of 92.6%. Specificity and sensitivity were 93.5% and 91.0%, respectively. By adjusting the abnormality detection threshold along the ROC curve, the false negative rate reduced from 19% to 7% while the accuracy slight reduced from 91.5% to 90.3%. The average time from fcs file upload to prediction result displayed on the cloud platform were 21.3 seconds for single-fcs-file upload and 47.3

seconds for five-fcs-files uploaded simultaneously. The prediction results displayed include an interactive three-dimensional plot comparing the sample to the training database at specimen level and four-interactive scatter plots showing predicted T, B and NK cells of the sample in question.

Conclusions: The study demonstrated that the machine-learning approach can develop models using fcs files acquired across institutions and instrument. The preliminary results showed consistent identification of B-cell abnormal phenotype in PB specimens. Furthermore, the cloud-based model can detect specimen level abnormality and lymphocyte classification in less than a minute per sample.

HEM-39

ROLE OF FLOW CYTOMETRY DETECTION OF BLAST CELLS IN CEREBROSPINAL FLUID FOR NEUROLEUKEMIA DIAGNOSIS OF PATIENTS WITH T-ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

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Introduction: Russian treatment protocol ALL2016 for acute lymphoblastic leukemia (ALL) includes broad intrathecal prophylaxis of neuroleukemia. Diagnosis of neuroleukemia in the start of protocol was defined as indication of at least 1 blast cell in conventional cytospine (CC). We decided to find a role of flow cytometry (FC) detection of blast cells for neuroleukemia diagnosis for patients with T-acute lymphoblastic leukemia/lymphoma as the most often reason for central nervous system involvement in ALL.

Methods: we prospectively analyzed 29 patients with T-ALL who was enter the National Research center for Hematology for treatment from December 2018 to October 2022. Investigation was submitted by ethical committee. All patients enrolled in treatment protocol ALL 2016. Male/Female ratio was 19/9, Median of age was 32 years. Cerebrospinal fluid (CSF) was checked in the onset of disease by CC and FC for all patients. We used Thermo Scientific Cytospin 4 Centrifuge and 10 color BD Canto flow cytometer with BD reagents for investigation. Treatment of neuroleukemia was implemented respectively to CC data.

Results: we divided all patients in 3 groups. The 1st group contained patients with diagnosed neuroleukemia by both methods (8 persons), the 2d – patients without blast cells by both methods (16 persons) and the 3d one included 5 patients when blast cells was found by FC but not by CC. For the last group there were statistically significantly lower cell count of CSF (4,15; 1,35; 1,10 cells/mkl respectively (p=0,017)); lower white blood cells count (71,45; 14,2; $2,6*10^9$/l respectively (p=0.071)); but at the same time statistically significantly there were worse results of measurable residual disease on the 70^{th} day of treatment (positive MRD result in 37,5%; 21,4%; 100% for groups respectively (p=0,010)) and worse results of overall survival (100%; 90%; 50% respectively (p=0.028)).

Conclusions: we found occult neuroleukemia in patients with T-ALL by flow cytometry in 5 of 29 patients (17%). For all these patients the treatment of neuroleukemia was not performed but results of MRD on 70^{th} day of treatment and overall survival was statistically significantly lower. Inspite of

small group of investigation we suppose that occult neuroleukemia could have impact in outcome of T-ALL and have to be a reason for appropriate treatment changes.

HEM-40

ECLIPSE: A MULTIVARIATE APPROACH FOR ENHANCED DETECTION OF MINIMAL RESIDUAL DISEASE IN MULTIPLE MYELOMA USING MULTICOLOUR FLOW CYTOMETRY

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Introduction: The detection of Minimal Residual Disease (MRD) in haematological malignancies, such as Multiple Myeloma (MM), is crucial for evaluating treatment effectiveness and assessing the risk of relapse. Conventional analysis of MRD using manual gating in multicolour flow cytometry (MFC) is subjective, labour-intensive, and requires expertise. This study aims to introduce ECLIPSE (Elimination of Cells Lying in Patterns Similar to Endogeneity), a multivariate method designed to improve the detection of rare disease-specific cell populations in MM MRD by eliminating normal cells.

Methods: We applied the ECLIPSE method to 8-color MFC data obtained from bone marrow samples of 7 MM patients and 13 control bone marrow samples. ECLIPSE employs an automated elimination step for normal cells in patient samples. Firstly, ECLIPSE uses multiset Simultaneous Component Analysis (SCA) to describe the cell distribution of control bone marrow measurements. Subsequently, the measurements of patients samples at diagnosis and follow-up were projected into the SCA model and the residuals of each cell was calculated. Cells with a low residual error were classified as normal-like and removed, while cells with a high residual error were classified as aberrant and kept.

Results: ECLIPSE enabled fast and automated identification of MRD in all MM patients, with a lower limit of detection of 0.001%. Furthermore, in 5 out of 7 cases, ECLIPSE outperformed conventional manual gating by detecting a larger number of monoclonal plasma cells.

Conclusions: Our findings demonstrate that ECLIPSE, the multivariate approach developed for rare cell detection, is particularly well-suited for the identification of MM MRD. The method offers improved sensitivity, faster analysis, and requires less expert effort compared to conventional MFC analysis. By eliminating normal cells from analysis, ECLIPSE enhances the accuracy and reliability of MRD detection. The implementation of ECLIPSE in clinical settings has the potential to significantly impact the evaluation of treatment response and prediction of relapse in MM patients.

HEM-41

REAL-WORLD APPLICATION OF MODERN MACHINE LEARNING FOR FAST AND RELIABLE DECISION SUPPORT IN CLINICAL CYTOMETRY OF HEMATOLOGICAL NEOPLASMS

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Introduction: Flow cytometry is an integral part of routine diagnostics for hematological malignancies. The skill of flow cytometry analysis isn't just increasingly hard to find, it also results in inter- and intra-observer variability. To reduce labor time, dependency on expert knowledge and interpretation variability, we aimed to build and validate clinical-grade decision support software using machine learning (ML) for clinical cytometry.

Methods: We combined >80k flow cytometry files from multiple centers, taken from clinical routine and annotated by expert users using multiple diagnostic methods. We trained supervised ML algorithms to classify the B-NHL sub-type or non-malignancy, eight classes in total. Data pooling was used to combine data from different protocols. We built a web-application ("hema.to") in which selected users could upload cytometry data and diagnose new samples. Both speed and accuracy of the diagnostic recommendations were tested in a two-arm, four-center blinded clinical study, where annotations against the ground truth were tested and compared to the conventional workflow. Each of the two arms contained 96 non-overlapping randomly selected samples.

Results: The clinical study revealed that hema.to made the process faster and maintained diagnostic quality. Compared to the control arm, analysis time was reduced by >2x, and accuracy was increased by >6%. Following this success, we fine-tuned the model for routine use at two labs. A worst-case performance was computed by testing the model against historic data without expert supervision, and found an f1 score of >90% and sensitivity of 96%. This demonstrates that the software can be used for both screening and sub-typing. We therefore integrated hema.to into the routine for B-NHL diagnosis at the HpH (Hamburg) since January, including a deep integration into the laboratory database with automated reporting. The quality of the diagnostic recommendations has been stable over time despite a change of device type. We've expanded the models to classify acute leukaemia samples and found initial f1 scores of ≈90% and are starting development for detection of measurable residual disease.

Conclusions: We've shown that machine learning can offer decision support for screening and classifying blood cancers from flow cytometry in a routine setting. Data pooling was necessary to achieve clinical-grade performance. These results represent major strides towards decision support software for any lab and all hematological neoplasms. Such a software will not only speed up and simplify diagnostic workflows, but also improve the quality of the analysis.

HEM-42

CSF PERIPHERAL BLOOD CONTAMINATION FLOW CYTOMETRY ANALYSIS: IMPROVED METHOD

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Introduction: Cerebrospinal fluid (CSF) cell count is an important clinical procedure for diagnostic and prognostic classification of a wide variety of diseases. Flow cytometric immunophenotyping (FCM) of CSF is a recommended method for hematological malignancies patients with suspicion of leptomeningeal disease. However, the presence of peripheral blood cells in CSF, due to contamination during the lumbar puncture, can complicate the interpretation of the results.

Thus, evaluation of CSF peripheral blood contamination should be performed in all cases, especially when malignant cells are present in peripheral blood. Current methods for CSF peripheral blood contamination analysis consist of absolute cell counting of red blood cells (RBC) and/or neutrophils, however, these approaches lack sensitivity enough, particularly in those samples where even visibly undetectable contamination by peripheral blood drastically alters the CSF content.

A new method for evaluation of CSF contamination, that allow bypassing these disadvantages, would help to interpret diagnostic results.

Methods: A total of 117 CSF lysis supernatants from patients with infiltrating leukemia and lymphomas of which 34 showed traumatic punctures, were analyzed for peripheral blood contamination, comparing both classical neutrophil count vs. a new quantitative method developed by Immunostep. The new method is a sandwich bead-based flow cytometric RBC-specific analyte assay.

Results: Here, we evaluate the sensitivity of the new method versus conventional granulocyte count CSF blood contamination. Of the total CSF samples analyzed (117), in 23.08% (27) of peripheral blood contamination was detected by neutrophil count, while the new method was able to detect contamination a 90.06% (106). Interestingly neutrophil count detected contamination only in 55.88% (19) of the samples identified as traumatic punctures, while the new method was able to detect contamination in 100% (34) of these samples.

Conclusions: The new method improves very significantly the cell count sensitivity for CSF peripheral blood contamination analysis, improving the interpretation of diagnostic results.

HEM-43

FLOW CYTOMETRY VERSUS GDNA-BASED PCR DATA FOR MEASURABLE RESIDUAL DISEASE IN PEDIATRIC NON-STANDARD RISK ACUTE MYELOID LEUKEMIA

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Introduction: Measurable residual disease (MRD) assessment in pediatric acute myeloid leukemia (pedAML) is clinically crucial for patient treatment and highly demanding due to the disease heterogeneity without a single internationally applicable golden standard method. In this scope, we aimed to compare the performance of flow cytometry (FC) and a patient-specific DNA-based assay in non-standard risk (non-SR) patients with pedAML.

Methods: 41/77 consecutively recruited patients carried a chromosomal translocation providing a specific DNA sequence for genomic breakpoint analysis. A FC dual tube approach ("leukemia associated immunophenotype"- "LAIP" and "colony formation unit"- "CFU" tube)with customized dried format antibody cocktails was also implemented allowing AML MRD detection by immunophenotype as well as different from normal assessment. MRD levels were evaluated concomitantly by FC and gDNA-PCR in 183 bone marrow (BM) follow-up (FUP) samples from 36 patients at various timepoints during or after therapy.

Results: For the FC-MRD clinically relevant cut-off of ≥0.1%: 1. FC-MRD and gDNA-PCR data presented a high concordance level of 90.2%; negative FC-MRD, but positive gDNA-PCR MRD values, were detected in 7.7% samples, 2. maturation (M2 and M4 FAB type) led to reduced, but not significant, concordance of FC and gDNA-PCR MRD (80.0% vs. 91.8% in M2/M4 and M0/M1/M5/M7 FAB subtypes, respectively, p=0.066). For any positivity (i.e.,no threshold and ≥50 clustered leukemia cellular events): 1.overall concordance dropped to 68.9%, 2.maturation (M2 and M4 FAB type) led to reduced concordance of FC-MRD and gDNA-PCR MRD(44.0% vs. 72.8% in M2/M4 and M0/M1/M5/M7 FAB subtypes, respectively, p=0.0039). Irrespective of threshold application, FC-MRD and gDNA-PCR showed:1. increased sensitivity compared to morphologic assessment, 2. no significant differences among genetic subtypes' groups ("*KMT2Ar*", n=136; "*NUP98*", n=25; "*Miscellaneous*", n=22), although a marginal p value was recorded when the 0.1% threshold was applied.

Conclusions: FC and gDNA-PCR MRD results correlate well. The gDNA-PCR method is feasible in pediatric AML with traceable genetic rearrangements and is characterized by the advantages of a DNA-based methodology, such as sensitive quantification and stability. Altogether, the 2 methodologies can be applied complementarily in order to ameliorate patient assessment and stratification, especially in FAB types with maturation.

HEM-44

THE EXPRESSION OF SAP AND EAT-2 IS DECREASED IN CHRONIC LYMPHOCYTIC LEUKEMIA.

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Introduction: SAP (signaling lymphocytic activation molecule (SLAM) - associated protein) and EAT-2 (Ewing's sarcoma-associated transcript 2) are Src homology 2 (SH2) domain-containing intracellular adaptor proteins which couple SLAM family receptors to their intracellular signaling pathways.

Interestingly, SAP was also shown to interfere with programmed cell death-1 (PD-1) function. SAP and EAT-2 are expressed in T, NK, NKT and B cells and regulate their activation. The expression of SAP and EAT-2 in chronic lymhocytic leukemia (CLL) has not been studied yet.

Methods: Here, we analyzed the expression of SAP and EAT-2 intracellular adaptor proteins in B, T and NK cells of 69 patients with CLL by means of multiparametric multicolor flow cytometry.

Results: The strongest expression of SAP was observed in T cells followed by NK and B cells whereas the strongest expression of EAT-2 was detected in NK cells followed by T and B cells. The expression of EAT-2 was higher in T and NK cells from peripheral blood as compared to the bone marrow. Pathological CLL B cells showed significantly decreased expression of both SAP and EAT-2 in comparison with normal mature B cells ($p<0.001$). Higher expression of SAP in B cells was observed in patients with trisomy 12 ($p<0.05$) and relapsed vs. newly diagnosed patients ($p<0.05$). Lower SAP expression in B cells was associated with 11q deletion ($p<0.05$). The expression of SAP in T (both CD4+ and CD8+) and NK cells and the expression of EAT-2 in NK cells were higher in relapsed vs. newly diagnosed patients ($p<0.01$) and patients with poorer therapeutic response (PD/SD vs. CR/PR, $p<0.01$). On the other hand, no relationship was observed between SAP or EAT-2 expression and Rai clinical staging, CLL-IPI, and overall survival of CLL patients.

Conclusions: We observed a decreased expresion of both SAP and EAT-2 intracellular adaptor proteins in pathological vs. normal B cells of patients with CLL. The expression of SAP and EAT-2 in B, T and NK cells may play an important role in the biology of CLL.

HEM-45

BD ONEFLOW™ ALOT PERFORMANCE: INTERFERING SUBSTANCES STUDY

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Introduction: The BD OneFlow™ Acute Leukemia Orientation Tube (ALOT) is intended for flow cytometric immunophenotyping of aberrant immature populations of hematopoietic cells (lymphoid and nonlymphoid lineage) in bone marrow (BM) and peripheral blood (PB). This air dried down 8-color immunophenotyping panel aids in the diagnosis of acute lymphoblastic leukemia and non-lymphoid acute leukemia. Substances commonly found within BM and PB may interfere with biochemical and fluorescence assays and consequently cause significant difference in the assay test results and interpretation.

Methods: We evaluated the performance of the BD OneFlow™ ALOT in the presence of substances potentially found within PB or BM that may interfere with immunofluorescence staining and interpretation of cell scatter or fluorescence signal during evaluation by flow cytometry. The study design was based on the CLSI guidelines (EP07-A3). We used healthy BM spiked with endogenous and exogenous substances. Endogenous substances included hemoglobin, albumin, bilirubin (conjugated and unconjugated), triglycerides, and erythrocytes. The exogenous compounds were acetaminophen, acetylsalicylic acid (aspirin), ibuprofen, oseltamivir phosphate, ondansetron, dexamethasone, prednisolone, albuterol, guaifenesin, promethazine, cefotaxime, meropenem, and vancomycin. Test concentrations for exogenous substances were at 3X the highest concentration reported following a

drug therapeutic dose (acute peak concentration). For the endogenous substances, the test concentrations were at the highest expected concentration. For each interferent sample type, solvent controls were run in parallel. Spiked interference samples were compared to its solvent control for qualitative and quantitative evaluation by paired-difference analysis.

Results: There was 100% agreement of qualitative assessment for populations present in the sample when compared between test and reference. The mean bias between test and control, as quantitative assessment for populations present in the sample, ranged between 0.9 to -0.82.

Conclusion: The presence of interferent substances evaluated in this study did not significantly affect the performance of the BD OneFlow™ ALOT.

This research is scientific in nature. Products NOT for diagnostic use.

HEM-46

BD ONEFLOW™ ASSAYS PERFORMANCE: DETECTION CAPABILITY OF LST, B-CLPD T1, ALOT, PCST, PCD

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Introduction: The BD OneFlow™ Assays are intended for flow cytometric immunophenotyping of normal and aberrant populations in bone marrow (BM), peripheral blood (PB), and lymphoid tissues as an aid in the diagnosis of leukemia, lymphoma, and plasma cell disorders. The assays are LST (Lymphoid Screening Tube), B-CLPD T1 (B-cell Chronic Lymphoproliferative Diseases Tube 1), ALOT (Acute Leukemia Orientation Tube), PCST (Plasma Cell Screening Tube) and PCD (Plasma Cell Disorders Tube). As qualitative assays, their detection capability refers to the ability to distinguish abnormal cells from normal cells.

Methods: We evaluated Limit of Blank (LoB) and Limit of Detection (LoD) of these BD OneFlow™ Assays. The study design was referenced from ICCS and ICSH Practice Guidelines. We spiked diseased specimen into normal specimen (BM/PB) at three known LoD ranges of abnormal cells: 0.02%, 0.05% and 0.1% of All Events and stained using each of the five BD OneFlow™ Reagents. Normal unspiked specimens were used as a control and for LoB assessment. Analysis was based on the phenotype of the abnormal specimen. The differences in immunophenotype of the normal and abnormal specimens enabled the ability to distinguish the normal and abnormal populations within a spiked sample. LoB was determined based on the maximum value of the two metrics i.e., the maximum value observed and the 95th percentile of the observed background events in normal unspiked specimen. Trueness and standard deviation of the bias were calculated along with 95% lower and upper bound values to determine LoD. Trueness was calculated as the ratio of the actual number of abnormal phenotype readings over the expected number.

Results: All five BD OneFlow™ Reagent Tubes i.e., LST, B-CLPD T1, ALOT, PCST and PCD, could precisely identify abnormal population in spiked samples at abnormal cell ranges of 0.02%, 0.05% and 0.1% of All Events. The trueness was 100% for all the candidate concentrations for all the five BD OneFlow™ Tubes.

Conclusions: The study results suggest that the Limit of Blank (LoB) and the Limit of Detection (LoD) of the five BD OneFlow™ Assays is 0.03% of All Events and 0.1% of All Events, respectively.

This research is scientific in nature. Products NOT for diagnostic use.

HEM-47

DEEP CHARACTERIZATION OF IMMUNE DYSFUNCTION IN PATIENTS WITH MULTIPLE MYELOMA (MM) AND IDENTIFICATION OF CELLULAR BIOMARKERS FOR TAILORED VACCINATION STRATEGIES

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Introduction: Infection is the leading cause of death in MM. However, the extent of immune dysfunction compared to other B-cell lymphoproliferative disorders (B-CLPD) and healthy adults remains unexplored. Aim: Generate an atlas of immune dysfunction in MM vs B-CLPD patients and age-matched health-care practitioners (HCP) using COVID-19 mRNA-vaccines as a case study.

Methods: A total of 1,099 blood and serum samples from 28 MM, 53 B-CLPD and 96 HCP were collected before vaccination, at days 7 and 14 post-first dose, at days 7 and 62 post-second dose, as well as before and at day 17 post-booster. Immune profiling was performed using multidimensional and computational flow cytometry that systematically analyzed 56 immune cell-types per sample and time point. Serum levels of IgM, IgG and IgA against the receptor-binding domain (RBD) of the spike (S) glycoprotein, S-glycoprotein, nucleocapsid (N) and main protease were quantified using a multiplex-microsphere-based assay. SARS-CoV-2-specific CD8 T-cells were quantified using a dextramer panel of S, N, membrane, and ORF3 proteins.

Results: When compared to HCP and/or B-CLPD, MM patients showed abnormal basal distribution of 17/17 B, 22/30 T, 4/6 antigen-presenting and 1/3 granulocytic cell-subsets. During vaccination, they were unable to expand the B-cell compartment, and antibody titers were lower after the second dose. Importantly, the booster increased anti-RBD IgG levels in HCP, but not in MM and B-CLPD. The T-cell compartment expansion was also altered in MM, and virus-specific CD8 T-cells after second and booster doses did not increase in MM and B-CLPD, contrary to HCP. Furthermore, the booster induced effector-memory differentiation in HCP, but not in MM and B-CLPD. In addition, MM patients showed abnormal kinetics of antigen-presenting and granulocytic subsets. An immune dysregulation longitudinal cumulative score based on differences in immune-cell distribution vs HCP showed high immune-dysregulation in 34% MM and 17% B-CLPD patients. Among them, 75% and 33%, respectively, had low seroconversion after second dose. We found 8/30 T- and 14/17 B-cell subsets associated with poor vaccine-response in MM, namely CD127lowPD1+CXCR5+ effector-memory CD8 T-cells, as well as CD21+ naïve and transitional B-cells.

Conclusion: We provide an atlas of immune dysfunction in MM patients and how it affects the efficacy of vaccination strategies such as for COVID-19. The schedule of vaccine doses may thus benefit from individualization according to patients' immune status, which reflects host, tumor and treatment-

related immune dysfunction, and can be readily monitored using key cell types identified here by routinely available flow cytometry.

HEM-48

LEUKEMIA IMMUNOPHENOTYPING PROFILE IN YEMEN

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Background: Leukemia is a clonal disease derived from a single cell in bone marrow or peripheral lymphoid tissues.

Objectives: The aim of this study was to use flow cytometry to correctly classify acute and chronic leukemias using 3-color antibody panels.

Methods: The immunophenotype of 2327 leukemia cases that presented over six years were assessed using a single laser FACSCalibur flow cytometer. Bone marrow or peripheral blood cells were analyzed using CD45 vs side scatter gating to identify the leukemia cells. The cells were further characterized using antibodies against CD1a, CD2, CD3 ,CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13 ,CD14, CD15, CD16, CD19, CD20, CD22, CD23, CD25, CD33, CD34, CD38, CD41a, CD45, CD56, CD64, CD79a, CD117, CD235a, anti-HLA-DR, anti-kappa and anti-lambda as appropriate.

Results: Of the 2327 leukemia cases, 1293 (55.6%) were adults. 1938(83.3%) of cases had an immunophenotype consistent with acute leukemia (AL) and 389 (16.7%) with chronic leukemia (CL),
Amongst the ALs, there were 1048/1949 (56.3%) with acute lymphoblastic leukemia (ALL) including 825 (%) with B lineage (B-ALL) and 226 with T lineage (T-ALL). 834 (36.7%) showed myeloid differentiation (AML) and a further 57 (1.2%) were ALs of ambiguous lineage.
All 378 patients with immunophenotypes consistent with CL were adults. Amongst these cases there were 335 (89%) with chronic lymphocytic leukemia, 26 (9%) with hairy cell leukemia (HCL) and 32 (2%) with other CLs.
Conclusion: Using flow cytometry and 3 color antibody panels, we found that AL is the most common type of leukemia diagnosed in Yemen. CLL is uncommon, but it is not known how many patients remain undiagnosed. ALL is more common than AML in children and AML is more common in adults in keeping with the literatures.

Key Words: Flow cytometry, Leukemia, Immunophenotyping.

IMM-01

FLOW CYTOMETRIC EVALUATION OF NORMAL RANGES OF SURFACE IMMUNOGLOBULIN B-CELL SUBSETS IN HEALTHY CONTROLS

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Introduction: Common variable immunodeficiency (CVID) is a rare but clinically important inborn errors of immunity (IEI) characterised by significantly reduced immunoglobulin levels with low to absent antibody production. Flow cytometry is a rapid and highly sensitive screening tool to diagnose and characterize IEI. Recently Euroflow has demonstrated the utility of levels of B-cell subsets expressing different surface heavy-chain immunoglobulins (sIg) such as IgG, IgA, IgM, IgD and IgE in the diagnosis of IEI. However, data on the normal range of sIg subsets in healthy controls is limited and there is no data from Indian population. Hence, we aimed to evaluate normal ranges of sIg subsets in healthy controls.

Methods: We studied sIg B-subsets in peripheral blood from 36 healthy volunteers. 11-color multicolor flow cytometry (MFC) assay with antibodies against IgG1 (clone SAG1), IgG2 (clone SAG2), IgG3 (clone SAG3), IgG4 (clone SAG4) IgA1 (clone SAA1), IgA2 (clone SAA2), IgM (clone, G0-127) and IgD (clone IA6-2) along with backbone B -cell markers (CD38, CD27, CD45, CD20, CD19, IgM, IgD). Data was acquired on LSR Fortessa (BD Biosciences) and analysed using Kaluza software version2.2.

Results: Median age of participating volunteers was 27 years (range 22-45 years) and included 25 females and 11 males. The median percentage of B-lymphocytes was 4.95% (range 1.89-18.62). Median (range) of naive and memory B-cells were 70.65% (43.67-86.14) and 25.19% (5.02-53.09) respectively. The median percentage of IgG1+B was 5.78% (range 0.76-15.33), IgG2+B 2.16% (range 0.35-5.63), IgG3+B 1.35% (range 0.10-8.36), IgG4+B 0.31% (range 0.02-1.17), IgA1+B 3.44% (range 0.29-7.73), IgA2+B 1.65% (range 0.20-6.13), IgM+B 84.20% (range 64.32-93.99) and IgD+B 79.18% (range 56.66-96.30).

Conclusion: We have evaluated the normal range of different surface immunoglobulin expressing B-cell subsets in healthy volunteers from India. These ranges will be useful to enhance the utility of flow cytometric diagnosis inborn errors of immunity including CVID.

IMM-02

THE PROPENSITY OF B CELLS TO INFILTRATE THE MS BRAIN: IMPACT OF GENETIC VARIATION AND ENVIRONMENT

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Introduction: The interplay between Epstein-Barr virus (EBV) and specific genetic risk variants is assumed to alter the functional program of B cells in patients with multiple sclerosis (MS). Very recently, our group revealed that CXCR3$^+$ B cells are preferentially recruited to the central nervous system (CNS), correspond with local antibody production and are promising targets for the Bruton's tyrosine kinase (BTK) inhibitor evobrutinib (EVO) in MS. In this study, we explored the contribution of environmental and genetic risk factors to the inducibility of brain-homing CXCR3$^+$ B cells in MS and examined whether this could be targeted by EVO.

Methods: B-cell subsets were analyzed in blood of monozygotic twin pairs discordant for MS using mass cytometry to study the impact of environmental factors. In addition to this, human B-cell lines and primary B cells were selected based on the presence of an MS risk SNP in *IFNGR2* (rs9808753) to examine the IFN-γ signaling pathway using flow cytometry, phosphoflow and immunoblotting. *In vitro*-stimulated blood B cells were compared between MS patients and controls and treated with or without EVO.

Results: Amongst all B-cell subsets analyzed, the frequency of CXCR3$^+$ class-switched memory B cells was reduced in genetically identical twins with MS vs unaffected co-twins. This reduction was not seen in twins treated with natalizumab (anti-VLA-4 antibody, inhibiting CNS immune cell entry). These data support our previous findings for CXCR3$^+$ B cells in blood from patients with MS vs healthy controls, emphasizing their selective induction by EBV and recruitment to the CNS. In B-cell lines carrying the *IFNGR2* risk SNP, STAT1 phosphorylation was more pronounced after addition of IFN-γ, the main trigger of CXCR3$^+$ B cells. The IFN-γ pathway was constitutively activated in EBV-infected B cells, showing upregulation of IFNGR1, IFNGR2 and phosphorylated STAT1. In primary B cells, CD40 ligand-induced surface expression of IFNGR2 and not IFNGR1 was attenuated by EVO. In B cells from patients with MS, specifically for risk SNP carriers, STAT1 phosphorylation was highest in the transitional subset (CD38^{high}CD27$^-$) at baseline and T-bet transcription was more induced by IFN-γ.

Conclusion: The twin data show that circulating CXCR3$^+$ B cells are influenced by environmental factors in patients with MS. The work also provides further evidence that EBV interacts with genetic risk variants to induce these types of B cells to enter the CNS, a process that may be prevented by the BTK inhibitor EVO in MS.

IMM-03

COMPARISON OF AUTOMATED VS. MANUAL PBMC ISOLATION

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Peripheral blood mononuclear cells (PBMCs) isolated from human blood are often used in *in-vitro* functional assays to evaluate cellular responses.

The first aim of this work was to compare T cell functionality based on INFγ readout from PBMCs isolated by two different isolation procedures. One procedure is using an automated instrument

designed to target unwanted cells for removal with antibody complexes and magnetic particles by negative selection. The other procedure is the well-known traditional and manual density gradient centrifugation procedure that can separate PBMCs from other components of whole blood based on differential cell densities. The switch to automated PBMC isolation would reduce the inter-operator variation to a minimum and would simplify the PBMC isolation training at multiple collection sites.

The second aim of this work was to compare cryopreservation using FBS/DMSO versus a synthetic animal-free cryopreservation media. Furthermore, freezing of the isolated PBMCs was performed using the two different media and after thawing, assessed by specific T cell functionality after stimulation with specific peptide mixes. The advantage of replacing FBS with a synthetic media is to reduce operational burden and for ethical reasons.

IMM-04

PREVALENCE OF TH17.1 AND ACTIVATED TREG CELLS IN BILIARY ATRESIA PATIENTS AND ITS CORRELATION WITH LIVER INJURY INDICES

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Introduction: Biliary atresia (BA) is a progressive fibro-inflammatory cholangiopathy of infancy associated with excessive adaptive and innate immune reactions targeting bile ducts with still unclear effects on liver injury status. The aim of the study was to identify immune characteristics of peripheral leukocytes which would be associated with clinical outcome parameters and eventually would be use as BA biomarkers.

Methods: We tested: peripheral T (naïve, memory, Th1, Th2, Th17, Th17.1 and Tregs) and B (naïve, memory and Bregs) lymphocyte subsets distribution and expression of metalloproteinase (MMP) -2 and -9 (by flow cytometry), leukocyte gene expression pattern (by RT-PCR) of *MMP-2, MMP-9,* tissue inhibitors of MMPs (*TIMP-1* and *TIMP-2*)*, IL-1β, IL-6, IL-17A, IL-21, IL-ra, TGF-β, T-bet, CD161, GATA-3, FoxP3, RORγT* and plasma MMP-9, TIMP-1, TIMP-2, IL-6, IL-17 concentration levels (ELISA) in blood samples of 25 BA children and 12 age-matched controls.

Results: BA children had: A) lower frequency of total Tregs but higher proportion of activated latency associated peptide $(LAP)^+$ Tregs, Th17.1 cells, and MMP-2 and MMP-9 bearing lymphocytes, B) increased plasma TIMP-1 level and leukocyte expression of *MMP-9*, *TIMP-1*, *TIMP-2*, *IL-6*, and *TGF-β* but decreased expression of *IL-21* and *T-bet*, C) negative correlations between LAP^+ Tregs vs hemoglobin, but positive vs total and direct bilirubin and PT/INT, D) positive correlations of: 1) frequency of Th17.1 cells vs ALT, AST and PT/INR, 2) plasma IL-6 level with direct bilirubin and ALT, 3) *IL-6* and *TIMP1* with γ-glutamyltransferase, and 4) *MMP-9* with total billirubin and PT/INR. Among plasma mediators tested, only TIMP-1 levels increased significantly, and IL-6 concentration correlated positively with billirubin direct and ALT levels.

Conclusion: Poor outcomes parameters in BA infants correlated with higher novel Th17 subpopulation (Th17.1 cells), activated LAP^+ Tregs subset, and increased plasma IL-6 level as well as leukocyte expression of *IL-6, MMP-9, and TIMP-1*.

IMM-05

EVALUATION OF THE ANTIPROLIFERATIVE ACTIVITY OF NEW AMIDRAZONE DERIVATIVES CONTAINING THE CYCLOHEX-1-ENE-1-CARBOXYLIC ACID SYSTEM

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Introduction: In the search for new anti-inflammatory agents, 6 amidrazone derivatives containing the cyclohex-1-ene-1-carboxylic acid system were synthesized. The toxicity and antiproliferative activity of these compounds towards human peripheral blood mononuclear cells (PBMC) were experimentally evaluated.

Methods: PBMC were isolated from freshly drawn blood from volunteer healthy donors. The study was approved by the Bioethics Committee of the Nicolaus Copernicus University in Toruń (Poland). Compounds at concentrations of 10, 50 and 100 µg/mL were added to PBMC cultures. Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used to test the toxicity of compounds in PBMC cultures and VPD450 dye (BD Pharmingen) was used to evaluate their antiproliferative activity. The tests were performed on a cytometer CytoFLEX (Beckman Coulter). Data analysis was performed in the CytExpert 2.3 program.

Results: The percentages of viable, apoptotic and necrotic cells were determined. The toxicity of the tested compounds depended on the concentration used, in 10 µg/mL it was low, in 50-100 ug/mL it was moderate. One of the compounds at a concentration of 100 µg/ml caused apoptosis of about 74% of the cells. The tested compounds also showed a dose-dependent antiproliferative effect. Three compounds in the highest dose inhibited proliferation in about 81-85%, and one in about 90%.

Conclusions: Due to the low or moderate toxicity and strong antiproliferative potential, 4 compounds deserve further research in the direction of anti-inflammatory and anticancer activity.

IMM-06

DETECTION AND DIAGNOSIS OF CLINICALLY LATENT PULMONARY ALVEOLAR PROTEINOSIS - CASE SERIES

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Introduction: Pulmonary Alveolar Proteinosis (PAP) is a rare diffuse pulmonary syndrome and is characterized by progressive accumulation of surfactant lipoprotein material in the alveoli, resulting in impaired gas exchange and often respiratory failure. Bronchoalveolar lavage fluid (BALF) examination plays a central role in diagnosis in typical cases. The purpose of our study is to emphasize the importance of careful evaluation of specific findings in BALF sample analysis in a flow cytometry laboratory, which in clinically latent cases, taken together are suggestive of PAP.

Methods: In 2022 3 BALF samples from the same number of patients, without any clinical suspicion of PAP, were submitted to our laboratory for examination. During the procedure of their analysis, counting of total BALF cells in the body fluid (BF) mode of hematology analyzer (Body fluid, XN1000-SYSMEX, ROCHE), May Grünwald-Giemsa staining, flow cytometry analysis (FACSCalibur, BD) and PAS (Periodic Acid-Schiff) staining were applied. Macroscopically, none of them had the strongly milky appearance typical of PAP.

Results: During the processing of all 3 BALF samples the simultaneous presence of the following characteristic laboratory findings was observed: 1) inability of white blood cells classification, inaccurate counting of total BALF cells and marking of results with an asterisk, during the analysis in the BF mode of the hematology analyzer, 2) presence of acellular aggregates - amorphous debris with a 'dirty' background, during the study of the BALF smear, after cytocentrifugation and May Grünwald-Giemsa staining and 3) in the SSC/CD45 dot plot: a) appearance of a characteristic picture with a 'sickle-shaped' population, which cannot be standardized by monoclonal antibodies used to identify lymphocyte subpopulations and b) lymphocyte population less than 3%, when analyzed in the cytometer. After evaluation of the above findings, PAS staining, which is pathognomonic for PAP, was suggested. In all 3 cases, PAS staining was positive, establishing the diagnosis of PAP.

Conclusions: Pulmonary Alveolar Proteinosis (PAP) is usually diagnosed by patient's symptoms, radiographic findings and/or during bronchoalveolar lavage, but in some cases the diagnosis may be missed for a long time. The simultaneous presence of specific laboratory data suggestive of PAP during the processing of BALF samples, should raise the suspicion of the existence of the syndrome. Therefore, the flow cytometry laboratory can play an important role in the diagnosis of PAP in these cases, helping to treat patients early and correctly.

IMM-07

MONITORING DIFFERENTIATION, MEMORY AND SENESCENCE IN CIRCULATING T CELLS OF IMMUNOSUPRESSED PATIENTS AFTER COVID-19 VACCINATION

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Background: T cells are deeply involved in protection against infection by natural- and vaccine-induced immunity. Monitoring T-cell function is crucial to follow-up COVID-19 vaccination in immunosuppressed patients. In a preliminary phase of an ongoing study, we have found that patients present lower and heterogeneous antigen-specific CD8 cell response after vaccination. Patients showed persistent decrease of total lymphocytes, T cells and CD4 lymphocytes, but TEMRA CD4 cells markedly increased. Interestingly, CD28-CD27+ CD4 cells increased, while CD28+CD27+ CD8 cells decreased. These findings suggested impaired T cell co-stimulatory function.

Aims: To apply FCM for assessing the efficiency of COVID-19 vaccines in immunosuppressed patients, based on the expression of surface markers of cell differentiation, function and exhaustion on T-cell subpopulations.

Methods: Twenty-two immunosuppressed patients and 21 controls were studied at months one, three, six and twelve along mRNA vaccination. DuraClone panels (Beckman Coulter) were used for measuring the expression of CD27, CD28, CD57 and PD-1 (CD279) on the CD4 and CD8 T-cell subpopulations defined by the co-expression pattern of CD45RA and CCR-7 (CD197) markers, namely naïve, central-memory (CM), effector-memory (EM) and terminally-differentiated memory (TEMRA) cells. For statistical analysis, Mann-Whitney and Spearman tests were applied.

Results: Patients showed increased percentage of $CD27^+/CD28^-$ cells within the CD4 naïve cell compartment and increased percentage of $CD27^-/CD28^+$ cells within the TEMRA cell compartment. The expression of exhaustion marker PD-1 in naïve and CM compartments of CD4 cells was increased in terms of $PD\text{-}1^+$ percentage and antigen density. Naïve CD8 cells showed increased frequency of $CD28^-$ $CD27^+$ cells and increased PD-1 antigen density. In CD8 CM compartment, $CD27^-CD28^-$ cells increased, while $CD27^+CD28^+$ cells decreased. In this compartment, the antigen density of the senescence marker CD57 increased, but PD-1 decreased both in percentage and antigen density. In CD8 EM compartment $CD27^-CD28^-$ cells increased markedly, while $CD27^+CD28^+$ cells decreased. In CD8 TEMRA, both $CD27^-$ $CD28^+$ cells and $CD27^+CD28^+$ cells increased, while the antigen density of CD57 decreased. No clear differences in the subpopulations studied were observed when comparing good and poor responders according to the amount of antigen-specific circulating CD8 cells.

Conclusions: Our results are suggestive of increased T-cell exhaustion in immunosuppressed patients undergoing COVID-19 vaccination. Our FCM approach may be applied for personalized follow-up of high-risk patients. Sponsored by Fundación Mutua Madrileña (IRAS-VAC Project). JEO is a member of the Spanish Network of Inflammatory Diseases (REI-RICORS: RD21/0002/0032), Institute of Health Carlos III, Madrid, Spain.

IMM-08

MARKERS OF T-CELL DIFFERENTIATION, MEMORY AND SENESCENCE IN YOUNG PATIENTS WITH IDIC15 SYNDROME, A NEUROLOGICAL RARE DISEASE

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Introduction: Idic15 syndrome is a rare neurodevelopmental disease caused by variable duplications in the q11-q13 region of chromosome 15. Previously, we have found immune alterations and susceptibility to infections in young patients of Idic-15. Immune dysfunction is correlated to accelerated immunosenescence in neurological disorders. On this basis, we have assessed the changes in relevant T-cell markers along age in a cohort of young Idic15 patients (age: 2-20 years) and in age- and sex-matched healthy controls.

Methods: We have used flow cytometry to quantify the expression of biomarkers of cell differentiation and function on the surface of helper (TH) and cytotoxic (TC) T cells. DuraClone panels (Beckman Coulter) were used for assessing the expression of CD27, CD28, CD57 and PD-1 (CD279) on the T-cell subpopulations defined by the co-expression pattern of CD45RA and CCR-7 (CD197) markers, namely

naïve, central-memory (CM), effector-memory (EM) and terminally-differentiated memory (TEMRA) cells. For statistical analysis, Mann-Whitney and Spearman tests were applied.

Results: The only significant changes correlated to age in the controls were observed in the TC compartent, with increased frequency of both $CD8^+CD28^-CD27^-$ and $CD8^+CD57^+$ cells, suggesting TC cell differentiation and exhaustion along age in this group. The Idic15 cohort showed larger number of immune changes correlated to age. Together with an age-dependent reduction of total counts of T cells, most changes affected TH cells. The co-expression pattern of CD45RA, CD197, CD27 and CD28 revealed significant decrease of naïve cells and a general increase of the CM, EM and TEMRA memory-cell subpopulations, as well as a decrease of $CD28^+CD27^+$ cells refflecting the changes in naïve TH cells. In the TC compartment, the only significant changes were age-dependent increases of $CD8^+CD28^+CD27^-$ cells, pointing to increased naïve TC engagement, and of $CD8^+CD57^+$ cells, suggestive of increased TC exhaustion.

Conclusions: In a previous study of the cohorts, we found no significant differences between controls and patients in the abundance of TH or TC cells expressing PD-1, nor in the intensity of PD-1 expression per cell. However, an age-dependent decrease of the intensity of expression of PD-1+ TH cells in both controls and patients was observed. Our present findings suggest that Idic15 patients may undergo accelerated T-cell immunosenescence, as shown for other neurological conditions. Sponsored by donations to the "One House One Life" Initiative promoted by Great Chance SLU. JEO is a member of the Spanish Network of Inflammatory Diseases (REI-RICORS: RD21/0002/0032), Institute of Health Carlos III, Madrid, Spain.

IMM-09

CD45RA VERSUS CD45RO AN ATYPICAL MATURATION PATTERN IN A TEEN AGE WITH DIGEORGE SYNDROME

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Introduction: CD45RA and CD45RO are isoforms of the CD45 antigen that are expressed on T Cells in identifiable patterns using flow cytometry. The CD45RA/RO assay is used in the evaluation of immunodeficiency as well as various immune disorders. The 22q11.2 deletion syndrome (formerly called DiGeorge) is a frequent chromosomal microdeletion (1/ 4000 births) identified by deletion of 1·5–3 Mb. We report a case of an atypical maturation pattern resulting in a coexpression of CD45RA and CD45RO on CD4+ and CD8+ T cells in a teen ager affects by DiGeorge syndrome and referred to our clinic for immunological evaluation.

Methods: Lymphocyte populations were analyzed by multiparameter flow cytometry to assess CD45RO, CD45RA, CD8, CD3 and CD4 antigens. Data was analyzed in BD FACSuite software. CD3 by side-scatter was used to establish a lymphocyte gate, CD4+ or CD8+ were input gate for CD45RA by CD45RO plots.

Results: During two hospitalization, one year apart, we observed an atypical phenotypic pattern in a fourteen years old boy. The first appearance of an anomalous pattern of CD45RA/RO espression was in coincidence of a SARS CoV2 active infection (despite COVID vaccination with dose boosting). One year later during an admission for severe pain after a surgery procedure for scoliosis. The quantitation

of T cells subset showed: CD3+ 58.3%, 58.4%; CD4+ 36.0%, 39.2%; CD4+CD45RA+CD45RO- 10.3%, 10.3%; CD4+CD45RA+CD45RO+ 25.3%, 28.5%; CD4+CD45RA-CD45RO+ 0.4%, 0.4%; CD8+ 15.5%, 15.3%; CD8+CD45RA+CD45RO- 5.1%, 5.0%; CD8+CD45RA+CD45RO+ 8.7%, 8.6%; CD8+ CD45RA-CD45RO+ 1.7%, 1.7%, at the first and second evaluation respectively. In particular, our attention fell on the morphology expressed by the atypical maturation pattern in which the acquisition of CD45RO expression was deficiency and occurring without the gradual loss of CD45RA expression. The retained or the reaquisition of CD45RA expression resulted in a “double-positive” population in both CD4+ and CD8+ lymphocytes.

Conclusion: The function of double-positive (CD45RA+CD45RO+) T limphocytes in the immunological response is not well undenstand yet. The T cell population expressed could be interpretated as a transient form due to recently activated T cells by an important inflammatory stimulus or it could represent a distint subset. In the absence of the baseline assessment of T cell differentiation this population it is necessary further investigated the immune status of the patient by functional assays. The CD45 gene polymorphism associated at CD45RA-CD45RO+ deficiency and with high percentage of double-positive CD45RA+CD45RO+ have been reported, investigate the presence of this polymorphisms could be carry out.

IMM-10

DIAGNOSTIC EVALUATION OF ANTI-TRBC1 ANTIBODY BY COMBINED ANALYSIS OF CD57 AND T-MEMORY CELLS IN A VARIETY OF CLINICAL SAMPLES

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Introduction: The purpose of the study was to compare the anti-TRBC1 antibody JOVI-1 in the assessment of T cell clonality with a 24 epitope TCR beta chain repertoire, in a multiparametric cytometry protocol combining T memory subpopulations with CD57.

Methods: 63 samples were studied, including 43 peripheral blood samples, 5 bone marrow samples, 2 lymph nodes, 3 effusions and 10 paucicellular samples, 6 CSF and 4 vitreous fluids. The clinical indications were lymphocytosis (18), anemia or other cytopenia (thrombocytopenia, neutropenia, lymphopenia) (10), infections (3), lymphomas (including 3 peripheral T lymphomas and one Sezary) (11) and miscellaneous (21). A NAVIOS (Beckman-Coulter) analyzer with a protocol of 8 antibodies (CD3, CD4, CD8, CD27, CD45RA, CD45, CD57 and JOVI-1) was performed. TRBC1+/TRBC1- ratios were calculated, based on previously suggested reference values [Cancers 2021, 13(17), 4379]. If the ratios were out of the recommended range (>+/-3SD), further confirmation was made with a 24 TCRVβ chain repertoire Kit (Beckman Coulter, USA). A modified protocol was applied, which focused on Vb epitope combined with CD27, CD45RA and CD57, discriminating the T memory subpopulations in combination with CD57 expression. If the percentage of Vb was >50%, population was confirmed as clonal. In the cases of paucicellular vitreous fluids, clonality was assessed by PCR analysis of TCRγ and TCRβ genes.

Results: 41 samples with ratios of TRBC1+/TRBC1- within +/3SD were not indicative of clonality by TCRVb repertoire or PCR assessment. In 22 cases of significant deviating ratios of TRBC1+/TRBC1- the identification of >50% Vβ population or silent repertoire confirmed clonality. In one case of paucicellular vitreous fluid with abnormal TRBC1+/TRBC1- ratio PCR confirmed clonality. The analysis was not possible in an ATLL lymphoma not expressing surface TCRαβ, whereas another AITL lymphoma had a clone of double negative CD4-CD8- cells. 6 T8-LGL and 4 T4-LGL cases were diagnosed, with clonal CD57+ T4CD8dim and CD57+ T4 terminal effective memory (EMRA).

Conclusions: In all cases with discrepant TRBC1+/TRBC1- ratios clonality was confirmed by TCR Vβ epitopes while negative evaluations were in agreement with both methods. The diagnosis of T8-LGL and T4-LGL populations was amplified by focusing on the clonality assessment in CD57+ memory subpopulations. The feasibility of accurate anti-TRBC1 clonality examination was shown in a variety of clinical samples other than blood and marrow, like effusions, lymph nodes as well as paucicellular samples like CSF and vitreous fluid.

IMM-11

TRACKING THE TRACKERS : WHEN FLOW CYTOMETRY MEETS IMMUNOLOGY

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Introduction: The immune system is complex and highly regulated. The analysis of the immune composition is required in various settings enabling to identify potential immune dysregulations. The analysis of the immune system in cross-sectional as well longitudinal studies is facilitated by flow cytometry. Still, many errors and significant variability can be introduced in a flow cytometry experiment. We are describing here key examples of clinical research studies where immunomonitoring was employed to track the immune cell phenotypes and functions.

Methods: A compilation of studies related to immune deficiencies, transplantation and cancer immunotherapy research utilizing methods to improve reproducibility of flow cytometry results is described. The combination of sample preparation tools such as dry reagents, as well as improved sensitivity of flow cytometers combined with multi-parametric analysis are described. The throughput of flow cytometry experiments has also been tested with various platforms.

Results: Whole blood immunomonitoring is modulated by various elements including (i) time of sampling (ii) choice of anti-coagulant (iii) sample preparation procedures prior to staining (iv) panel design (v) dry versus liquid reagents utilization (vi) calibration and sensitivity of the cytometer (vii) data analysis. The research studies presented highlight the benefits of a better control of the variability inherent to flow cytometry experiments.

Conclusions: Immune cells are constantly tracking for pathogens and cancer cells. Immunomonitoring enables to identify potential pitfalls in the composition and functionality of the immune system. Especially in the context of longitudinal research studies, where variation of the immune phenotypes may be subtle, the accuracy of the measurement is key. Hence, the data presented shows the importance of introducing solutions to reduce the variability often observed. This should enable to improve reproducibility and generalization of clinical research findings.

IMM-12

SPECIFIC T CELL RESPONSE TO SARS-COV-2 IN VOLUNTEERS BEFORE AND AFTER VACCINATION

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Introduction: SARS-CoV-2 is a highly contagious virus that has caused a global pandemic since its emergence in late 2019. An effective immune response is crucial for successful defense against the virus. In this study, we aimed to introduce a reliable method to determine specific T cell responses to SARS-CoV-2. The specific T cell response to SARS-CoV-2 was measured in individuals exposed to viral antigens either after recovery from COVID-19 disease or after vaccination. The T cell response may also be present in individuals who are unable to develop antibodies due to various diseases or therapies.

Method: We recruited 59 healthy volunteers who donated blood before and after vaccination against COVID-19. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples and stimulated with the SARS-CoV-2 viral peptides S and N. PBMCs were then analyzed for intracellular interferon-gamma (IFN-γ) production and cytotoxic capabilities of antigen-specific CD8 T cells, which we confirmed by labeling the CD107a molecule (part of the cytotoxic secreted granules) with a FACSCanto II flow cytometer.

Results: We found that both methods, cytokine production and cytotoxic assay, were effective in measuring T cell response against SARS-CoV-2 after vaccination. Intracellular production of IFN-γ results in a Th1 matched antiviral defense mechanism. Statistically significant normal values for the proportion of cells expressing IFN-γ ranged from 0,25% to 3,15% in all T lymphocytes, from 0,24% to 4,26% in helper T cells , and from 0.28% and 2.99% on activated (CD69+CD4+) T lymphocytes . Cytotoxic T lymphocytes are also activated, as confirmed by expression of the CD107a molecule after secretion of cytotoxic granules. The statistically significant normal values of cytotoxic response after vaccination ranged from 3.85% to 21.5%.

Conclusion: Our study demonstrates that measurement of specific T cell responses to SARS-CoV-2 is a reliable method to evaluate the immune response to the virus. Our results suggest that the T cell response may play an important role in defense against SARS-CoV-2 infection in individuals who have not developed an antibody response or who are unable to develop antibodies because of various diseases or therapies. Further studies are needed to determine the long-term protective effect of the T cell response against SARS-CoV-2.

IMM-13

AN OPTIMIZED FLOW-CYTOMETRY PROTOCOL FOR SIMULTANEOUS DETECTION OF T-CELL ACTIVATION INDUCED MARKERS AND INTRACELLULAR CYTOKINES: APPLICATION TO SARS-COV2 IMMUNE RESPONSE

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Introduction: antigen-specific T cell analysis by multicolor flow cytometry is an important step for investigating cellular immunity in many settings, such as infectious diseases, cancer and vaccines. In the cellular immunologist's toolbox, the expression of activation-induced markers (AIM) following antigen exposure allows the study and sorting of ag-specific T cells without using human leukocyte antigen (HLA)-multimers. In parallel, assessing the cytokine profile of responding T cells would support a more comprehensive description of the ongoing immune response by providing information related to cell function, such as polarization and effector activity.

Methods: a protocol and a 12-color flow cytometry panel were optimized to combine the detection of activated CD4+ and CD8+ T cells in a TCR-dependent manner with the evaluation of cytokine production by intracellular staining, without affecting the positivity of activation markers. In particular, the expression of CD134 and CD69 have been tested in conjunction with intracellular (ic) CD137 to detect SARS-CoV2 Spike protein-specific activated T cells. Panel design and data analysis were optimized for resolution and reporting results. Antigen-specific T cell responses to Spike and CMV were then assessed by using the optimized protocol.

Results: in our setting, CD134 provided minimal contribution to detect the pool of AIM+ T cells, whereas a key role was described for ic-CD69 which was co-expressed with ic-CD137 in both CD4+ and CD8+ lymphocytes. The analysis of TCR-triggered cytokine-producing T cells (IFNγ, TNFα and IL-2) further confirmed the capacity of ic-CD69 to identify functionally responsive antigen-specific T cells which were often largely negative or weakly positive for CD134 expression. In parallel, the use of CD45RA, CCR7 and CXCR5 allowed characterization of the T cell maturation curve and detection of T follicular helper CD4+ cells, including the antigen specific activated subsets. 24hr PBMC stimulation of 10 donors either with Spike or CMV peptide pools resulted in detection of antigen-specific T cell activation in 9/10 donors for Spike and 5/10 donors for CMV. Triplicate assessments on 4 donors highlighted antigen responding T cells %CV ranging from 5% to 44%.

Conclusions: This method allows AIM testing in conjunction with intracellular cytokine evaluation and T cell maturation to provide a detailed description of antigen-specific T cell response. It has been applied for studying responses during SARS-CoV2 infection or following vaccination, and further to assess responses to CMV. The method is potentially applicable to any setting requiring investigation of antigen-specific T cell activation.

IMM-14

NOVEL MAIT CELL POPULATION IN ATOPIC DERMATITIS AND UNCONVENTIONAL T CELLS REFERENCE RANGES

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Introduction: Unconventional T cells are non-MHC restricted T cells, bearing αβ and γδ T cell receptors (TCR). They have been associated with immune-mediated disorders. However, reference ranges were not established. Atopic dermatitis (AD) is an inflammatory skin disorder with immune dysregulation. Since mucosa-associated invariant T (MAIT) cells are abundant in mucosal tissues and show memory

phenotypes that led us to investigate MAIT cells in AD. Also, we have established age and gender-based frequency of invariant natural killer T cells (iNKT), γδ T cells and MAIT cells in healthy adults.

Methods: We used an 8-color flow cytometric panel. 18 AD patients' and 11 age and gender matched healthy controls' (HC) peripheral blood (PB) samples were obtained. Cytokine measurements were carried our using intracellular staining, PBMCs were stimulated with PMA/ionomycin in the presence of Brefeldin-A. Total of 203 PB of healthy adults were collected to establish the reference range of iNKT, γδ T, and MAIT. Samples were categorised by age and gender (18-90 years), (94 males, 109 females). T-distributed stochastic neighbourhood embedding and unsupervised clustering (FlowSOM) algorithm were used to identify meta clusters. Then these meta clusters were compared between AD and HC.

Results: Cluster phenotype (CD45/$CD3^+$ in all phenotype); $V\alpha7.2^+/CD161^{dim}$, $V\alpha7.2^-/CD161^+$, $V\alpha7.2^-/CD161^{++}$, $V\alpha7.2^+/CD161^{++}$, $V\alpha7.2^+/CD161^{++}/CD69^+$, $V\alpha7.2^+/CD161^{dim}/CD8^+$, $V\alpha7.2^-/CD161^+/CD8^+$ and $V\alpha7.2^+/CD161^{dim}/IFN\gamma^+$ showed lower representation in AD than HC (p = 0.0101, 0.0303 and 0.0060, 0.0257, 0.0233, 0.0370, 0.0487 respectively). Phenotype $CD3^+/PD\text{-}1^+$, $CD161^+/PD\text{-}1^+$, $CD3^+/CD4^-/CD8^-/CD38^+$ showed higher representation in AD than HC (p = 0.0452, 0.0427, 0.0233 respectively). MAIT cells showed significant upregulation of GzB in AD patients (p = 0.0083). The reference ranges of iNKT, γδ T and MAIT cells in males were 0.0087 – 0.56%, 0.43 - 14.2% and 0.20 - 10.2% and in females, 0.015 – 2.1%, 0.11 - 11.6% and 0.28 - 13% respectively. iNKT cells in females were higher 0.114%, than in males 0.076%. Whereas γδ T cell in males were higher 2.52%, than in females 1.79%. γδ and MAIT cell frequency were higher in younger age groups than elderly and was negatively correlated with age, supporting immunosenescence, unlike iNKT cells.

Conclusion: We have found MAIT cells producing higher GzB and identified a $CD161^{dim}$ novel phenotype in AD patients. These results suggest that MAIT cells may be suitable therapeutic targets in AD. Our reference range finding provides age and gender-wise comparison of unconventional T cells in pathological conditions.

IMM-15

ROLE OF THE IMMUNE CELL MICROENVIRONMENT IN THE PERSISTENCE OF MEASURABLE RESIDUAL DISEASE IN CHILDHOOD T-CELL LYMPHOBLASTIC LEUKEMIA

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Introduction: Weakened immune-cell surveillance has been a well-known factor responsible for the recurrence/progression of cancer. Studies have demonstrated the association of immune-cell profile with clinical outcomes in B-cell acute lymphoblastic leukemia (ALL). However, there is no immune profile data on T-cell ALL, which is a high-risk leukemia. We and others have shown that post-induction measurable residual disease (PI-MRD) predicts relapse-free survival in T-ALL. Herein, we prospectively studied deep immune cell profiles, including immune checkpoint protein expression from children with T-ALL and with an aim to study their association with PI-MRD status.

Methods: A 16-color four-tube antibody panel including antibodies against CD1c, CD3, CD4, CD7, CD8, CD10, CD11c, CD14, CD15, CD16, CD19, CD20, CD25, CD27, CD33, CD38, CD45, CD45RA, CD56, CD83, CD123, CD127, CD141, CD226, CXCR3, CCR4, CCR6, CCR7, CCR10, TCRγδ, LAMP-14-1BB, NKp46, HLADR, and immune checkpoint proteins Tim-3, PD-1, PDL-1, CTLA4, Lag3, OX40, OX40L, and Tigit was studied for deep immune-cell profile analysis. Data were acquired on BD-LSR-Fortessa and analyzed using Kaluza-v2.0. The immune-cell subsets' percentages and absolute counts were studied in peripheral blood at diagnosis.

Results: We studied the levels of 220 immune-cell subsets (after exclusion of leukemic cells) in peripheral blood from 37 childhood T-ALL (age, 0-14 years and M:F-6:1). PI-MRD was positive in 15/37 (41.7%) patients (median-0.06%; range, 0.005-39%). Out of 220, 54 immune subsets have shown an association with PI-MRD status (positive versus negative) using the Mann-Whitney-U test. These predominantly included NK-cells and immune checkpoint protein-expressing helper/cytotoxic effector/memory T-cell subsets. The top 10 immune-cell subsets demonstrating a strong association (AUC>0.70) were analyzed further for multivariate analysis. Finally, increased levels of absolute counts of LAG3-expressing terminal effector cytotoxic CD8+T-cells, cytotoxic TC2-CD8T-cells, and LAG3-expressing terminal effector CD4+T-cells, helper TH2-CD4T-cells were found to be associated with PI-MRD positive status.

Conclusion: To our knowledge, this is the first study to evaluate deep immune-cell profiles in patients with T-ALL. Our data showed the association between immune cell subsets with LAG-3 immune checkpoint protein expression. Lymphocyte Activation Gene 3 (LAG3) is one of the immune checkpoint receptors expressed by T-cells. It significantly contributes to the inhibitory signals regulating immune cell functions, including T-cell activation, proliferation, cytokine production, cytolytic activity, etc. leading to T-cell exhaustion. Although limited to a small cohort, our data indicate the role of LAG3-expressing T-cells in the immunobiological mechanism of the persistence of residual disease in T-ALL patients and raises the possibility of the potential role of anti-LAG-3 immunotherapy.

IMM-16

T CELL CHEMOKINE RECEPTOR PROFILING FOR THE DIAGNOSIS OF CHRONIC MUCOCUTANEOUS CANDIDIASIS. A MACHINE LEARNING APPROACH

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Introduction: Diagnosis of Chronic Mucocutaneous Candidiasis (CMC) is challenging and relies mostly on genetic testing for *STAT1* mutations post clinical suspicion and on functional Th17 assays, the latter displaying low levels of IL-17 production post stimulation. Due to the lack of universal availability of these assays, the disease remains highly underdiagnosed. As effector T helper cell subsets, such as Th17, may now be rapidly identified through their chemokine receptor profile, we aimed to set up a simple, chemokine receptor-based method for screening and diagnosis of CMC.

Methods: Blood was collected in EDTA tubes from 5 patients with CMC (median age 4.4 years) , along with 7 normal controls and 22 patients with other Inborn Errors of Immunity (mostly Common Variable Immunodeficiency, median age 14.6 years). The following panel was used: CD183 (CXCR3)-AF 488/ CD197 (CCR7)-PE/ CD45RA-ECD/ CD19-PC5.5/ CD196 (CCR6)-PC7/ CD279 (PD1)-APC/ CD161-APC-AF 750/ CD194 (CCR4)-BV421/ CD8-KRO/ CD4- BV605. DXFlex instrument was used for data acquisition and Kaluza2.2 plus Cytobank softwares were used for analysis. An 8 parameter viSNE analysis on CD4+ memory cells (iterations 1000, perplexity 30) was followed by Flow SOM clustering (225 clusters, 25 metaclusters, 10 iterations). Additionally, Th17 were enumerated through intracellular cytokine staining post PMA/Ionomycin stimulation in the CMC patients and their respective normal controls.

Results: Using functional assays, all five CMC patients had very low Th17 cell percentages, as compared to normal controls (<0.05% and 0.9%- 5.3% on CD3+ cells respectively) a finding compatible with the diagnosis. Upon Cytokine receptor profiling, percentages of total CXCR5-CD161+CXCR3- memory CD4+ cells (theoretically corresponding to Th17 cells) did not differ significantly among groups. However, a FlowSOM metacluster was detected (CD45RA-CCR7+ CXCR5-CD161+CXCR3-CCR4+), whose percentage on memory CD4+ cells was significantly lower in CMC patients.

Conclusions: A Th cell subset with central memory, Th17 phenotype, additionally expressing skin mucosa homing receptors (CCR4) was found as a potential marker for rapid screening for CMC. Further studies on larger patient cohorts, including other entities with low Th17 count (such as Job's syndrome) are needed to establish its clinical utility.

IMM-17

FEATURES AND FUNCTIONS OF REGULATORY T CELL DERIVED EXTRACELLULAR VESICLES IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

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In the last years, regulatory T (Tregs) cell therapies emerged as a novel approach in the treatment of autoimmune diseases, transplant rejection and graft-versus-host disease (GvHD). Treg derived extracellular vesicles (Treg EVs) may represent a valid alternative in the treatment of immune-related disorders. This mainly because it is well-known that EVs produce scarce side effects and are not sensitive to the inflammatory environment, that induces cell differentiation. Here we characterized Treg derived EVs, with the final aim to study their potential as immunomodulatory agents in the prevention of GvHD.

Tregs from 10 patients enrolled by the Hematological Unit, "S. Spirito" hospital of Pescara (Italy) were isolated by a CD4+ CD25+ positive selection and expanded with a mix of cytokines as already reported. EVs were isolated by fluorescence activated cell sorting or ultracentrifugation and further precipitated in polyethylene glycol (PEG). Flow cytometry, morphological (nanotracking analysis, NTA) and proteomic analyses were carried out to assess the inhibition activity and protein cargo of Treg-EVs.

The phenotypic analysis of selected Tregs displayed a purity of at least 60%. The NTA characterization of Treg derived EVs revealed that their size was in the range of small EVs (50-150nm). Functionality tests (n=3) on activated autologous T effector cells revealed that the optimal EV concentration to

achieve best results in terms of inhibition of t-cell activation (97-98%) was between 4-6x10^6 EVs/1x10^5 cells after 24 hours of treatment. In addition, EVs purified by FACS displayed a 3-fold higher inhibition capacity compared to the ultracentrifuged ones. Furthermore, the inhibition effects of Treg EVs were comparable to that induced by the related parental cells (P<0.05). The immunomodulatory functions of EVs stemming from expanded Tregs was confirmed by the functional analysis of their protein cargo.

We demonstrated that Treg derived EVs have clear immunomodulatory functions and their well-known low toxicity strongly points out their potential as new therapeutic agents in the GvHD prevention.

IMM-18

IMPROVEMENT OF THE SENSITIVITY OF THE BD™ HLA-B27 KIT BY EXTENSION OF THE MANUFACTURERS RECOMMENDED NEGATIVE CUT-OFF RANGE

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Introduction: Spondyloarthritis is a group of inflammatory conditions which includes ankylosing spondylitis (AS) and reactive arthritis (ReA) and are characterized by a strong association with the major histocompatibility complex allele HLA-B27. The BD™ HLA-B27 Kit is a two-colour flow cytometry-based assay which measures expression of HLA B27 on the surface of CD3 positive T cells. HLA B27 antibody within this kit cross reacts with HLA B antigens, most commonly HLA B7 which can result in false positive results. The BD™ HLA-B27 Kit recommend a 10 channel gray zone in which results are reported as inconclusive with secondary confirmational analysis required. In this current study we aimed to verify the manufacturers' recommended gray zone.

Methods: A total of 61 excess peripheral blood samples were sourced from the Immunology Laboratory at the University Hospitals of Leicester NHS Trust. Following setup and technical validation, samples were analysed using BD™ HLA-B27 Kit on the BD FACS Lyric. Data was analysed for HLA B27 positivity as per manufacturers recommendations and compared to results from a predicate Beckman Coulter Navios assay and a molecular typing assay. A receiver-operating curve was used to verify the negative cut-off for this assay.

Results: Of the 61 samples tested, 3 samples were HLA B27 positive, 17 samples were inconclusive, and 41 samples were negative by the BD™ HLA-B27 Kit flow cytometry methods. Agreement between BD™ HLA-B27 Kit flow cytometry analysis and molecular typing was 88.5% with specificity at 100% and sensitivity at 74%. This sensitivity was lower compared to the predicate Beckman Coulter Navios assay (86.6%). A receiver-operating curve was used to improve sensitivity by lowering the manufacturer's negative cut-off by 10 MdFI units. This improved agreement between the BD™ HLA-B27 Kit flow cytometry-based method and molecular method to 98%, improved sensitivity to 96% and maintained specificity at 100%.

Conclusion: This study confirms the utility the BD™ HLA-B27 Kit for assessment of HLA B27 positivity. Adjustment of the manufacturers negative cut-off by 10 MdFI units resulted in better concordance of the BD™ HLA-B27 Kit flow cytometry assay with molecular typing analysis and improved sensitivity and specificity.

IMM-19 Abstract has been cancelled

IMM-20

IMMUNOPHENOTYPING USING DRIED REAGENT COCKTAIL ON THE BD FACSDUET™ PREMIUM SAMPLE PREPARATION SYSTEM INTEGRATED WITH THE BD FACSLYRIC™ FLOW CYTOMETER

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Traditional workflows in flow cytometry laboratories involve multiple manual processing steps, such as pipetting reagents to generate multicolor reagent cocktails, pre-washing samples, staining samples followed by lysing and washing steps. Manual reagent and sample processing workflow is time consuming and is increasingly challenging as the number of parameters increases in flow cytometry applications. Manual processes inherently introduce human error and contribute to the overall variability in experimental data. The BD FACSDuet™ Premium Sample Preparation System integrated with the BD FACSLyric™ Flow Cytometer can reduce error-prone steps while also decreasing the overall hands-on time spent preparing samples for acquisition. BD Horizon™ Chroma Panel reagents provide pre-aliquoted, performance-optimized multicolor panels in a dried down and ready-to-use format with increased shelf lifewhen compared to traditional liquid cocktails.

In this study, we evaluated a 12-color BD Horizon™ Chroma Panel (CD7 FITC/Anti-Lambda PE/CD34 PerCP-Cy5.5/CD19 PE-Cy7/Anti-Kappa APC/CD5 APC-R700/CD20 APC-H7/CD3 V450/CD45 V500-C/CD8 BV605/CD10 BV711/CD4 BV786) on the BD FACSDuet™ Premium System integrated with the BD FACSLyric™ Flow Cytometer. The BD FACSLyric™ Flow Cytometer setup and compensation was completed using BD® CS&T Beads, BD® FC Beads and six batch-matched BD driedsingle-color reagents (CD19 PE-Cy7, CD5 APC-R700, CD20 APC-H7, CD8 BV605, CD10 BV711, CD4 BV786). The completely automated workflow on the system was accomplished using a two-tube preparation method on BD FACSDuet™ Premium System. The BD FACSDuet™ Premium System first did an automatic prewash of specimen (300 µL) in the first tube to remove soluble kappa and lambda in the whole blood sample. The second tube contained the dried reagent cocktail, i.e., the 12-color BD Horizon™ Chroma Panel tube. The system rehydrated the dried reagent cocktail with 50 µL of BD Horizon™ Brilliant Stain Buffer, then transferred 100 µL of the pre-washed specimen into the reagent cocktail followed by vortexing, incubating, and finally lysing and washing the stained samples. The prepared samples placed on a sample carrier were automatically transferred to the BD FACSLyric™ Flow Cytometer for data acquisition and analysis.

Immunophenotyping and characterization was performed for T cells and subsets (CD4+ and CD8+ T cells), B cells and subsets (Kappa and Lambda B cells) and CD34+ cells. In this study, we demonstrate an automated sample-to-results workflow for the new 12-color BD Horizon™ Chroma Panel using the BD FACSDuet™ Premium Sample Preparation System integrated with the BD FACSLyric™ Flow Cytometer.

This research is scientific in nature. Products NOT for diagnostic use.

IMM-21

EVALUATION BY CONVENTIONAL- AND IMAGING-FLOW CYTOMETRY OF SYNTHETIC CELLS MIMICKING LEUKOCYTES

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Introduction: Traditionally, setup and optimization in flow cytometry (FCM) are performed using fresh cells, cell preparations and solid-core microbeads. Recently, a series of synthetic cells that match the optical and immunophenotypical characteristics of human blood cells have been made commercially available. FlowCytes™ and TruCytes™ are synthetic cells assembled into a three-dimensional spherical matrix, physically resembling a cell. FlowCytes™ are synthetic white blood cells (WBC) that mimic the optical properties of human blood populations for fixed or unfixed lymphocytes, monocytes, and granulocytes. FlowCytes ™ are scatter controls for the identification of non-fixed whole blood cells. TruCytes™ synthetic cells are are lyophilized cell mimics that feature WBC biomarkers with scatter properties that match lymphocyte and monocyte populations. TruCytes™ provide positive and negative level for surface biomarkers. Here we present the results of the evaluation of different synthetic mimic cells.

Material and Methods: This study was performed by conventional FCM using Gallios (Beckman Coulter) and Fortessa X-20 (Becton Dickinson) cytometers and by imaging FCM (IFCM) with an Amnis ImageStream (Luminex) instrument. The following WBC mimics (Slingshot Bioscience, Inc.) were evaluated: FlowCytes™ non-fixed WBC mimics, FlowCytes™ Fixed WBC Scatter mimics; TruCytes™ TBMNK Control, TruCytes™ TBNK Control, TruCytes™ T-Cell $CD4^+$ Mimics, TruCytes™ T-Cell $CD8^+$ Mimics and TruCytes™ B-Cell $CD19^+$ Mimics. TruCytes™ and blood WBC were analyzed using TetraONE 1 (CD45-FITC/CD4-RD1/CD8-EDC/CD3-PC5) and TetraONE 2 (CD45-FITC/CD56+CD16-RD1/CD19-ECD/CD3-PC5), both from Beckman Coulter.

Results: FlowCytes™ products showed similar percentage of lymphocytes in both fixed- cells and non-fixed products. However, the percentage of mimic granulocytess was higher in fixed than in non-fixed FlowCytes™, while percentage of monocytes was very high in non-fixed mimics. For the TruCytes™ products assessed by tetraONE panels, the percentages of mimic cell subpopulations were comparable to peripheral WBC and no differences were observed between both FCM instruments. By IFCM, TruCytes™ T-Cell $CD8^+$ Mimics and TruCytes™ B-Cell $CD19^+$ Mimics had larger size than peripheral blood lymphocytes. However, the immunophenotypic features of the mimics after staining with the tetraONE panels were similar to those observed in blood lymphocytes.

Conclusions: FlowCytes™ and TruCytes™ WBC Mimics are suitable as controls for instrument performance or for gating and standardization in immunophenotype in FCM and IFCM. The immunophenotype of TruCytes™ and the scatter pattern of FlowCytes™ are comparable to those of WBCs in FCM and IFCM, but IFCM reveals a significant difference in cell size between mimic- and real lymphocytes. We thank Palex for the gift of the FlowCytes™ and TruCytes™ WBC mimics.

IMM-22

CD45RA/CD45RO "U" SHAPE MATURATION PATTERN IN FLOW CYTOMETRY WHETHER IT MAY BE ASSOCIATED WITH INFECTIOUS SYMPTOMS?

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Introduction: Immunophenotyping of T lymphocytes is performed in the assessment of immunological disorders. Based on the expression of CD45 isoforms, naïve (CD45RA) and memory (CD45RO) T cells are distinguished. In flow cytometry assays using antibodies against CD45RA and CD45RO, a rare "u-shape" pattern can be observed. It was described that U-shape is associated with CD45R C77G polymorphism, however, it has not been established whether this phenomenon may affect the clinical outcome of patients suspected with inborn errors of immunity (IEI).

Methods: Flow cytometric analysis was performed using a Canto-10colour analyzer using the following antibodies: CD45RO-APC, CD45RA-PE-Cy7, CD3-PerCP, and CD45-APC-H7. Data was analyzed using BD FACS Dive software v 9.0. Pediatric patients were diagnosed for IEI in the Immunology Department of University Children's Hospital of Cracow. The patients underwent a comprehensive immunological diagnosis and infectious and autoimmune symptoms were analyzed.

Results: Over a 2-year period, 336 cytometry CD45RA/CD45RO tests were performed, of which 4 patients had an atypical U-shape pattern. 5-year-old girl with pneumococcal sepsis (serotype19A) who was properly vaccinated with a conjugate vaccine. She had a history of hospitalization due to severe pneumonia at the age of 2. All other tested immunological parameters were normal. 7-year-old boy with Bloom syndrome, with recurrent, moderate to severe, infections of the upper and lower respiratory tract, requiring frequent antibiotics hospitalizations. In immunological evaluation, a slight decrease IgA concentration was found. 5-year-old boy with a serious course of molluscum contagiosum on the lower limb and not related bacterial inflammation of the skin and subcutaneous tissue. Other tested immunological results were normal, CGD was excluded. 15-month-old boy with protracted and recurrent diarrheas. The concentration of IgG have been slightly decreased temporarily, currently normal.

Conclusions: Based on our observation it is difficult to state whether patients with U-shape are prone to immunodeficiencies, as we have examined a specific group of patients, who already at admission displayed immunodeficiency symptoms. Determining whether CD45 polymorphisms play a significant role in susceptibility to infection requires larger-scale studies, including healthy controls. However, during diagnosing process of IEI, attention should be paid at information such as non-classical distribution of cells in the cytometer, and not just to cell numbers. Such observations will make it easier to make decisions about further genetic tests, the availability of which in some countries, including Poland, is limited.

IMM-23

BARCODING SAMPLES TO FACILITATE MULTI-SAMPLE MEASUREMENT IN ONE FLOW CYTOMETRY TUBE: A PROOF-OF-CONCEPT STUDY

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Introduction: Flow cytometric assessment of marker expression or cellular responses has revolutionized drug development and health care. However, increasingly complex sample analysis significantly increases reagent costs, analysis time, and sample variation upon staining. Therefore, we wanted to establish a method to combine individual donors into one sample while allowing donor delineation. Here, we present the results of a barcoding matrix that allows for the distinction of 9 individual donors by flow cytometry.

Methods: Peripheral blood mononuclear cells were isolated from healthy donor buffy coats and cryopreserved. Samples were thawed and stained with cell tracker dyes (CFSE, Cell Trace Yellow [CTY], Cell Trace Far Red [CTFR] or Violet Proliferation Dye [VPD]) in a titration experiment. In the final set-up, cells from each donor were stained with a unique combination of two suitable dyes (three concentrations Dye-A x three concentrations Dye-B = nine unique barcodes) according to manufacturer's instructions. Subsequently, stained cells from all donors were combined in one sample, and cell subsets were stained with phenotypic markers (e.g. CD3/CD19). Cells were fixed and measured directly after fixation, or after storage for 1 day at 4°C to determine dye/sample stability. All samples were measured on a FACSLyric flow cytometer. Data were analysed with Kaluza software.

Results: First, different dyes were titrated. CFSE and CTY were unsuitable for barcoding due to dye characteristics. 0.5 mM and 2.5 mM VDP and/or CTFR allowed for clear distinction of different donors and were suitable for use in a combinatorial barcoding matrix. After barcoding, we characterized multiple cell subsets via the addition of fluorescent antibodies directed against surface markers. Stability experiments confirmed that dyed samples are stable for at least 18 hours at 4°C without a loss in staining intensity, and without alterations to the subset distribution for subsets assessed.

Conclusions: We show that combinatorial coding with two different cell tracking dyes allows for delineation of at least 9 samples on a clinical flow cytometer and allows for identification of multiple subsets through addition of fluorescent antibodies directed against surface markers. This approach minimizes differences introduced during staining, as all individual samples are stained simultaneously in the same well. In practice, this approach could be used to directly compare donors, or compare (drug) treatment within one donor, with fewer technical influences. We are currently undertaking efforts to expand the phenotyping panel and performing assay transfer to a flow cytometer that allows measuring larger antibody panels.

IMM-24

DISTORTED NON-TRANSFORMED B CELL COMPARTMENT AND REDUCED IMMUNOGLOBULIN LEVELS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Secondary immunodeficiency characterises chronic lymphocytic leukemia (CLL) patients, however the remaining non-transformed B cell landscape and how it shapes B cell functional capacity remain to be uncovered. Here we characterize the residual non-malignant B cell compartment in untreated CLL and its relation to immunoglobulin (Ig) production.

Methods: Peripheral blood (PB) samples were collected from healthy controls (HC) and untreated CLL patients. B cell subpopulations were evaluated within isolated PB mononuclear cells by flow cytometry using a monoclonal antibody panel: IgD-PECy7, CD27-PEDazzle594, CD19-AF700, CD38-PECy5, CD5-APC, CD3-APCCy7. Non-transformed B cells were defined as $CD5^-CD19^+$. After gating out plasmablasts ($CD27^+CD38^{hi}$), we established the counts of antigen-inexperienced B cells (IgD^+CD27^-), unswitched memory (IgD^+CD27^+), switched memory (IgD^-CD27^+) and double negative (DN) (IgD^-CD27^-) B cell subpopulations. Serum levels of IgG, IgM, and IgA were determined using immunoturbidimetric method. This work was funded by the Latvian Council of Science Fundamental and Applied Research Project Grant No Izp-2022/1-0080.

Results: Data from 11 HC (8 males, 3 females; median age 67 years; interquartile range (IQR) 6.0) and 21 CLL patients (16 males, 5 females; median age 69 years; IQR 11.0) were analysed. CLL patients had significantly reduced IgG and IgM levels while the amount of IgA was comparable (IgG_{CLL} 873.0 mg/dL; IQR 425.5; IgG_{HC} 1041.0 mg/dL; IQR 267.0; p=0.023; IgM_{CLL} 45.0 mg/dL; IQR 61.0; IgG_{HC} 92.0 mg/dL; IQR 99.0; p=0.009; IgA_{CLL} 174.0 mg/dL; IQR 114.0; IgA_{HC} 235.0 mg/dL; IQR 154.0; p=0.219). Non-transformed B cell counts were comparable between CLL and HC (in CLL median 69.44 cells/µL; IQR 86.92; in HC 81.75 cells/µL; IQR 57.8; p=0.558). Nevertheless, $CD5^-CD19^+$ B cell composition in CLL patients was altered with significantly increased DN and reduced naive B cell number, compared to HC. However, we did not find correlations between B cell subpopulations and immunoglobulin levels.

Conclusions: Non-transformed $CD5^-CD19^+$ B cell counts in untreated CLL patients are comparable to HCs. However, signs of suppressed humoral immunity are already present with decreased IgG and IgM levels. The altered non-transformed B cell subsets in CLL with reduced naive and increased DN B cell counts do not directly correlate with Ig levels suggesting broader distortion of immune response.

IMM-25

CD45RA-EXPRESSING CELLS IN INTRATHORACIC LYMPH NODES, ALVEOLAR COMPARTMENT AND BLOODSTREAM OF PULMONARY SARCOIDOSIS PATIENTS

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Sarcoidosis is a granulomatous interstitial lung disease (ILD) mainly affecting the lungs and hilomediastinal lymph nodes. It is characterized by non-caseating epithelioid cell granulomas in lymph nodes and lungs. The present study aimed to evaluate and compare the distribution of CD45RA-expressing cells in the alveolar compartment, lymph nodes and the bloodstream simultaneously in the

same patients to elucidate the immune responses associated with the development and progression of sarcoidosis.

Patients with suspicious of sarcoidosis and who underwent bronchoscopy with bronchoalveolar lavage (BAL), lung-draining lymph node (LLN) biopsy by EBUS-TBNA and peripheral blood (PB) sampling were included in the study. They were monitored at the Regional Referral Centre of Siena University Hospital and the Respiratory Diseases Unit of Perugia Hospital. Multicolour flow cytometry analysis through FASCLyric (BD Biosciences) was performed to assess cell subsets.

Thirty-two patients (median age (IQR) 57 (52–58) years) were consecutively and prospectively enrolled. Cell proportions of TemRA ($p = 0.0416$), Th2 ($p = 0.0212$), Th17 ($p = 0.0177$) and T-naïve ($p = 0.0368$) were higher in PB than BAL samples, while T-reg were lower in PB than BAL ($p = 0.0329$). Conversely, Th1 ($p = 0.0322$) and CD4 ($p = 0.0486$) were more abundant in LLN than in BAL and PB.

Lower percentages of CD4 cells further substantiating the crucial role of T-cell interaction with antigen-presenting cells for granuloma formation and maintenance. Moreover, our study is the first to describe altered Th-CD45RA$^-$ subsets in three anatomical compartments of sarcoidosis patients with higher Th2 and Th17 cell percentages in PB and higher Th1 and Treg in LLN and BAL samples. The regulatory counterpart of Th cells accumulating in the alveolar compartment may be due to peripheral recruitment; nevertheless, the proportion of regulatory CD127-expressing cells (T-naïve) was lower in BAL, probably because pathogenic T-cells are resistant to suppression signalling by Treg cells.

Besides supporting the multisystem nature of the disease, these findings raise concerns about low cell immunity in peripheral blood of sarcoidosis patients. Changes in the relative content of peripheral blood cells may be due to changes in cell production and to selective redistribution to granulomatous foci. This implies that any changes to the spectrum of peripheral blood cells may reflect both pathogenic and systemic compensatory processes.

IMM-26

REGULATORY T CELLS EXHAUSTION IN SEVERE EOSINOPHILIC ASTHMA TREATED WITH ANTI-IL-5 MONOCLONAL ANTIBODIES

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Introduction: Biological treatments have redesigned the clinical management of severe eosinophilic asthmatic (SA) patients. Among these, there are two drugs specifically targeting the interleukin (IL)-5 axis: mepolizumab and benralizumab. Despite emerging evidence supporting the role of mature of regulatory T cell (Treg) subsets in the pathogenesis and severity of asthma, no data are available on the impact of anti-IL5/IL5R therapies.

Methods: We prospectively enrolled fourteen SA patients treated with benralizumab (n=7) or mepolizumab (n=7) and compared them with healthy controls (HC) (n=11) and mild to moderate asthmatic (MM) patients (n=9). Cellular analysis was determined through multicolor flow cytometry. The analysis of CD4$^+$CD25$^+$CD127^{low} Treg and T/NK cell subsets was evaluated. T cell subsets were detected on the basis of CD62L and CD45RA expression: T central memory cells (TCM) (CD62L$^+$CD45RA$^-$

), effector memory T cells (T_{EM}) ($CD62L^{-}CD45RA^{-}$), naive T cells (Tn) ($CD62L^{+}CD45RA^{+}$) and T effector memory RA cells (T_{EMRA}) ($CD62L^{-}CD45RA^{+}$). Moreover, the expression of PD-1 was detected in Treg and NK cells after the administration of drugs.

Clinical and functional parameters, blood eosinophil counts, and asthma control tests were collected at baseline (T0) and during follow-up after 1, 6, and 24 months.

Results: From the clinical point of view, all SA patients resulted in functional improvement, with increased FEV1 percentages and ACT score. At baseline, SA patients showed higher percentages of TEM and TCM than HC and MM patients. SA patients also reported lower percentages of regulatory T cells before the administration of the drug. During follow-up, an increase of these cells was reported concurrently with an increase in their expression of PD-1. With different timing, the two drugs induced a reduction of TEM. The change of $CD56^{dim}$ $PD\text{-}1^{+}$ and regulatory T cells expressing PD-1 significantly correlated with clinical and functional parameters.

Conclusions: Benralizumab and mepolizumab-treated patients showed an alteration of the immune system, with effects on regulatory and effector cell balancing. In particular, an exhausted phenotype of regulatory T cells was reported after therapies, supporting TregPD-1 as a biomarker of response in severe eosinophilic asthma patients treated with anti-IL-5.

IMM-27

DEVELOPMENT OF 27-COLOR INNATE IMMUNOPHENOTYPING AND INTRACELLULAR CYTOKINE STAINING PANEL

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Once our immune system is exposed to pathogens such as viruses, the innate arm is activated to identify and fight against them. At the same time, through antigen presentation, it informs the adaptive immune system to engage and to sustain long-term protection from the infectious agent. However, excessive innate immune responses such as cytokine release syndrome or reactogenicity of certain compounds can cause harmful effect to the host. Common ways of measuring the cytokine and chemokine response to an antigen are enzyme-linked immunosorbent assay and bead based multiplex assays. Although these assays provide an easy and quick quantification of the cytokines and chemokines response in different culture condition, it does not provide the information of which specific immune cell subset(s) contributed to a certain cytokine or chemokine response. Here, we describe a novel assay that links the innate immune cell subsets and cytokine/chemokine responses at the single cell level.

We successfully developed a 27-color flow cytometry antibody staining panel that provides deep immunophenotyping of activated human peripheral blood mononuclear cells (hPBMCs). The modulation of key activation markers (CD83; CD32; CD40; CD86; HLADR) on dendritic cells (pDC, cDC1, and cDC2), monocytes (classical, intermediate, and non-classical) and NK cells as well as intracellular cytokines (IL-6; IL1RA; IFN-a; IFN-g) and chemokine (IP-10; MIP-1b; MCP-1) were measured at the single cell level after exposure of PBMCs to various TLR agonists. This high throughput assay can prove insightful for the measurement of human innate cell responses to new compounds in early in vitro screening and contribute to the down selection of candidates with too high reactogenicity.

NOV-01

IMPLEMENTATION OF CYTEK® AURORA INSTRUMENTS IN CLINICAL TRIALS: A MULTI-STEP PROCESS INCLUDING PERFORMANCE QUALIFICATION AND STANDARDIZATION

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Introduction: Flow cytometry plays an important role for patient immune profiling in global clinical studies. Instrument standardization is critical to obtain transparent results across different instruments located in different labs. High parameter assay with deeper characterization of patients' immune subsets in clinical trials utilizing spectral flow cytometer requires developing new methods for instrument implementation and standardization. As no specific guidelines on how to perform spectral performance qualification (PQ) and instrument standardization are yet available, an in-house workflow was developed for this purpose.

Methods: For PQ, instrument optical system alignment, resolution and stability was assessed by running daily SpectroFlo® QC beads in the software's QC module and in a user-defined acquisition module, using default Cytek Assay Settings (CAS). MFI deviation from baseline was calculated for all detectors to identify optics errors, if any. Spillover Spread Matrix (SSM) was generated, as quality control and benchmarking tool, by single stained peripheral blood mononuclear cells (PBMCs) with anti-CD4 antibodies conjugated to fluorochromes spanning full emission spectrum. Lastly, analytical performance was evaluated by using an immunophenotyping Cytek® kit (14 colors) on PBMCs. To assess standardization between two Cytek® Aurora instruments in different locations, MFI target values, obtained by acquiring the same lot of SpectroFlo® QC beads with CAS in an acquisition module were compared. Next, instrument comparison was assessed by calculating the difference (delta matrix) between the SSM obtained from each instrument. The same lot of an 18-color pre-stained lyophilized PBMC kit was used to evaluate MFI and population frequencies as a measure of assay performance between instruments. Finally, the same immunophenotyping Cytek® kit was used on the same 3 PBMC donors to assess instrument standardization.

Results: Analytical performance qualification data with 14-color Cytek® kit showed precision for all primary populations were within acceptance criteria of ≤ 25% coefficient of variation (CV). Results from instrument standardization evaluation showed that 1) instruments are comparable as variation of the MFI target values was between 2-5 %CV and the SSM delta matrix values, calculated between the SSM obtained from each instrument, are within Cytek's specifications for instrument comparison; 2) the performance between the two instruments was consistent as MFI output and populations frequencies obtained from immunophenotyping assay showed ≤ 20% difference.

Conclusions: The process discussed here for PQ and to assess standardization of Cytek® Aurora instruments provide guidance on how to implement within clinical assay, and how to reliably monitor inter-instrument performance over time.

NOV-02

IMPAIRMENT OF CIRCULATING HEMATOPOIETIC STEM AND ENDOTHELIAL CELL COUNTS IN METASTATIC COLORECTAL CANCER PATIENTS

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Higher level of circulating endothelial cells (CEC) already represents a solid method to assess vascular functions in cancer patients. The balance between circulating Endothelial progenitor cells (EPC) and CEC is crucial in the endothelial homeostatic process contributing to cancer vasculogenesis. It has been hypothesized that it may exist a hemangioblast common progenitor that give rise to both hematopoietic and endothelial lineages. The characterization of circulating CEC and EPC and the putative hemangioblast may be of clinical relevance in cancer. Beside the well-known CEC phenotype, the consensus on the EPC and hemangioblast phenotypes has not been reached yet. Therefore, in this study we analyzed different circulating endothelial and hematopoietic stem cell subsets in a cohort of metastatic colorectal cancer patients with the aim of investigating their role as predictive biomarkers on the treatment follow-up.

35 advanced colorectal cancer patients and 185 matched healthy controls (HCs) were enrolled by the clinics of oncology of the “SS Annunziata" hospital Chieti, Italy from 2016 to 2023. Blood samples of patients were collected at treatment baseline and at the first radiological assessment. The characterization of different circulating cell phenotypes was carried out by using a polychromatic flow cytometry approach, based on the optimization of gating strategies and the evaluation of different cell subsets based on the surface expression of CD45, CD34, CD146, CD309, CD117 and Annexin-5. A dimensionality-reduction approach was finally carried out to understand the impact of the therapy on the subsets involved in patients’ outcomes.

No difference of median peripheral blood total CD34bright/CD45neg and CD34bright/CD45neg/CD146pos (CECs) cell concentration was observed between HCs and colorectal cancer patients (p-value=0.45; p-value=0.82). Otherwise, median HSC (CD34pos/CD45dim) blood level was significantly lower in the cancer group compared to HCs (487 vs 2361 cells/ml p-value=$2,47*10^{-15}$). Prognostic and predictive significance of blood concentration of CD34 positive cell subpopulations, phenotypically characterized by combined expression of cell surface markers like CD117, VEGFR2 and annexin-5, was investigated. Of note, patients with higher levels of CD34 bright/CD45neg/CD117pos and HSC cells presented worse overall survival and lower overall response rate to cancer treatment compared to those with lower blood counts of these cell subpopulations.

These data strongly suggest that HSC are potentially involved in colon cancer pathogenesis. Furthermore, CD117 expression on circulating CD34 positive cells is associated with unfavorable clinical outcomes in patients with advanced colorectal cancer.

NOV-03

EFFECTIVE DETECTION OF ABERRANT CELLS IN ACUTE MYELOID LEUKEMIA USING A SINGLE-TUBE, 20-COLOR PANEL ON A SPECTRAL CYTOMETER

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Introduction: Multiparameter flow cytometry is widely and routinely used in acute myeloid leukemia (AML) diagnosis and for measurable residual disease (MRD) detection and monitoring. We have developed a single-tube, 20-color panel for AML analysis, taking advantage of the full spectrum profiling technology, allowing for increased reagent and sample efficiency.

Methods: Normal and AML bone marrow samples were stained with the 20-color panel, acquired, and analyzed using SpectroFlo® software. As part of the panel optimization, the resolution of each marker was compared between single-color stained controls and the 20-color stained, matched samples. Once the assay was fully optimized, the analytical precision for cell populations of interest and antibody cocktail stability were assessed. The limit of detection (LoD) and lower limit of quantification (LLoQ) of the 20-color assay were determined using limiting dilution analysis.

Results: The resolution of each marker in the full 20-color panel was comparable to that seen with the single-color stained controls, indicating that the resolution of each individual marker has been optimized. Normal versus aberrant myeloid cell populations were clearly identified and the coefficient of variation (CV) of cell percentage for defined populations in replicate runs were all less than 25%. The pre-mixed 20-antibody cocktail was stable at 4ºC for 21 days. The single-tube, 20-color panel easily achieved a LLoQ of 0.01% for residual aberrant cells in AML, better than the minimum required LLoQ of 0.1%.

Conclusions: Cytek's single-tube, 20-color panel demonstrated an effective, high sensitivity spectral flow cytometry approach that can be used for AML testing, for identification and characterization of normal and aberrant cells, immunophenotypic classification, and MRD evaluation in translational research.

NOV-04

CREATING A FLOW CYTOMETRY SAMPLE MANAGEMENT AND REPORTING SYSTEM STARTING FROM INSTRUMENT MANAGER™

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Introduction: Flow Cytometry is becoming the preferred method for monitoring immune cell populations at single cell level. To perform a flow cytometric analysis, a sample follows different steps from arrival in the lab to result reporting. Having a user-friendly and low maintenance tool to track the status of samples throughout this process is essential in a high-throughput lab.

Methods: Instrument Manager™ (IM) software (Data Innovations®) is an off-the-shelf middleware system, allowing ordering from and resulting to the laboratory information system (LIS), yet lacks sample management functionality. To obtain this, we configured multiple workspaces to track the

entire path of a sample. These let us distinguish samples that 1) need processing, 2) are acquired, 3) need (re)analysis and 4) need review. By adding filters and applying rules, we visualize data of interest within one workspace, generating a digital overview of pending tasks for each step in the process. Each workspace is set up in a way that easily allows to identify which assays need to be performed per sample and provides the option to add comments per sample and assay. In addition, each user group has its own workspace. A feature that is lacking in most other middleware systems. To avoid alteration of a workspace, we applied different authority levels through user rights.

Results: We developed a personalized and paperless system where users can track the status of a sample and adjust based on authority levels. The different workspaces divide samples into groups dedicated to the task at hand. Lab technicians and analysts can update the status of a sample and add additional comments, as needed. Our configuration enables reviewers to choose which set of results to report to LIS, in case multiple data sets are present *e.g.*, due to re-analysis. Having all information in one place increased efficiency of communication and cooperation between lab techs, data analysts and reviewers. Different operators can take over tasks from one another, without losing oversight during sample preparation, data analysis or reporting. Combined with the IM audit trail, this set up also greatly reduced errors in sample workflow follow-up.

Conclusions: Using the flexibility of IM to our advantage resulted in a tool to track sample status within the Flow Cytometry department. It resulted in an efficient, automated, and traceable workflow from request to report, which is indispensable considering the increasing number of samples flow cytometry labs are facing.

NOV-05

STANDARDIZATION OF 25-COLOR CYTEK NORTHERN LIGHT FLOW CYTOMETER AND ITS UTILIZATION IN COMPREHENSIVE IMMUNOPHENOTYPING OF IMMUNE CELL SUBSETS

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Introduction: Multicolour flow cytometry (MFC) is a powerful tool used for studying immune system in health and disease. A major advantage of MFC is the capability to study multiple markers simultaneously. Recently, Full Spectrum Flow Cytometry (FSFC) has introduced a more efficient technology to evaluate simultaneously more than 25 proteins in each cell. Experience in the instrument-set up and validation of immune monitoring assay in FSFC is still limited. We prospectively validated a 25-color immune-cell profile using FSFC and compared the results with 16-color immune cell profile assay performed on conventional MFC (CMFC).

Materials and Methods: We performed 25-color and 16-color flow-cytometry on 15 peripheral blood samples in post-bone marrow transplant patients (as a part of immune reconstitution study). A 25-colorsingle-tube FSFC immune profiling assaywas studied using CYTEK-Northern Lights and a 16-colorsingle-tube CMFC immune subset assay was studied using BD-LSR Fortessa. Initially, instrument setup for CYTEKwas done by acquiring single stained 25c tubes for the reference spectral signature and these signatures were applied to 25c immune subset panel for unmixing phenomenon with unstained cells for every sample. The data was analysed on Kaluza version 2.1 software.

Results: We identified 35 different immune subsetsusing 25-color FSFC assay. Out of 25, comparison with conventional 16-color CMFC assay was possible in 19 subsetsapplying similar gating strategies for both the instrument data. It included Median (range) of 25-colorFSFC and 16-color CMFC of 19 immune subsets were respectively **Tcells** 66.3% (1.07-83.9%) and 59.0%(23.12-81.8%) **Bcells** 5.33% (0.11-30.71%) and 9.1% (0.16-50.07%) **NKcells** 18.06% (2.5-40.2%) and 18.5%(5.7-41.4%) **Monocytes** 5.69%(2.24-20.5% and 5.8%(2.98-21.02%) **Basophils** 0.59%(1.36-0.08%) and 0.3%(0.11-2.13%) **Eosinophils** 0.01%(0-0.23%) and 0.2%(0-3.9%) **Granulocytes** 50.22% (21.25-78.17%) and 47.6%(0.47-76) **Plasmacytoid Dendritic Cells** 0.17%(0.009-1.01%) and 0.1%(0.004-1%) **Memory Bcells** 1.11%(0.012-3.07%) and 1.7%(0.07-19.24%) **Naive B cells** 10.04%(0.95-30.25%) and 5.4%(0.07-72.9%) **Transitional-B** 0.83%(0.03-34.75%) and 1.3% (0.02-51.7%) **CD4+ T cells** 33.99%(2.51-49.97%) and 32.4%(3.58-49.8%) **CD8+ T cells** 27.34%(8.77-35.54%) and 22.2%(6.7-72.22%) **Early-NKcells** 0.91%(0.14-35.26%) and 0.8%(0.08-32.81%) **Effector-NKcells** 11.9%(0.76-34.44%) and 11.6%(0.6-74.3%) **Terminal-NKcells** 1.92% (0.93-34.75%) and 3.8%(0.4-35.5%) **Classical-Monocytes** 4.24%(1.16-16.64%) and 5.8%(1.54-84.8%) **Intermediate-Monocytes** 0.68%(0.24-4.46%) and 0.3%(0.1-31.7%) **NonClassical-Monocytes** 0.45%(0.03-1.21%) and 0.4%(0.04-9.83%)

The data from BD LSR Fortessa and CYTEK for the immune subsets were compared using Mann Whitney test and was found to be similar with no statistical significance (p= >0.05).

Conclusion: This is the first study in India where standardization of 25-color CYTEKNorthern Light flow cytometer was done. Using this approach of 25-color full spectrum flow cytometry we can broadly phenotype much of the cellular composition of the human peripheral immune system.

NOV-06

THE FEASIBILITY OF TRANSFERRING A CUSTOMIZED FLOW CYTOMETRY ASSAY FROM A CONVENTIONAL TO A SPECTRAL FLOW CYTOMETER

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Introduction: Since 2010, >80 OMIPS (Optimized Multicolor Immunofluorescence Panels) have been published[1], describing methods for analyzing up to 40 markers on a single cell level. These panels can be used for the immunophenotypic characterization of various cell subsets from human, rodent, and non-human primates. The differences in the configurations of flow cytometers present a major challenge while transferring the assay developed from one instrument to another.

Methods: The OMIP publications published until March 2023, providing all the necessary details for setting up a flow cytometry assay, were retrospectively analyzed. We assessed the transferability of all OMIPs developed on a conventional flow cytometer to the spectral flow cytometer, Cytek Aurora, which can be equipped with 3 to 5 lasers and 38 to 64 fluorescence detection channels, respectively. To assess the transferability, we determined the complexity index (CompIDX), which evaluates how distinguishable a collection of spectral signatures are from each other when unmixed together.

Results: The majority of the OMIPS (78 out of 82) have been developed using conventional flow cytometers such as BD LSR II (40%), BD LSR Fortessa (25%), and BD Symphony A5 (24%). The mean CompIDX for OMIPS (developed on the conventional flow cytometers) with 6-10, 11-20, and 21-30 fluorochromes were 2.81 (n=12), 4.67 (n=47), and 8.94 (n=19) respectively. These values were lower than the CompIDX values for panels developed using Cytek Aurora (mean CompIDX: 3.24, 10.64, and

17.46 for the panels including same ranges of fluorochromes). The lower CompIDX for OMIPS developed on conventional flow cytometers suggests that these panels can be easily transferred to the spectral flow cytometer. Based on the CompIDX values, the Cytek Aurora with 3-laser (Violet/Blue/Red) or 4-laser (UV/Violet/Blue/Red) configuration can onboard 36 or 39 OMIPS, respectively. As anticipated, the 5-laser (UV/Violet/Blue/YellowGreen/Red) instrument could onboard all 78 OMIPs.

Conclusions: Our retrospective analysis indicates that, regardless of the primary instrument used for panel optimization, all OMIPs are transferable to the 5-laser, 64-detector Cytek Aurora. In our laboratory, we could readily transfer four multi-parameter (up to 12 colors) flow cytometry assays developed on a conventional flow cytometer to our 5-laser Crytek Aurora. We also discuss here further conditions that need to be assessed for successful flow cytometry assay transfer from conventional to spectral flow cytometers.

https://medicine.uiowa.edu/flowcytometry/resources/omips-optimized-panels

NOV-07

STANDARDIZATION AND IMPLEMENTATION OF THE 5-LASER ZE5 CELL ANALYZER FOR GLOBAL CLINICAL TRIALS

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Introduction: With the recent advances in cancer vaccines and the use of immunotherapy for treatment of multiple tumor types, immune monitoring has become increasingly critical to define patient immunological profiles and guide treatment decisions. Multi-parameter flow cytometry allows the fine characterization of single cells in solution, and is ubiquitously used in pre-clinical tumor immunology and in cancer immunotherapy trials. However, one of the biggest challenges for the use of flow cytometry in multicenter clinical trials is to ensure cross-site data combinability with robust analytical processes and cross-instrument standardization.

Methods: During the deployment of the Bio-Rad® 5-laser ZE5 Cell Analyzer platform across our Central Laboratory Services (CLS) sites of Geneva, Indianapolis and Singapore, the 3-intensity AccuCheck™ ERF Reference Particles from Thermo Fisher Scientific were used to setup and standardize instruments. To verify standardized instrument settings and robust performance, beads were monitored longitudinally along with two levels of quality control (QC) material stained with a 13-color T cell panel that was designed and validated to assess the expression profiles of immune checkpoint molecules, co-inhibitory and co-stimulatory receptors, as well as activation and proliferation markers of T lymphocyte subsets during tumor immunotherapy clinical trials.

Results: A lot-specific median fluorescence intensity (MdFl) target value was established for the 3rd AccuCheck™ bead population in all 27 channels of the ZE5 Cell Analyzer and used for instrument standardization across CLS sites. Longitudinal MdFl monitoring of this bead population in all fluorescent channels indicated intra- and inter-instrument %CV values below 5% and, when compared to the lot-specific MdFl targets, %Difference values below 20%. Linear regression parameters calculated from all 3 bead intensities confirmed robust and comparable instrument standardization and performance across sites. The globally implemented instrument settings were further verified with

two levels of QC material (Immuno-Trol™ Normal Cells and Immuno-Trol™ Low Cells) stained for the above mentioned 13-color T cell panel. Intra-assay and inter-instrument precision (i.e. %CV values below 20%) were obtained for various MdFl as well as numbers of equivalent reference fluorophores (ERF) readouts of functional markers such as CD69, Ki67, PD-L1, OX-40 or 4-1BB monitored on T cells and their subsets.

Conclusion: We successfully standardized and deployed the 5-laser ZE5 Cell Analyzer platform in global CLS sites. Cross-site standardization of the instruments with hard-dyed reference particles yielded robust, reliable and comparable analysis of biological QC samples qualifying the instruments and their setup for application in global clinical trials.

NOV-08

METACLEAN2.0: ROBUST AND ACCURATE REMOVAL OF LOW-QUALITY MEASUREMENTS IN FLOW, MASS, AND SPECTRAL CYTOMETRY

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Introduction: Despite quality procedures in place including instrument standardization approximately 14% of flow cytometry sample acquisition files contain low-quality data. The main reasons for that are clogged fluidic lines, air bubbles, or instabilities in instrument performance in the light path (lasers or detectors). If such anomalous measurements are left unremoved, they may impact the quality of the results and lead to drawing incorrect conclusions from the study. This preprocessing of the files has been a good practice in manual analysis of FCS files, but analysts have been looking at one or just a few channels at a time, to eliminate the “corrupted” events in their files. Herein, we propose the automated removal of these events from the FCS file to improve the quality of analyses and results. Incorporating such preprocessing tools in the diagnostic cytometry can improve diagnosis accuracy and aid in delivering appropriate treatment for the patient sooner.

Method: MetaClean2.0 is a method that detects and removes acquisition-borne anomalies from flow cytometry samples. Current methods (e.g. flowClean, flowAI, flowCut, and PeacoQC) detect anomalies based on values from all channels, a set of static hard-coded channels, or channels the user chooses. These assume that issues at acquisition affect all channels equally, most affect the same channels for all files, or the user already knows which channels are most affected beforehand, respectively. We show that these assumptions do not always hold. We test and verify an alternative hypothesis with MetaClean2.0, where we automatically select channels that best reflect flow obstruction to detect anomalies. We also notice that current methods assume that anomaly detection should be done using either time-agnostic or time-dependent methods. Herein, we hypothesize that we can account for all types of flow obstructions if we engineer a method that combines the two method archetypes. MetaClean2.0 does this by grouping flow patterns with clustering (time-agnostic) and evaluating their quality across time using changepoint analysis (time-dependent).

Results: MetaClean2.0 achieves the highest F1 score out of all existing state-of-the-art methods. We test MetaClean2.0 on a clinically relevant heterogeneous set of flow, mass, and spectral files. Each file is analyzed by at least five flow cytometrists to establish a ground truth reference for calculating accuracy.

Conclusion: MetaClean2.0 is a robust and accurate method for removing low-quality measurements to improve downstream analysis. For future work, we want to continually enhance MetaClean2.0 for a more extensive variety of real-world cytometry data.

NOV-09

VALIDATION OF A FLOW CYTOMETRY ASSAY ON CYTEK® AURORA TO MONITOR IMMUNE CELLS IN PERIPHERAL WHOLE BLOOD FOR CLINICAL TRIALS

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Introduction: In patients enrolled in clinical trials for drug development, immune monitoring by flow cytometry (FCM) is pivotal to provide data that support the evaluation of drug safety, efficacy, and exploratory endpoints, safety, and toxicity. Spectral FCM and the ability to develop high parameter panels, allows for a deeper characterization of patient samples and a more comprehensive picture of immune system dynamic in response to a particular drug. Here, we describe the validation workflow of a 14-color one tube assay designed to characterize T-cells, B-cells, NK cells, monocytes, and subsets thereof to be used for immune monitoring of patients enrolled in global clinical trials.

Methods: A 14-color Immunoprofiling kit developed by Cytek® was further optimized in whole blood collected in Cyto-Chex® tubes (Streck), by performing titration of viability dye and identifying optimal reference controls. To use the panel in patient routine testing, validation was performed according to CLSI H62 guidelines. Whole blood from three apparently healthy donors collected in Cyto-Chex® tubes was tested in triplicate by two operators for a total of 2 runs. Evaluation of repeatability, intermediate precision, and inter-operator variability were performed. Sample stability was additionally assessed on whole blood from six apparently healthy donors, stability calculation was based on the percentage of change from baseline within 2 hours after collection.

Results: Accurate titration of the viability dye showed that live-dead cells can be discriminated without nonspecific staining in Cyto-Chex® tubes. SpectraComp® beads from SlingShot Biosciences were selected as reference controls that resulted in the best alternative to cells for unmixing. Analytical validation data with 14-color Cytek® kit showed that precision for all primary populations were within acceptance criteria of ≤ 25% CV and inter-operator variability below ≤ 20% difference. Stability in Cyto-Chex® tubes for primary populations was established at 72 hours.

Conclusion: The workflow discussed here provides guidance on how to optimize and properly validate a high parameter panel to be implemented for patient immune profiling in global clinical trials.

NOV-10

MERGING AND IMPUTATION OF FLOW CYTOMETRY DATA: A CRITICAL ASSESSMENT

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Introduction: In most laboratories, multi-parameter flow cytometry (MFC) applications still depend on flow cytometers measuring relatively small (<16) numbers of parameters. When the number of markers that need to be assessed exceeds the available parameters that can be acquired, these are commonly distributed over multiple tubes that include a backbone of common markers. Several methods have been proposed to calculate (i.e., "impute") values for combinations of markers that were not measured simultaneously. These imputation methods are frequently used without proper validation and knowledge of their effects on data analysis. Therefore, we assessed the accuracy of the currently available methods.

Methods: We evaluated the performance of four software packages: Infinicyt, CyTOFmerge, CytoBackBone, and cyCombine. Both laboratory and *in silico* MFC datasets were generated in which a full combination of markers existed over multiple tubes (ground truth) or one or more markers were deliberately left out. The software packages were then used to impute the expression of those missing markers. We evaluated how well the imputed marker expression values resembled the ground truth data in terms of similarity in the distribution of expression levels, matching immunophenotypes of individual cells and manually gated populations.

Results: Performance was insufficient to poor for all methods; pairwise comparison of imputed and ground truth expression showed changes in the range of 5-25%, with some markers imputed close to random (Pearson correlation coefficient < 0.1). CyTOFmerge showed the highest concordance, with a mean Pearson correlation coefficient of 0.67, 0.94, and 0.80 in three different datasets. However, even with the best method, manually gated populations were retrieved with low sensitivity (0.58), with an average change in population size of 39%. Additionally, we showed an unexpected mechanism by which imputation with Infinicyt caused high numbers of duplicate cells, causing notable distortions in commonly used read-outs such as mean fluorescent intensities (MFIs), as well as in computational analyses such as clustering, often without apparent visual distortions.

Conclusions: Imputation software that is used to calculate non-existing marker combinations from markers distributed over multiple tubes may cause extensive distortions in MFC data and subsequent biological interpretation. These distortions were previously also shown in published bivariate plots, highlighting the need for a critical assessment of imputation methods. Although imputation may be beneficial in exploratory studies, the limitations that we describe here should be taken into account. Independent validation experiments should be performed to justify conclusions, in particular when used in clinical diagnostics.

NOV-11

LIVE-CELL IMAGING ANALYSIS AND ACOUSTIC FLOW CYTOMETRY FOR CLINICAL RESEARCH APPLICATIONS

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Background: High-throughput flow cytometry and high-resolution brightfield imaging allow the measurement of morphology parameters concurrently with classical flow cytometric parameters. As the optical focus is maintained regardless of the sample flow rate, it can be easily adapted for clinical research to confirm the identity of target cells. The aim of this study is to evaluate the combination of

high speed bright-field imaging and precision flow cytometry to identify rare and elusive cells using clinical specimens.

Methods: Peripheral blood and bone marrow specimens were obtained in EDTA-anticoagulated tubes and fixed and lysed using CyLyse™ (Sysmex) and stained with FxCycle™ Violet (Invitrogen). For TransFix™ treated samples, DAPI was used to discriminate nucleated cells from debris. Unlysed cells were stained with Hoechst 33342 or alternatively with DyeCycle™ Violet stain (Invitrogen). Samples were acquired on the Invitrogen™ Attune™ CytPix™ Flow Cytometer (Thermo Fisher Scientific), using the Attune™ NxT™ No-Wash, No-Lyse Filter Kit for violet laser side scatter detection, which offers a robust assay with minimal sample manipulation.

Results: The imaging system combined with the acoustic flow cytometer makes it possible to identify cells of interest, especially when dealing with rare events, to confirm small clonal populations in ploidy analysis or to rule out possible artifacts due to non-specific antibody binding, as well as to discriminate true cells and apoptotic populations from background noise. The images obtained allow us to confirm the non-hematopoietic nature of circulating tumor cells in blood, the interaction of cells with cytotoxic activity and their reactivity with tumor cells, as well as to identify submicron vesicles at the single vesicle level and discriminate them from aggregates.

Conclusions: Here we show the potential of acoustic imaging cytometers for the development of new approaches for the evaluation of human health. The combination of acoustic focusing, light scattering and live-cell imaging could be a useful tool to be applied to the immediate evaluation of clinical specimens for research use.

NOV-12

SAMPLE PREPARATION METHODS FOR FLOW CYTOMETRY INVOLVING BULK LYSIS PROCEDURES ALTER THE COUNTS OF RARE CELL POPULATIONS

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Background: Flow cytometry (FCM) immunophenotyping is a technology of extreme importance in the evaluation of measurable residual disease (MRD). To achieve high sensitivity for rare cell detection (<1 in 10^5 cells), the minimum number of acquired cells must be considered, and conventional immunophenotyping protocols involving a classical erythrocyte lysis fall short of reaching these numbers. To overcome this, a pre-erythrocyte lysis prior to immunophenotyping called “bulk” lysis (BL) is a standardized approach that allows the analysis of the millions of cells required for high-sensitivity MRD detection. Despite its advantages, this additional step has been associated with significant cell loss, along with other adverse effects. We hypothesize that BL protocols can specifically alter target cell numbers in MRD evaluation, because these cells may be more resistant or sensitive to these protocols. In this study, we aim to evaluate BL protocols and compare them with minimal sample perturbation (MSP) protocols to detect potential over or underestimates of rare cells when using these methods.

Methods: We first generated an MRD model by mixing fresh peripheral blood obtained from healthy donors with a K562 cell line, expressing stable EGFP, at known frequencies of EGFP+ cells over leukocytes (from 10 to 0.01%). Samples were then prepared with BL and MSP protocols and evaluated

with FCM, excluding necrotic cells with propidium iodide and detecting K562 cell frequencies through its EGFP fluorescence. Samples were acquired in triplicate on the Attune™ NxT Flow Cytometer (Thermo Fisher Scientific).

Results: For all frequencies of K562 cells stablished and evaluated, a significant decrease of this population was detected in BL samples compared with MSP samples, even at low K562 cell frequencies (e.g., for 0.01%: 0.009±0.0.0006% EGFP cells for MSP samples and 0.0007±0.0005% EGFP cells for BL samples; p-value: <0.0001, 95% CI: -0.010 to -0.007). We also found significant differences in frequency when evaluating non-necrotic cells, being lower in BL samples (e.g., for 0.01%: 99.8±0.04% non-necrotic cells for MSP samples and 19.7±0.8% non-necrotic cells for BL samples; p-value: <0.0001, 95% CI: -82.11 to -78.04).

Conclusions: Evaluation of the potential effects of "bulk" lysis protocols in obtaining the final count is of great interest, especially for over or underestimation, as in the case of measurable residual disease. Since conventional flow cytometry methods or minimal sample perturbation assays fall short in obtaining the minimum numbers required to reach high sensitivity measurements, significant efforts may be needed to improve bulk lysis solutions reagents.

NOV-13

CROSS-VALIDATION OF STANDARDIZED EUROFLOW 8-COLOR PROTOCOLS ON THE XF-1600 FLOW CYTOMETER

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Background: Flow cytometry (FCM) immunophenotyping has become a mainstream application in the diagnosis, classification, and follow up of hematological diseases, including leukemias, lymphomas and myelomas. In order to reduce variability and subjectivity of results, different expert groups have stablished consensus guidelines and recommendations to ensure standardization and reproducibility of FCM results between different instruments and centers. The EU-supported EuroFlow Consortium developed a project to provide optimal multicolor combinations of fluorochrome-conjugated antibodies together with adequate standard operation procedures (SOPs) that would allow reproducible and time-consistent FCM measurements by applying predefined values of light scattering and mean fluorescence intensity (MFI) using specific reference beads. The performance and reproducibility of these results were evaluated in multiple clinical diagnostic centers. Importantly, it is necessary to evaluate the performance of currently available flow cytometers, especially those made by different manufacturers, and to determine the procedures and settings that provide the most comparable results. The objective of this work is to develop a setup procedure based on EuroFlow guidelines for the XF-1600™ flow cytometer (Sysmex) in order to define reference fluorescence values comparable to those of a cross-calibration instrument (Navios™ EX flow cytometer, Beckman Coulter).

Methods: XF-1600™ (evaluated instrument) and Navios EX (reference instrument) were the flow cytometers used in this study. Sphero™ Rainbow Calibration Particles 8-peaks (Spherotech, ref. RCP-30-50A, lot EAP01) were used to obtain reference target values. CyFlow™ CompSet (Sysmex, ref. BD055531, lot. CO0204) labeled with monoclonal antibodies were used to prepare single controls for FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7, Pacific Blue and Pacific Orange fluorochromes.

Results: Both flow cytometers used the same excitation lines (violet, blue and red), but differed in their optical configuration and, consequently, the Navios™ EX reference target values could not be directly applied for the XF-1600™. To calculate the specific MFI correction factor, unique controls for each fluorochrome prepared with compensation beads were first acquired in Navios™ EX and adjusted in XF-1600™ to fully match the reference flow cytometer. PMT values obtained on XF-1600™ were used to acquire Rainbow Calibration Particles and calculate adjusted MFI values to determine XF-1600™ target values.

Conclusions: In this study we have demonstrated that the standardized EuroFlow approach based on specific target MFIs can be used on other flow cytometers equipped with >8 colors, such as the XF-1600™. Further experiments will be necessary to evaluate the performance of this setup providing superimposable data files when analyzing samples labeled with specific monoclonal antibodies.

NOV-14

PLATELET IMMUNOPHENOTYPE ANALYSIS IN PATIENTS WITH RUNX1 MUTATIONS

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Introduction: Some platelet functional abnormalities are caused by genetic defects that affect protein expression or functionality on the platelet membrane. In this case, genetic testing remains the main tool for confirming the diagnosis of hereditary thrombocytopathy. However, currently, flow cytometry is becoming more and more relevant in the diagnosis of these pathologies. Platelet flow cytometry is eminently suited to monitor platelet function in whole blood even under thrombocytopenic conditions. The patients with RUNX1 mutation have a phenotype characterized by several platelet function defects, which include impaired platelet aggregation and secretion upon activation, dense and/or α-granule storage pool deficiency and impaired αIIbβ3 activation. We hypothesized that the occurrence of pathogenic mutation in the RUNX1 gene could be suspected immediately after flow cytometric evaluation of changes in the patient's platelet phenotype.

Methods: Flow cytometry was used to examine markers of platelet adhesion (CD42a, CD42b, CD31, CD36, GPVI, CD49b), aggregation (CD41a, CD61), activation (PAC-1) and secretion/degranulation (P-selectin, CD63) in resting platelets. Platelet activation and secretory capacities were assessed by measuring the expression PAC-1, P-selectin and CD63 upon stimulation of platelet with ADP (for PAC-1) and TRAP (for P-selectin and CD63). The results were collected by quantifying the % of positive marker expression on platelets, which was compared to the percentage of same marker expression in healthy adult donors.

Results: Out of five cases in which a mutation in the RUNX1 gene was confirmed by genetic testing, the mutation identified in 3 cases was interpreted as pathogenic. In these three patients, flow cytometry revealed decreased expression of CD49b and CD31 markers, hyperexpression of CD36 marker, increased basal expression of P-selectin and CD63 markers, but decreased response of these two markers to stimulation by TRAP agonists. The detected changes in the expression of platelet markers differed between these patients only in the intensity of marker expression. Other two identified RUNX1 mutations were defined as likely non-pathogenic and as of uncertain significance. In the latter cases changes in the platelet marker expression only partially correlated to the expression pattern in patients with pathogenic mutation.

Conclusions: We can effectively conclude that the analysis of platelet by flow cytometry can help successfully suspect the presence of a pathogenic RUNX1 mutation before performing confirmatory genetic testing.

NOV-15

RAPID AND LOW-COST T-CELL EPITOPE MAPPING BY FLOW CYTOMETRY USING PRE-CONFIGURED 96-WELL PLATES CONTAINING PEPTIDE MATRIX POOLS

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Introduction: A huge number of protein sequences are available for mapping T-cell epitopes that may be used as new targets for immune therapy and vaccination. Peptide matrix pools have been used for mapping T-cell epitopes for many years, however, using conventional peptides the approach is relatively costly. By using SPOT-peptides synthesised on cellulose in small quantities and at low cost, micro-scaling the activation assay in 96-well plates, and using an automated plate reader, the approach becomes both more affordable and efficient. A major advantage over the Elispot technology is that information on the phenotype of responding T-cells is provided. Here, we demonstrate the usefulness of the technology by identifying T-cell epitopes in two human Cytomegalovirus (HCMV) proteins, one of which is latency-associated.

Methods: PBMC of HCMV-seropositive healthy donors were isolated and stimulated overnight (37°C, 5%CO_2) followed by extracellular staining, permeabilization, and intracellular cytokine staining (ICS) to detect responding T-cells. The remaining PBMC were stored in complete media over 2 further days (4°C) and rested for 4 hours (37°C, 5%CO_2) prior to each of several overnight stimulations. Initially, responders to these proteins were identified by stimulation with peptide pools spanning the entire proteins (peptide configuration: 15 amino acids, 11 overlaps). Cells from responders were subsequently submitted to a second overnight stimulation with the same peptides arranged in cross-matrix pools arranged in such a way that each peptide was contained in exactly two otherwise non-overlapping pools. For this step, plates had been prepared prior to use and stored at -80°C. They were removed from the freezer to equilibrate to room temperature 1h before stimulation. PBMCs were added to each peptide pool-containing well (200,000 cells per well), positive (SEB), and negative control wells (media alone). After stimulation, all staining steps were performed in the same plates, which were also used for sample acquisition using the integrated plate reader of a Beckman Coulter Cytoflex LX cytometer. The detection of positive responses for cross-matrix pools determined candidate peptides that were used in a third overnight stimulation to confirm individual peptide reactivity.

Results: This efficient and systematic approach allowed the detection of a range of new and confirmed CD4 and CD8 T-cell stimulating peptides as well as phenotypic characteristics of interest among responding cells.

Conclusions: Our streamlined approach proved to be a straightforward and efficient method suitable for large scale mapping of CD4 and CD8 T-cell reactivity to proteins of interest.

NOV-16

RECEPTOR OCCUPANCY (RO) ASSAY VIA FLOW CYTOMETRY SUPPORTING DOSE SELECTION

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Introduction: One of the key questions in the phase one of a clinical trial is how the drug interacts with its target. Flow cytometry is one technology, which is answering these kind of questions. The most important perquisite to use flow cytometry is that the target itself is available on peripheral cells. In addition, the cell subset and abundance of the target is relevant to develop such a RO assay.

Methods: There are two main approaches used to determine the occupancy of a certain cell surface marker. The so called free vs. total approach does not require self-made reagents most of the time. However, a competing and a non-competing antibody are necessary for this approach. The anti-drug approach on the other hand does require special self-made reagents and/or a certain mutation of the drug to target the drug directly, which is the key aspect of this approach. Both approaches do have their advantages and disadvantages in certain circumstances. Generally said the anti-drug approach is the preferred one in most of the cases as there is only one antibody used and due to that no normalization is needed. In contrast, the free versus total approach needs a certain kind of normalization, as two different antibodies are used, which will never give the same signal in terms of MFI.

Results: We successfully implemented a 6-color flow cytometry RO assay in a clinical phase one trial in the oncology field using the anti-drug approach. The results provided a clear dose-dependent effect supporting dose selection and providing insight in the mode of action and occupancy on the peripheral target.

Conclusions: Although successful implementation, every molecule is different and not every time the favorable anti-drug approach can be used due to possible drug interference or limitations in the availability of specialized reagents . We experience more interest in RO assays, which do not only have the target occupancy assessed. So called target competent RO assays would not only prove the occupancy of a certain surface marker, it even would prove the intactness of the drug. This is especially important for bispecific molecules. We would like to give some insights with the generated data and share the lessons learned.

NOV-17

ACCURATE AND COMPREHENSIVE LEUKOCYTE ENUMERATION WITH A 15-COLOR LYSE NO WASH ASSAY ON A CYTEK SPECTRAL FLOW CYTOMETER

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Introduction: A white blood cell (WBC) differential is routinely performed on automated hematology analyzers as a general health check to evaluate immune status of patients. The recent advances in high parameter flow cytometry, especially full spectral flow cytometry, enable the analysis of more comprehensive leukocyte subsets to meet the increased demands in clinical research. Here we designed a 15-color, single-tube panel to identify and enumerate all major subsets of leukocytes on a full spectrum flow cytometer.

Methods: Human peripheral blood samples were stained with 15 cFluor® dye conjugated mAbs with a lyse-no-wash method and analyzed on a Cytek® spectral flow cytometer. The 15-color panel identifies more than 20 leukocyte subpopulations: including neutrophils, eosinophils, basophils, lymphocytes, hematopoietic stem cells, monocyte subsets, T cell subsets, B cells, NK cell subsets. The performance of this assay was evaluated side by side with BD 6-color TBNK assay on BD FACSCanto II on healthy donor samples. Assay precision was examined on 12 donor samples in triplicates. Assay was also tested with blood samples collected in K_2EDTA, Heparin, ACD and Cyto-Chex® anticoagulants.

Results: The results are comparable to that from BD 6-Color TBNK. The coefficient of variations (CV) of intra assay precision for all cell populations of interest are less than 25%.

Conclusions: Cytek's single-tube 15-color pan leukocyte panel demonstrated an effective, high sensitivity flow cytometry approach that can be used for monitoring all immune cell subsets in peripheral blood in clinical and translational research.

NOV-18

A HIGH PARAMETER FLOW CYTOMETRIC ASSAY TO ANALYZE HUMAN MYELOID DERIVED SUPPRESSIVE CELLS

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Introduction: Myeloid derived suppressive cells (MDSCs) are a group of immuture myeloid cells that pocess potent immunosuppressive activity. These cells expand under pathologic conditions and suppress the immune functions of T cells, dendritic cells, macrophages, and natural killer cells. They have been shown to be involved in the pathological states, such as cancer. MDSCs are subset into PMN (polymorphonuclear)- and M (monocytic)- MDSCs. Phenotypic markers of MDSCs have been elucidated in recent years. Here Cytek® developed a 13-color, 15-marker, single-tube flow cytometric assay to analyzed MDSCs.

Methods: The panel includes markers to exclude lymphoid lineage cells, and markers to identify PMN-MDSC and M-MDSC populations, such as CD15, CD14, CD11b, CD33 and HLA-DR. In addition, PMN- and M- MDSC specific markers such as LOX-1, and CXCR-1 are also included to better distinguish the MDSC subsets. The panel performance was evaluated on a Cytek® Aurora or 3-laser Northern Light™ instrument with blood samples collected in EDTA, Heparin, ACD, and Cyto-Chex® blood tubes as well as peripheral blood mononuclear cell (PBMC) samples. Assay precision was assessed by testing on 12 donor samples in triplicates.

Results: Targeted cell populations are well identified in peripheral blood and PBMC samples with good resolution and assay precision with %CV less than 25% for all cell populations of interests.

Conclusions: This 13-color 15-marker preoptimized flow cytometric assay can be used in clinical research and drug discovery in cancer, autoimmune diseases, infections and other inflammatory diseases.

NOV-19

EVALUATION OF A DRY MONOCLONAL ANTIBODY LYMPHOCYTE SUBSET KIT USING THE XF-1600 FLOW CYTOMETER

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Introduction: Exbio (Prague), have recently launched the DryFlowEx TBNK 6-color dry tubes (DFE) for lymphocyte subset analysis. Sysmex Europe SE (SEU) evaluated the use and comparability between DFE tubes and KOMBITEST™ TBNK 6-color liquid cocktail (Exbio, Prague) using XF-1600 flow cytometer TBNK automated volumetric counting software.

A limited study was performed comparing the methodologies, results, and difference in stain index (SI) between the predicate SEU process, XN-Series haematology analyser and the DFE tubes on normal patient samples and CD Chex quality control blood.

Methods: The evaluation was performed at SEU in Hamburg, Germany. 40 samples were tested - 10 CD Chex CD4 Low, 10 CD Chex CD4 Normal and 20 normal patients. 2 sample preparation protocols were applied for the flow cytometry portion, the predicate method which consisted of 50ml blood, 20ml KOMBITEST™ TBNK 6-color cocktail, and 500ml CyLyse™ FX, this provides a volume of 570ml which is required for accurate volumetric counting on the XF-1600 flow cytometer. Preparation of the DFE tubes consisted of 50ml blood and 520ml CyLyse™ FX. The patient samples were also placed on the XN-Series haematology analyser for WBC and lymphocyte analysis, CD Chex could not be analysed on the XN-Series due to the incompatible matrix of the qc blood. Results were obtained as percentage and absolute parameters, SI for all flow cytometric parameters, WBC and total lymphocyte parameters were also compared for the haematology portion.

Results: Regression analysis illustrated that all parameters were comparable with R^2 values >0.95. Bland-Altman analysis showed a negative bias towards all dry tube parameters. All CD Chex parameters were within range with a P value of <0.05 in all cases, showing that the results were of no significant difference. For the flow cytometric portion SI information showed a significant difference for CD16/56 (Mean percentage difference of -39.5%) for CD Chex QC, and CD3 and CD16/56 (Mean percentage difference of -55.2% and -37.3% respectively) for fresh patient samples, all other parameters were comparable.

Conclusion: All parameter results were comparable and statistical analysis showed no significant difference. However, the SI data for CD16/56 and CD3 a significant change to the predicate for all samples. This is likely due to the XF-1600 being set up specifically for the KOMBITEST™ TBNK 6-color liquid cocktail and not optimised for the DFE. Further testing should include specific set up criteria and the use of patient samples with a low CD4 count.

NOV-20

HIGH-PARAMETER PANELS FOR ACUTE LEUKEMIA DIAGNOSIS ON CYTEK NL-CLC FLOW CYTOMETER WITH IMPROVED SAMPLE EFFICIENCY

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Introduction: Multi-parameter flow cytometry offers fast detection and high sensitivity for clinical diagnosis and minimal residual disease （MRD） monitoring of acute leukemia (AL). However, traditional flow cytometry is limited in terms of laser channel settings, resulting in AL immunophenotypic analysis panels mostly utilizing 6 to 10-color channels. The Cytek Northern Lights (NL-CLC) full-spectrum flow cytometer overcomes these limitations by providing up to 38 color channels and automatic fluorescence interference compensation. This allows for obtaining extensive information even from precious samples.

Methods: Bone marrow samples were collected from a variety of 22 AL patients and 8 non-AL individuals. Based on common immunophenotype of AL and the channel settings, 2 standard panels including in total 35-antibody (HLA-DR, CD1a, CD2,CD3, CD4, CD5, CD7, CD8, CD9, CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD33, CD34, CD38, CD56, CD64, CD65, CD66c, CD71, CD99, CD117, CD123, CD300e, cMPO, cCD79a, cCD3, nuTdT, CD45) were used to label the membrane and cytoplasmic antigens of bone marrow cells. Two different cytometers were utilized: Cytek NL-CLC with 23-color and 16-color panels and Canto^{TM} II with 6-color panels. The cell percentage and intensity of antigen expression were analyzed and compared using Diva 9.0.1 and SPSS.

Results: Both Cytek NL-CLC and Canto^{TM} II accurately identified different types of AL patients. The classifications of the cell populations showed no significant difference ($P > 0.05$, $CV < 7.5\%$). In both panels, abnormal cells were identified in all 22 AL patients (abnormal cells range from 7% to 96.5%) and were negative in 8 non-AL patients, resulting in a 100 % coincidence rate. There were no statistical differences in the cell proportions of AL patients ($P > 0.05$). The types, rates and intensities of antigen expression were consistent between two panels ($P > 0.05$).

Conclusions: The Cytek's 23-color and 16-color panels are very effective in the diagnosis of AL. There was no difference in detection accuracy and sensitivity between Cytek NL-CLC and Canto^{TM} II. Moreover, Cytek NL-CLC offers several advantages, including more parameters in a single tube, reduced compensation interference, and beneficial to small-volume samples. These advantages would promote its application in clinical practice.

NOV-21

CYTOMETRIC EVALUATION OF THE ACTIVE DNA DEMETHYLATION PATHWAY: 5-HYDROXYMETHYLURACIL AS A NEW VALUABLE RESEARCH AND DIAGNOSTIC TOOL

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Introduction: There are plethora of studies which support the link between epigenetic processes and carcinogenesis. Cellular DNA is exposed to various endogenous and exogenous factors. Exposure to them results in DNA damage and the formation of modified DNA bases. The 5-hydroxymethyluracil was originally identified as an oxidatively modified DNA base derivative. Recent evidence suggests its formation from thymine in the reaction catalyzed by TET proteins, the family of dioxygenases involved in an active DNA demethylation process. A current „gold standard" in 5-hydroxymethyluracil

determination is liquid chromatography with tandem mass spectrometry Despite many advantages, it has one great limitation–it is not able to measure compounds at a single-cell level. Our main goal was therefore to develop and optimize method for the 5-hydroxymethyluracil content assessment with using flow cytometry - the only one technique allowing such determinations.

Methods: Based on available literature data and own experience, we initially tested three procedures. The best results were obtained after using high temperature and this protocol was the starting point for further standardization. Due to the lack of a flow cytometric recommended anti-5-hydroxymethyluracil antibody, an unconjugated antibody tested for histochemical staining was selected along with a compatible flow cytometric recommended secondary antibody labeled Alexa Fluor 647. Both antibodies were titrated. Samples were analyzed using a BD FACSCantoII flow cytometer. The 5-hydroxymethyluracil content was calculated as the fold change in fluorescence intensity relative to the negative control. To provide further evidence that the observed signal originated from genomic DNA, we also performed a study using the Cytek Amnis® ImageStream®X Mk II imaging flow cytometer.

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

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

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NOV-22

FULLY AUTOMATED POINT-OF-CARE FLOW CYTOMETRY COMBINED WITH INTELLIGENT SOFTWARE ALLOWS FOR ON-SITE DIAGNOSIS OF BACTERIAL AND/OR VIRAL INFECTIONS

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Introduction: The host response to infection and inflammation is based on complex and heterogeneous mechanisms. This complicates the interpretation of the expression of single immune system markers like cytokines. Innate immune effector cells, such as neutrophils and monocytes, integrate these heterogeneous signals and change their phenotype accordingly. These subtle phenotypic changes can be picked up by flow cytometry combined with intelligent software. We tested whether fast point-of-care automated flow cytometry combined with analysis with two intelligent multidimensional algorithms could discriminate between absence or presence of viral and/or bacterial infections in patients at risk of infection at the emergency department.

Methods: a fully automated AQUIOS flow cytometer (Beckman Coulter), located at the emergency department of our university medical center, automatically prepared, stained and analyzed whole blood samples within 25 minutes. For each sample a specific neutrophil and monocyte panel were analyzed comprising 7 parameters (forward/sideward scatter and 5 surface markers). Two

multidimensional algorithms were applied to semi-automatically analyze the flow cytometry data: 1) the FlowSOM algorithm (Cytobank platform, Beckman Coulter) was used to automatically gate neutrophils and monocyte subsets; 2) The new “ASUDES” algorithm (Flowview diagnostics) was used to highlight disease specific signals, by subtracting information normally expressed by healthy cells.

Results: 86 patients suspected of infection were enrolled for analysis. The combination of automated flow and application of the algorithms offered a very good discrimination between bacterial vs. viral and no infection (AUC 0.86) as well as viral vs. bacterial and no infection (AUC 0.93) with intuitive graphical representations of disease specific signals.

Conclusions: Automated point-of-care flow cytometry allows fast, on-site diagnostics and discrimination of bacterial vs viral vs no infections. The next step is the automated integration of these algorithms with the flow data allowing the automated analysis independently of human interference. This would result in a fast and fully automated diagnostic platform that can be performed and analyzed 24/7, even by non-specialists such as clinical support personnel.

OTHER (OTH)

OTH-01

PHARMACODYNAMIC MONITORING OF MEMBRANE-BOUND BCMA BY FLOW CYTOMETRY IN HEALTHY PARTICIPANTS UPON TREATMENT WITH A Γ-SECRETASE INHIBITOR

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Introduction: B cell maturation antigen (BCMA) is a membrane protein expressed on normal plasma cells (PCs) and often overexpressed on malignant PCs in multiple myeloma. Recently, BCMA has become a target for many immunotherapies, which are at various stages of clinical development. Membrane-bound BCMA (mbBCMA) is continuously shed from the cell surface through γ-secretase activity resulting in the soluble BCMA (sBCMA) formation. Shedding BCMA reduces mbBCMA levels and may affect the response to BCMA-targeted therapies. Inhibition of the BCMA shedding through the addition of a γ-secretase inhibitor (GSI), such as nirogacestat, maintains higher levels of mbBCMA. In this study, dynamic changes in mbBCMA upon GSI treatment were assessed in healthy participants using a validated flow cytometry (FC) assay.

Methods: An eight-color antibody panel was designed for the analysis of PC populations and for the assessment of mbBCMA in whole blood (WB) and bone marrow (BM) samples from healthy participants. The assay was validated based on precision, stability, and sensitivity. Importantly, this assay was used to evaluate the PD effects of nirogacestat on mbBCMA levels on PCs in healthy subjects. The time-dependent effects of GSI addition on mbBCMA receptor density were also measured on PCs from WB and BM at baseline and various time points post-dose.

Results: Analysis of rare PC populations were influenced by multiple factors, including short sample stability, the temperature sensitivity of both mbBCMA and PCs, limited data analysis reproducibility due to the complex gating scheme for PCs, and the donor-donor variability in PC number. Regardless, the validated assay was applied in the clinical trial, and analysis of fresh samples was achieved due to the proximity between the clinical site and the laboratory. Treatment with nirogacestat resulted in a rapid increase of mbBCMA on PCs both in WB and BM, with a maximal 8-12-fold increase in mbBCMA occurring between 4 to 8 hours post-dose. The detectable levels of mbBCMA returned to baseline after 24 to 48 h post-single dose of GSI. Of note, the dynamic changes in mbBCMA were similar between WB and BM, but the overall response in BM was lower.

Conclusions: The assessment of mbBCMA levels on PCs using FC was challenging due to several factors, including limited stability, rare population of interest, and the complex gating strategy required for analysis. In spite of these challenges, the assay was validated and, with proper staff training, successfully implemented to assess the primary endpoint in this clinical trial.

OTH-02

UPSCALING OF A CLINICAL FLOW CYTOMETRY LABORATORY

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Introduction: As flow cytometry is one of the go-to methods for fast and in-depth monitoring of immune cell populations at single cell level, clinical flow laboratories are observing an increase in assay complexity and number of samples to process. We discuss here how we scaled up our operations in a qualitative and cost-effective manner.

Methods: Several aspects of the laboratory design were evaluated. A lean floor plan organized from sample receipt to data analysis was implemented. Sample and reagent management was altered to increase efficiency and automation. Antibody cocktail preparation and QC was streamlined and improved. We switched from FACSCanto™ to FACSLyric™ instruments and enhanced our data management systems on a global scale.

Results: We observed improved efficiency, quality, and ease of work in sample preparation due to the implemented changes. Automated sample preparation instruments (FACSDuet™ and Lyse-Wash Assistant™ (BD Biosciences); PS-10™ and Rotolavit™ (Sysmex)) were evaluated. We found these were not yet able to manage the versatility in staining methods or reagents needed for higher parameter flow cytometry assays. Moreover, loss of cells was often a trade-off for automation of washing steps Therefore, we decided not to install these systems yet. Given the variety in reagents needed for higher parameter assays in clinical trials, custom commercial antibody cocktails are not available. Therefore, we validated stability of multicolor antibody cocktail up to 7 days. Depending on antibody combinations, cocktails could be used with a stability of 3 to 7 days. This enhanced efficiency and assay performance, while reducing waste. Another improvement was switching from FACSCanto™ to FACSLyric™ instruments. We observed faster, more efficient, and more controlled measurements with improved standardization within and between laboratories in our global laboratory network. Implementing semi-automated data cover sheets and a global repository for data storage combined with a global middleware, resulted in efficient and fully automated reporting, enabling remote data analysis and review without manual steps in data reporting.

Conclusion: All the above steps greatly enhanced quality, assay performance, productivity, and turn-around-times of our operations. Further ongoing efforts to implement automated reagent and sample preparation systems and automated gating software by vendors, as well as tools to track productivity (KPI) and automated data collection for analytical reports will enhance the efficiency of lab operations to handle large numbers of clinical samples. Additionally, electronic signatures for all steps from bench to reporting will increase traceability and minimize errors.

OTH-03

DIFFERENTIAL PLATELET-MONOCYTE AGGREGATES (PMA) INCIDENCE BETWEEN HEALTHY SUBJECTS AND DIABETES TYPE 1 PATIENTS

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Introduction: Platelet reactivity is higher in patients suffering from different cardiovascular and metabolic conditions[1]. Particular case represents diabetes mellitus type 1 (DM T1). Platelet hyperreactivity is conditioned in these patients due to multiple factors, such as hyperglycemia, insulin resistance, and chronic inflammation, which increase the risk of developing cardiovascular diseases.

Platelet-monocyte aggregates (PMA) have emerged as a promising early biomarker in various cardiovascular diseases as they may contribute to the worsening of cardiovascular conditions by supporting endothelial dysfunction, plaque formation and increasing thrombotic risk. Therefore, the aim of the project was to test the hypothesis, that higher incidence of PMA is associated with risk factors of cardiovascular diseases.

Methods: A total of 50 healthy volunteers and 50 DM T1 patients were enrolled. Blood samples from each subject was spiked with vehicle, rivaroxaban, apixaban, dabigatran, or argatroban at a final concentration of 1 μM, and the samples were stained with fluorescently-conjugated CD41-FITC (for platelets), CD45-PerCP-Cy5.5 (pan-leukocyte marker) and CD14-PE/Cy7 (monocyte marker) to specifically detect PMAs. Samples were immediately analyzed by flow cytometry and results analyzed with FCS Express 6 software. PMA incidence was measured and values were analyzed according to different anthropological and other factors (age, sex etc.).

Results: In relation to gender, no significant differences in PMA incidence were found. The differences were neither observed in healthy persons nor in DM T1 patients although women usually presented a higher number of PMA. Comparing healthy individuals and DM T1 patients, PMA incidence was higher in DM T1 patients reaching almost double than that of healthy subjects. Smokers had a higher incidence of PMA, but this reached significance only in DM T1 patients. In healthy donors, a trend between PMA and increasing age was observed, whereas this effect was not found in DMT1 patients, probably because of higher basal levels of PMA. No clear relationships were detected between body mass index (BMI) and PMA incidence.

Conclusions: DM T1 was associated with higher PMA levels and this might reflect their higher cardiovascular risks. The formed suggestion that women had higher PMA levels was not confirmed in our group of healthy persons and patients. Although a clear relationship between PMA incidence and increasing age was found in healthy volunteers, such an effect was not observed in DM T1 patients.

Reference:
[1]Barale C, Russo I. Int. J. Mol. Sci., 2020, 21, 623

OTH-04

DEVELOPMENT, VALIDATION AND CLINICAL APPLICATION OF FLOW CYTOMETRY BASED RECEPTOR OCCUPANCY ASSAYS

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Introduction: The assessment of target receptor occupancy (RO) by biological compounds provides critical information in determining an optimal dosing regimen during pre-clinical and clinical development. Flow cytometry is a powerful and widely adopted tool for this type of analysis in clinical research. With the multiplex capabilities of flow cytometry, it is possible to determine RO on the specific cell subset of interest. It also enables simultaneous determination of total and free receptors. Several strategies can be taken for the development of an RO assay, depending on the characteristics of the compound, availability of materials, as well as the context of use. Here, we present an overview of various possibilities and challenges when developing and validating an RO assay according to clinical research standards.

Methods: We describe various approaches to RO determination by flow cytometry. A common approach is the use of secondary antibodies for the detection of receptor-bound IgG1 and IgG4

subclass monoclonal antibodies. Also, the use of fluorophore-labelled compound is a well-known approach, and a non-competing antibody binding to the receptor may be added to monitor total receptor levels. Combinations of the above-mentioned methods are possible and will be discussed. We will also present approaches in the preservation of samples for RO determination, as for most flow cytometry assays in (multi-center) clinical studies, sample stability is one of the biggest challenges.

Results: Results of various methods for RO determination will be presented. Advantages of the methods will be discussed, as well as limitations. In addition, best practices for the preparation of RO assays for clinical trials, including extending sample stability, and assay validation, will be discussed.

Conclusions: Developing an RO assay can be challenging, but a well-developed assay provides highly valuable information during drug development in support of clinical trial design and dose selection. Here we discuss multiple approaches to develop RO assays, and how to validate these for use in clinical trials.

OTH-05

THE IMPACT ON HUMAN RESOURCE, FLEXIBILITY AND CONSISTENCY WITH THE BD FACSDUET™ PREMIUM SAMPLE PREPARATION SYSTEM VERSUS MANUAL PROCESSING

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Introduction: Laboratory workflow and throughput may be influenced by method of sample preparation and antibody panel format. Automation may optimize human resource utilization, provide more consistency while retaining flexibility in antibody choices.

Methods: Three different antibody formats were tested using (a)single dispensed reagents, (b)pre-cocktailed reagents, and (c)dry reagent tubes using both traditional manual and automated BD FACSDuet™ Premium Sample Preparation System physically integrated with the BD FACSLyric™ Flow Cytometer. Total process time (TPT), hands-on time (HOT), and error prone tasks (EPT) were assessed. Manual cocktail creation data was also collected for assessment.

Results: For all three antibody format workflows, the automated BD FACSDuet™ Premium System HOT was consistent (5.4%-6.8%) with 0%EPT. In contrast to manual workflow, automated cocktailing had: 80.2% less HOT than (a)single reagent dispensing (54.0%EPT); 66.7% less HOT than (b)pre-cocktailed (26.7%EPT); and 47.6% less HOT than (c)dry reagent utilization (9.1%EPT). Manual cocktail creation had 51.6% more HOT with 100% more EPT than the BD FACSDuet™ Premium System. Manual cocktailing also requires 100% manual entries, whereas the automated system provides a complete searchable audit report for cocktailing with 0% manual documentation.

Conclusions: When compared to manual processing, automation allows significant saving of hands-on-time, and reduction of error prone tasks whilst retaining maintaining flexibility with a complete audit trail. Automation aids in the reduction of potential errors and subsequent repeat testing, which can further streamline workflow in the laboratory. Manually prepared antibody cocktails may have

potential downstream error impact (remediation for mis-constructed cocktails) was not captured in this data set.

This research is scientific in nature. Products NOT for diagnostic use.

OTH-06

WORKFLOW ASSESSMENT CASE STUDY IN BATCHED VERSUS CONSECUTIVE RUNS IN MULTIPLE SITES ON THE BD FACSDUET™ PREMIUM SAMPLE PREPARATION SYSTEM

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Introduction: Increasingly, flow laboratories are performing lab developed tests using automation. Throughput considerations requiring human resourcing may include factors such automating pre-washing of specimens or batch processing.

Methods: Specimen processing was completed at two sites using (a)full automation, inclusive of sample pre-washes, compared to (b)manual prewashing with process completed on automation. All manual pre-wash steps were matched on the automation. Total processing times (TPT), hands-on time (HOT), and number of error prone tasks (EPT) were assessed on the following: (1) single specimen with two tubes; (2) three consecutive worklists with a single specimen of two tubes; and (3) batch of 8 specimens with 16 tubes.

Results: For both sites, across all runs, fully automated processing had lowest TPT differences (0.2-3.7%) providing predictable throughput due to low manual interventions, whereas specimens prewashed prior to completion on automation had more variable TPT differences of 2.3%-23.8%. Full automation HOT was 1-6% compared to preparing pre-washed specimens where HOT increased to 5-13% and more error prone steps were observed. While samples could be pre-washed off-board, increasing possible throughput by 34.6% to 42.2%, potential qualitative impact on data (e.g. scatter, MFI) could be introduced due to lack of standardized processing times, specifically washed-specimen to start-of-stain times.

Conclusions: Automation can be flexible in how it is utilized and can provide more reproducible throughput for laboratories. Maintaining manual components of sample processing may provide opportunities for increasing throughput but may also increase throughput variability and have a higher number of tasks and error prone tasks.

This research is scientific in nature. Products NOT for diagnostic use.

OTH-07

FLEXIBILITY IN SAMPLE PREPARATION FOR MIXED LABORATORIES RUNNING ON A SINGLE SAMPLE PREPARATION DEVICE WITH ALTERNATIVES IN POST-STAIN WASHING

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Introduction: Advanced sample preparation automation in flow cytometry laboratories utilize onboard centrifugation to process samples, similar to bench processing. For methods that require processing off-board in comes cases, for example incubation at 37 °C or stimulation steps, samples can be completed off-board. In addition, doing a post-stain off-board wash might be advantageous for workflow purposes.

Methods: Two sites participated in this study to evaluate various off-board post-stain wash methods. Three conditions were assessed: (1) full processing on the BD FACSDuet™ Premium Sample Preparation System through to automated loading onto the flow cytometer; and, loading to flow cytometer through the BD FACSDuet™ Premium System after (2) off-board removal of samples for washing on BD FACS™ Lyse Wash Assistant; and (3) Off-board removal of samples for post-stain washing using traditional centrifugation.

Results: Comparisons at both sites demonstrated no difference between preparation using (1) full processing with 0.0% Hands on time (HOT) and Total Process Time (TPT). (2) Off-board post stain wash using LWA preparation showed 0.6% faster TPT, with average 1.5% HOT. (3) Off-board post stain wash using Traditional centrifugation was 13.7% longer TPT with average HOT of 29.5%.

Conclusions: Predictable workflows were achieved with the help of automation in the full processing on the BD FACSDuet™ Premium System or with off-boarding for a post-stain wash using BD FACS™ Lyse Wash Assistant. Traditional centrifugation used for post-stain off-board washing was more variable between the sites, used more hands-on time, and could introduce errors in processing**.**

OTH-08

AUTOMATED SAMPLE PREPARATION FOR LABORATORIES STREAMLINING LYSE-NO-WASH (TBNK) AND LYSE-WASH (LDT'S) METHODS ON A SINGLE SYSTEM USING CARRIERS OR PLATES

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Introduction: In mixed laboratories, order of various workflows using automated sample preparation systems with flow cytometry integration may impact total processing time (TPT). Optimization can increase efficiency and throughput, with added flexibility in using tube carriers and plates.

Methods: Two sites with a BD FACSDuet™ Premium Sample Preparation System integrated with a BD FACSLyric™ Flow Cytometer explored different workflows. PartA: three different conditions using 2 specimens: (1) IVD-TBNK (lyse-no-wash[LNW]) assay; (2) 96-well plate TBNK-like (lyse-no-wash[LNW]) assay; and (3) LDT (lyse-wash[LW]) assay, examined in varying order. PartB: two different conditions running 96 specimens: (1) three consecutive carriers with 32 specimens each; and (2) a single 96-well plate. Time was measured from specimen loading onto the BD FACSDuet™ Premium System to the BD FACSLyric™ Flow Cytometer acquisition completion.

Results: PartA: Most efficient workflow with (3) LDT[LW] in the primary position (followed by (1) IVD-TBNK carrier then (2) TBNK-like plate) was 32.6% faster than LDT[LW] loaded last. PartB: Three consecutive carriers (96 specimen) throughput at Site 1 had 25.1% lower TPT compared to Site 2. Sample preparation was consistent (hands-on-time [HOT] at 5.9% and 9.5%) with TPT difference observed attributed to sample acquisition. Inter-site plate TPT was 1.2% with HOT of 2.0% and 2.9%.

Conclusions: The BD FACSDuet™ Premium System allows flexibility of processing and loading configurations. Automation can be optimized and customized for each mixed laboratory to achieve predictable TPT. Physical integration of a sample preparation system is advantageous as it increases throughput, removes transfer time and standardizes preparation.

This research is scientific in nature. Products NOT for diagnostic use.