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ABSTRACTS

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PLENARY PRESENTATIONS

PLE01-01

Leukemic Stem Cell as a Biomarker: Flow-Cytometric Insights and Clinical Application - From Bench to Bedside

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Leukemic stem cells (LSCs) are now recognized as a key players in the leukemogenesis, resistance to chemotherapy, and relapse of acute myeloid leukemia (AML). Defined by their self-renewal capacity, quiescence, stemness signature and functional properties as engraftment in mice PDX model, LSCs offer a powerful biological rationale for risk stratification and therapeutic targeting. Our ability to use advanced flow-cytometric technologies to detect and characterize these rare populations with increasing precision could help us to have a better understanding of LSC biology and their role on prognosis.

This keynote lecture will highlight the evolution of LSC detection using flow cytometry from early phenotypic identification in $CD34^{+}CD38^{-}$ space adding the incorporation of specific markers such as CD45RA, CLL-1, CD97, TIM-3, CD123, besides of CD90 (Thy-1), GPR56, CD81, CD36, CD200..., to better distinguish between nHSC (Hematopoietic Stem Cells) and LSCs and how using the high-dimensional multiparametric panels. The origin of AML LSC is still controversy, however all fundamental experience to elucidate the function of stem cells are based on CD34+CD38- selected populations, subsequent transplanted to immunodeficient mice, suggesting the importance of CD34/CD38 “space” analysis of all AML leukaemia cells at diagnosis, MRD time points and relapse. We will explore how these advances have improved our capacity to quantify LSC burden in CD34+CD38- during the time, but also to detect cells with stemness signature into CD34- leukemia fraction.

Beyond technical aspects, this talk will focus on the clinical translational application of LSC: how to integrate LSCs into the clinical trials and frontline diagnostics. Emerging data support LSC burden as an independent prognostic factor and a potential surrogate endpoint in early-phase studies. Furthermore, real-world applications are beginning to help guiding treatment intensification, transplant decisions, and post-remission monitoring.

While challenges remain, including standardization across flow cytometry platforms, ongoing international efforts are working toward harmonization according to ELN David LSCflow subgroup recommendations. The incorporation of LSC flow cytometry CD34+CD38- into routine practice opens a new paradigm in leukemia treatment, moving toward truly personalized, biology-driven therapy.

This lecture will present not only the current landscape but also future directions. In our view we are at the crossroads thanks to the accumulation of cognitive and clinical knowledge about the role of LSC in AML. It is time to move forward and integrate LSC quantification into future AML clinical trials, where flow cytometry and stem cell biology converge to redefine outcomes in leukemia moving precision medicine from “bench to bedside”.

PLE02-01

Role of flow cytometry in the multi-disciplinary diagnostic approach of MDS according to WHO classification 2022

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Myelodysplastic neoplasms (MDS) are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, cytopenias, and a risk of progression to acute myeloid leukemia. The 2022 World Health Organization (WHO) classification of MDS emphasizes a multi-disciplinary diagnostic approach, integrating clinical, morphological, cytogenetic, and molecular data to ensure accurate diagnosis and risk stratification. While not formally incorporated into the diagnostic criteria, flow cytometry has emerged as a pivotal tool in this framework, offering quantitative and qualitative insights into bone marrow and peripheral blood cell populations. This presentation explores the critical role of flow cytometry in the diagnosis of MDS by characterizing the myeloid stem cell compartment as well as maturing myeloid and monocytic cells.

Beyond diagnosis, flow cytometry plays a crucial role in disease monitoring, facilitating the detection of measurable residual disease (MRD) after treatment and enabling the early detection of relapse following stem cell transplantation.

This presentation will highlight standardized flow cytometry protocols, including the European LeukemiaNet recommendations, and discuss challenges such as distinguishing low-risk MDS from reactive conditions. Case studies will illustrate how flow cytometry integrates with cytogenetics, molecular testing, and bone marrow morphology to fulfill WHO 2022 diagnostic criteria.

PLE02-02

Flow Cytometry in the Diagnostic Work-Up of Myelodysplastic Neoplasms: the ELN perspectives

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Diagnosis of cytopenic patients suspected of myelodysplastic neoplasms (MDS) can be challenging. In normal haematopoiesis, antigen expression is tightly regulated; changes in expression patterns may therefore indicate dysplasia, the hallmark of MDS. Flow cytometry (FC) can identify aberrancies in antigen expression and maturation patterns not recognized by cytology. This technique is regarded instrumental in the diagnostic work-up of MDS [Van de Loosdrecht et al. Cytometry B Clin Cytom 2023]. FC should be performed according to recommendations defined by the ELNet-*i*MDS-flow working group -recently updated by Van der Velden et al. and Porwit et al.– that include guidelines on sample preparation, instrument set-up and quality assessments, antibodies, and gating strategies [Cytometry B Clin Cytometry 2023]. Defined abnormalities can be counted in FC scoring systems to provide a means to determine the extent of dysregulation of the maturation patterns, i.e. dysplasia according to FC. Ideally, scores should enable a categorization of FC results from bone marrow assessments in cytopenic patients as "normal", "low probability of", or “high probability of” MDS [Cremers et al. Haematologica 2017; Oelschlaegel et al. Cytometry B Clin Cytom 2023]. A multicentre study performed by Kern et al. identified a core set of 17 markers (CD45, CD34, CD117, HLA-DR, CD10, CD11b, CD13, CD16, CD15, CD14, CD33, CD64, CD123, CD7, CD19, CD56, and CD71) that enables identification of aberrancies in bone marrow cells independently related to the diagnosis of MDS by cytomorphology. Veenstra et al. recently confirmed the diagnostic validity of this marker set. They observed an overall diagnostic accuracy of 87% as compared to an integrated diagnostic approach at a cut-off of ≥3 aberrant markers. [HemaSphere 2025].

The sensitivity of the scoring system was 87% for lower risk and >90% for higher risk MDS cases (specificity 76%). More than 3% of myeloid progenitor cells was only seen in MDS, this concerned low as well as high risk cases. Hence, increased myeloid progenitor cells by FC may indicate MDS not only in cases with overt disease but also in cases with minimal dysplasia [HemaSphere 2025]. Notably, FC as a single technique is not sufficient for the diagnosis of MDS: FC results should always be evaluated as part of an integrated diagnostic work-up. In (near) future, application of computational analysis and artificial intelligence to FC data, or combinations of FC data and other biological parameters, may support diagnosis-making, prognostication, and/or treatment decisions.

PLE02-03

Tomorrow's Diagnostics for Myelodysplastic Neoplasms: The Potential of Flow Cytometric Detection of Aberrant Immunophenotypic Hematopoietic Stem Cells

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Introduction: Myelodysplastic neoplasms (MDS) are clonal hematologic malignancies marked by dysplasia and ineffective hematopoiesis. Despite their classification under one umbrella, MDS are biologically and clinically diverse, with outcomes ranging from near-normal survival to death from cytopenias or progression to acute myeloid leukemia (AML). Current prognostic models, including the Revised International Prognostic Scoring System (IPSS-R) and Molecular IPSS (IPSS-M), guide risk assessment but classify patients into numerous categories that nonetheless share the same composite endpoint of all-cause mortality and leukemic transformation. Our aim was to refine MDS risk stratification using flow cytometry markers, with the ultimate goal of linking each risk category to a single clinical endpoint (i.e., leukemic transformation, progressive marrow failure, or MDS-unrelated mortality).

Methods: We analyzed $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) from 68 MDS patients, alongside healthy donors and pathological controls, using the eight-color, thirteen-marker "leukemic stem cell (LSC) tube" validated for AML. Manual gating identified immunophenotypically aberrant hematopoietic stem cells (IA-HSCs) expressing membrane abnormalities such as CD7, CD11b, CD22, CD33, CD44, CD45RA, CD56, CD123, CD366, or CD371. Additionally, unsupervised clustering was applied to potentially identify previously unrecognized HSPC aberrancies.

Results: IA-HSCs were present in one-third of MDS patients but absent in controls. Their presence independently predicted leukemic progression (HR = 25, 95% CI: 2.9–218), irrespective of the IPSS-R. At 2-year follow-up, the presence of IA-HSCs predicted leukemic progression with 83% sensitivity and 71% specificity. Elevated human blast counts after xenotransplantation of MDS samples containing IA-HSCs, in contrast to those without, supported the neoplastic nature of IA-HSCs. Using AML patients and healthy controls as references, clustering analysis identified three MDS subtypes: (A) indolent, with preserved lymphoid and megakaryocytic-erythroid progenitors; (B) leukemic, with expanded LSCs and granulocyte-macrophage progenitors; and (C) unfavorable non-leukemic, characterized by increased HSCs and common myeloid progenitors (CMPs) with downregulated CD44, indicative of impaired hematopoietic differentiation.

Conclusion: The presence of IA-HSC is a promising biomarker for predicting leukemic progression in MDS. Additionally, CD44 downregulation on HSCs and CMPs may help identify an unfavorable non-leukemic MDS subtype. These findings demonstrate that flow cytometric analysis of HSPCs may have the potential to improve MDS risk stratification by linking distinct immunophenotypic profiles to specific clinical outcomes.

PLE03-01

The Studies of the CytHem-LOC French Group: Progress in the Diagnosis and Follow-Up of Oculocerebral Lymphoma

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Primary Central Nervous System Lymphoma (PCNSL) is a rare and aggressive subtype of non-Hodgkin lymphoma, most commonly represented by diffuse large B-cell lymphoma (DLBCL). The diagnosis and monitoring of PCNSL remains challenging due to its unique localisation within the central nervous system (CNS), often requiring invasive procedures such as stereotactic brain biopsy or vitrectomy.

Since 2018, the CytHem-LOC French Group has been actively engaged in improving diagnostic strategies and harmonising laboratory practices in flow cytometry (FCM) for oculocerebral lymphomas. This presentation highlights the collaborative work of the CytHem-LOC network, a group of the French CytHem association which now includes over 200 members across eight French-speaking countries.

The CytHem-LOC group initially focused on developing state-of-the-art recommendations for FCM analysis of cerebrospinal fluid (CSF). By comparing real-world practices to the ESCCA/ISCCA guidelines, the group identified discrepancies and areas requiring harmonisation in clinical workflows. In a subsequent study, CytHem-LOC compared cytology, FCM, interleukin assays, and the detection of the MYD88 L265P mutation in both the cellular fraction and circulating cell-free DNA (cfDNA) of CSF. These results support a comprehensive and integrative diagnostic approach, underscoring the importance of measuring interleukins IL-10 and IL-6 using FCM via the Cytometric Bead Array (CBA) technique, a method that has been shown to exhibit excellent sensitivity. Based on these findings, CytHem-LOC implemented a nationwide coordination programme in France to promote standardized CSF IL-10 and IL-6 analysis using the CBA method. However, CSF analysis does not always eliminate the need for brain biopsy. Consequently, the group is also working to standardize and provide recommendations for the analysis of brain biopsy rinse fluid by FCM. This fluid consists of a saline solution that is used to eject the biopsy from the trocar. This innovative approach, which has now been adopted in specialised centres, enables the diagnosis of B-cell lymphoma within 24 hours. When combined with histopathology, it supports a rapid, multidisciplinary diagnostic process.

The CytHem-LOC group remains committed to advancing these initiatives, continuing to refine diagnostic protocols and strengthen European collaborations to improve the management of PCNSL and related lymphomas.

PLE03-02

Flow Cytometry in CNS disease of Hematologic Malignancies: comparing ESCCA/ISCCA recommendations and survey results

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Central nervous system (CNS) involvement in hematologic malignancies (HM) remains a significant cause of mortality, despite improvements in systemic disease. Early detection of CNS infiltration can improve outcomes, with multiparameter flow cytometry (MFC) emerging as more sensitive than conventional cytology (CC) in identifying CNS involvement across various HM.

Challenges in cerebrospinal fluid (CSF) analysis include sample hypocellularity and poor cell viability. Previously, aposition paper by the European Society for Clinical Cell Analysis - ESCCA - and Italian Society for Clinical Cell Analysis - ISCCA - emphasized standardizing sample handling, processing, and interpretation. To assess global practices, a survey was conducted involving 113 cytometrists from multiple countries. Respondents included clinicians (30%), biologists (25%), biomedical scientists/technicians (13%), MD/PhDs (15%), and others (17%). Most had over 10 years of MFC experience and processed over 50 CSF samples annually. This survey confirmed the role of MFC in the diagnosis of CNS involvement. While CC remains the diagnostic gold standard, 43% of respondents are confident in diagnosing "occult CNS disease" using MFC alone. Most centers integrate MFC with CC for robust diagnosis. Sample quality varied, with 40% reporting all samples as adequate, and 46% stating only more than half were suitable. Most received <2 mL of CSF, though 30 mL is considered safe to collect. Regarding sample timing, only 25% processed CSF within 60 minutes. Though TransFix is recommended for delayed processing, just 60% of labs use it, with 17% noting changes in marker expression. Absolute cell counts were mostly manual (49%), with only 23% using MFC and beads.Red blood cell contamination was assessed via microscopy (40%) or MFC (6%). Parallel processing of PB and CSF was always done in 13%, and selectively in 48%. Thresholds for MFC positivity were inconsistent: 36% used ≥10 events, 44% ≥20, and 12% ≥30.While 34% used MFC only for HM patients, 48% applied it to unclear hypercellular CSF cases, and 13% to all CSF samples.

These findings underline the need for harmonized protocols and continued collaboration among laboratories worldwide.

PLE04-01

Old data, new tricks: comprehensive computational analysis of 10 years of multi-center EuroFlow acute myeloid leukemia diagnosis data

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Introduction: Acute myeloid leukemia (AML) is characterized by high inter- and intra-patient heterogeneity, both at the genotypic and immunophenotypic level, posing challenges in diagnosis, prognosis and treatment. The World Health Organization (WHO) classification divides AML into AML with defining genetic aberrancies and AML defined by differentiation, incorporating both molecular and genetic data alongside immunophenotypic data. AML genetic factors are associated with outcome, as described in the European LeukemiaNet (ELN) risk stratification, and some genetic aberrancies warrant specific types of treatment. The EuroFlow consortium has developed an AML/MDS flow cytometry panel, consisting of seven 8-color tubes. Here, we performed a comprehensive computational analysis on an extensive dataset of 5640 EuroFlow AML diagnosis files from 793 patients collected over ten years at eight centers, following EuroFlow standardized procedures.

Methods: Files from one center with a large and diverse patient population were used to build a FlowSOM model per tube of the panel. Metacluster (i.e. cell population) percentages and median fluorescence intensities (MFIs) were extracted as features for downstream analysis. A random forest machine learning model was trained to predict AML genetic aberrancies from immunophenotypic diagnosis data.

Results: Initial checks showed that EuroFlow standardization mitigated center-specific batch effects, eliminating the need for batch effect correction methods. Unsupervised hierarchical clustering on cell population percentages revealed roughly 12 groups of patients. These groups were not directly related to WHO classes, although some classes were enriched in one or more groups. Further sub-clustering of *NPM1*-mutated patients, the largest group in our dataset, uncovered six subgroups within this WHO class. Computational analysis allowed for comprehensive characterization of cell populations and marker expression levels unique for WHO classes, as well as correlation between expression of individual markers. Additionally, we characterized maturation stages based on normal bone marrow samples and investigated at which stages AML development was arrested. Some genetic aberrancies (e.g. inv(16) and t(8;21)) halted maturation at early CD34+ stages, whereas AML blasts in patients with *NPM1* mutations and t(15;17) translocation showed neutrophil-lineage differentiation. The machine learning model achieved high predictive performance for certain WHO classes, particularly those with unique immunophenotypes and lower interpatient heterogeneity (e.g. t(8;21)).

Conclusions: We present a comprehensive computational analysis describing differences in cell population frequencies and individual marker expression across WHO (sub)groups of AML patients. Furthermore, we demonstrate that employing EuroFlow standardized panels and protocols enables the analysis of multicenter data collected over an extended period of time.

PLE04-02

Multicentric study: flow cytometry of brain biopsy rinse fluid in four centers to diagnose lymphoma of the central nervous system

Agathe Debliquis [1], Bertrand Mathon [2], Jimmy Voirin [3], Fabien Rech [4], Walid Farah [5], Caroline Houiller [2], Luc Taillandier [4], Guillaume Gauchotte [4], Alina Nicolae [3], Camille Guibert [5], Sylvain Choquet [2], Bernard Drénou [1], Cedric Rossi [5], Julien Guy [5], Véronique Latger-Cannard [4], Jean-Francois Lesesve [4], Carole Rueff [1], Guido Ahle [6], Magali Le Garff-Tavernier [2]

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Introduction: Central nervous system lymphomas are rare, aggressive tumors requiring prompt diagnosis for effective treatment. The standard diagnostic procedure is stereotactic biopsy, but histopathology results often take over two days. In 2016, a faster method using flow cytometry (FCM) to analyze brain biopsy rinse fluid (BBRF) was published. However, this approach requires multicenter validation and assessment of feasibility.

Methods: A total of 179 patients with suspected brain tumors underwent brain biopsy over a 6-year period in four centers (age range 26–89 years; median 68 years; 100 males, 79 females). Neurosurgeons collected both biopsy tissue and BBRF, consisting of saline solution used to eject the biopsy from the trocar. Biopsies were analyzed by histology, while BBRF was assessed using FCM in each center. Histology was used as the reference. The FCM assay on BBRF consisted of lymphocyte screening, including analysis of morphologic parameters (FSC and SSC), kappa/lambda ratio, and sometimes CD10 and CD5 expression on B lymphocytes (CD19+, CD45+). A threshold of 20 lymphoma cell events was required for diagnosis. In suspected systemic lymphoma, peripheral blood was analyzed to rule out blood contamination.

Results: Histology reported 108 lymphomas: 100 diffuse large B-cell lymphomas (DLBCL), 1 Burkitt lymphoma, 1 lymphoblastic lymphoma, 1 polymorphic B lymphoma, 2 neurolymphomatoses, 1 follicular lymphoma, 1 mantle cell lymphoma, and 1 adult T-cell leukemia-lymphoma. Among DLBCL, the Hans algorithm classified 69 non-GC, 20 GC, and 11 unclassified. Nine DLBCL were EBV-induced and 8 occurred post-transplant. Seventy-one patients had non-lymphoma diagnoses, mostly glial tumors. Compared to histopathology, FCM achieved 85% sensitivity (92/108), 100% specificity (71/71), a positive predictive value of 100% (92/92), and a negative predictive value of 81.6% (71/87). B lymphomas showed monotypic kappa light chain in 53% (48/91), monotypic lambda in 27% (25/91), and were inconclusive in 20% (18/91). CD10 expression was found in 33% (30/90) and CD5 in 11% (6/56). The proportion of lymphoma cells detected ranged from 0.8 to 97% (32–854,170 events). Sixteen false negatives included two neurolymphomatoses, four post-transplant EBV-positive lymphomas, four cases under corticosteroid treatment, and cases affected by delays between bedside and benchtop, wich varied according to the organisation of each center.

Conclusion: This multicentric study confirms the feasibility and clinical value of BBRF analysis. Now implemented in specialized centers, this approach enables B-cell lymphoma diagnosis within 24 hours and, combined with histopathology, allows for a multidisciplinary diagnosis. Additionally, BBRF can be frozen, preserving cells for research in this rare disease.

PLE04-03

Full integration of flow cytometric data into a laboratory information system from an automated machine-learning pipeline

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Introduction: Flow cytometry is an essential methodology in the diagnosis and prognostication of leukemias and lymphomas. While flow cytometry data quality has improved with increasing performance of instrumentation and the availability of novel fluorophores, the analysis of this data is complex, requiring significant training and time. Here, we present detailed experience with the clinical validation and use of a machine learning based flow cytometry analysis paired with a hematopathologist trained decision support system. This automated analysis program was further developed for end-to-end automated analysis from cytometer to laboratory informatic system (LIS) resulting.

Methods: Custom machine learning models were developed for the analysis of clinical flow cytometry data, acquired on a Cytek Northern Lights system (31 markers, 29 fluorophores). These machines learning models were incorporated into a hematopathologist-trained clinical decision support system. This program was modified to run on a RedHat Linux AWS server with continuous ingestion of FCS file output from the Cytek Northern Lights cytometers. Automated results and interpretations are automatically parsed and ingested by the LIS for resulting by the responsible hematopathologist.

Results: Machine learning based cell identification resulted in >99% accuracy in the identification of cell types at clinically relevant annotation levels. The combination of machine learning based cell typing and the clinical decision support system had a 100% sensitivity and 93% specificity for the detection of monotypic B-cell populations at a 0.1% threshold on traditional flow cytometers, which was further improved using spectral flow cytometry. The full automation of clinical flow cytometry data saved an estimated 40 hours per week in technician and pathologist time, with a volume of ~400-500 cases per month.

Conclusions: We present a detailed pipeline for the integration of machine learning based cell typing and a clinical decision support system for flow cytometry data analysis. This method has been rigorously validation and implemented at our institution on both conventional and spectral platforms. The integration with an LIS further improves efficiency and mitigates errors while significantly improving turnaround time.

PLE04-04

The SALS Tube: A Novel Spectral Cytometry Approach for Rapid and Accurate Acute Leukemia Classification

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Introduction: Flow cytometry is essential for immunophenotypic diagnosis of acute leukemias. Conventional approaches require multi-tube panels, large sample volumes, and complex workflows. To address these limitations, we developed a 15-color spectral acute leukemia screening (SALS) tube for use on the Cytek Northern Lights™-CLC flow cytometer, enabling streamlined, single-tube assessment.

Methods: An analytical method validation was conducted using 62 clinical specimens comprising AML (n=26), B-ALL (n=15), T-ALL (n=7), acute undifferentiated leukemia (n=1), MPAL (n=1), mature B cell neoplasms (n=5), and non-malignant samples (n=7). The SALS tube includes 11 surface and 4 intracellular antibodies, targeting lineage-defining markers (e.g., CD34, CD19, cytoplasmic-CD79a, TdT). Fluorochromes include FITC, PE, BV421, and 12 different Cytek cFluor® dyes, spanning violet, blue, and red detectors on the Cytek Northern Lights™-CLC system. Using SpectroFlo® CLC software, autofluorescence extraction was performed with spectral unmixing to enhance resolution. For validation, each sample was assessed using our predicate 4-tube, 8-color conventional cytometry assay acquired on conventional clinical flow cytometers. Method comparison included qualitative and quantitative concordance analysis of diagnostic classifications and marker expression profiles. Assay precision was evaluated by replicate testing on two spectral instruments. Assay stability was evaluated using frozen antibody cocktails (stored at -20°C) tested over 22 days, with samples tested at multiple time points. Data was analyzed using FlowJo™ software (V10.10).

Results: The SALS tube demonstrated 100% concordance with the reference method in diagnostic classification across all 62 samples, supporting high analytical sensitivity and specificity for acute leukemia detection and subclassification. Verification of marker expression profiles revealed improved discrimination of several markers (such as CD34, CD117, and CD19) using the spectral platform, enhancing phenotypic clarity. Inter-instrument reproducibility testing showed consistent results across two Cytek Northern Lights™-CLC flow cytometers, with minimal variability in fluorescence intensity and gated population frequencies (CV <10%), confirming high analytical precision. Reagent stability testing demonstrated consistency of staining profiles and diagnostic performance for up to 22 days post-antibody cocktail preparation. Collectively, these data validate the SALS tube as a robust and reliable method for single-tube immunophenotyping of acute leukemias.

Conclusions: The SALS tube simplifies acute leukemia screening by consolidating multi-tube workflows into a single-tube assay, without compromising accuracy. It offers high performance, improved marker resolution, reduced sample requirements, and enhanced workflow efficiency. Currently in final validation as a lab developed test, SALS is positioned for implementation as a frontline diagnostic tool.

PLE06-01

Abstract has not been received

PLE06-02

Immunophenotypic and metabolic signatures of SARS-CoV-2 infection revealed by cytometry approaches

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Since the onset of the COVID-19 pandemic, flow cytometry has proven essential for dissecting the complex immune alterations induced by SARS-CoV-2. Through extensive cytometry studies, significant insights have been gained into both innate and adaptive immune responses during infection and recovery.

Patients with COVID-19 exhibit profound alterations in T-cell populations, including the expansion of senescent and exhausted $CD4^+$ and $CD8^+$ T cells, a shift toward pro-inflammatory Th17 phenotypes, and increased cytokine production. These findings support the role of T-cell dysfunction in disease progression and point to potential therapeutic targets, such as cytokine modulation.

B-cell compartments are also markedly affected, with reductions in naïve and memory B cells and a concomitant rise in plasmablasts, often associated with elevated inflammatory markers. This dysregulation highlights key mechanisms underlying humoral immune impairment and the development of severe pulmonary manifestations.

Furthermore, cytometric analysis of monocyte metabolism has revealed mitochondrial dysfunction and reduced oxidative phosphorylation in severe cases, suggesting that immune cell bioenergetics are closely linked to clinical outcomes.

More recent studies extended these analyses to vaccine responses, especially in immunocompromised patients. High-dimensional cytometry was used to track SARS-CoV-2-specific T and B cells several months after mRNA vaccination, revealing long-term antigen-specific immunity. Notably, patients under disease-modifying therapies displayed altered metabolic and phenotypic immune profiles, emphasizing the influence of immunomodulation on vaccine efficacy.

Altogether, these findings demonstrate the critical role of clinical cytometry in identifying immune signatures of COVID-19, supporting prognostic biomarker discovery, optimizing patient stratification, and guiding immunotherapeutic strategies in both acute infection and post-vaccination settings.

PLE07-01

High-dimensional single-cell analysis of human natural killer heterogeneity

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Natural Killer (NK) cells are critical effectors of immune surveillance. Single cell technologies have revolutionized our understanding of their heterogeneity. Yet, a harmonized classification based on these new insights is still pending and their diversity and function in health and disease remain incompletely understood. Here, we delineate a simple and robust classification of human NK cells into three major populations (NK1, NK2, NK3) and six sub-populations in healthy blood.

Transcriptional profiling of these subsets allows the prediction of distinct features in transcription factor usage, cytokine responsiveness, cytotoxic potential, metabolic state, and putative developmental origin, suggesting an ontological divergence between NK1 and NK2 cells that parallels findings in murine models. These populations are conserved across healthy tissues and persist within tumor microenvironments, where their relative abundances vary significantly across 22 different cancer types.

Integration of single-cell RNA and chromatin accessibility data (scRNA-seq and scATAC-seq) further uncovers key gene regulatory networks (GRNs) orchestrating NK cell states within non-small cell lung cancer (NSCLC). We identify a hypofunctional NK subset sharing features with tumor-reactive $CD8^+$ T cells, characterized by expression of CD39, inflammatory regulon activity, and tissue residency and dysfunction markers. Functionally, $CD39^+$ NK cells demonstrate heightened cytotoxicity and proliferation upon restimulation and preferentially expand following anti-NKG2A immunotherapy, positioning them as promising targets for therapeutic intervention.

Building on these insights, we evaluate NK cell-based therapeutic strategies leveraging their unique stress-sensing capabilities, favorable safety profile, synergy with T cells, and potent anti-metastatic activity. Three key therapeutic modalities emerge: immune checkpoint blockade, NK cell engagers, and adoptive NK cell therapies. Single-cell profiling of NK cells treated with ANKET4, a tetra-specific NK cell engager (CD20-NKCE-IL2v), reveals a biphasic NK response marked by an initial interferon-driven activation followed by sustained proliferation and the induction of cytokine-induced memory-like (CIML) NK cells. ANKET4 thus reprograms NK cell function and phenotype, providing a potent tool for enhancing NK cell-mediated immunity against cancer.

PLE08-01

Mechanical phenotyping of immune cells in whole blood using deformability cytometry

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Introduction: Deformability cytometry is a label-free, high-throughput microfluidic technique for characterizing single-cell physical phenotypes directly from whole blood. By capturing brightfield images of cells flowing through a constriction at high speed, it quantifies intrinsic biophysical properties including cell size and deformability at rates of 1 000 cells/s. We explore the potential of deformability cytometry for characterizing immune cell activation through changes in mechanical properties, a dimension not captured by conventional flow cytometry. We hypothesize that changes in immune cell mechanics can serve as sensitive biomarkers for hematological disorders and infections.

Methods: We applied deformability cytometry to analyze whole blood samples from adult donors. Each measurement required only 10 microliters of whole blood and took 10 minutes. Brightfield images were processed to extract physical features including deformation and calculated Young's modulus of lymphocytes, monocytes, neutrophils, and eosinophils. We studied 17 hospitalized COVID-19 patients, 24 healthy controls, and 14 recovered individuals. In a separate study, we analyzed samples from a cohort of over 6000 pediatric patients, focusing on detecting changes during infectious conditions.

Results: Significant disease-related alterations in cell mechanics were observed. In COVID-19 patients, we detected significant changes in neutrophil deformability and size, decreased lymphocyte stiffness, and enlarged monocytes. In the large pediatric cohort, we identified consistent correlations between immune cell mechanical properties and standard inflammatory biomarkers such as C-reactive protein (CRP) and procalcitonin (PCT).

Conclusions: Deformability cytometry of whole blood provides a fast, marker-free, and sensitive approach to detect pathological alterations in immune cell mechanics. It is a powerful complement to molecular and immunophenotypic assays. We demonstrate that immune activation and disease states are accompanied by robust, cell-type-specific mechanical changes. The method's speed, simplicity, and label-free nature make it particularly attractive as a complementary diagnostic tool in haematology and immunology, with potential to enhance early detection and immune monitoring in clinical practice.

PLE08-02

Lymphocyte Subpopulations as Potential Immunological Biomarkers in ANCA-Associated Glomerulonephritis

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Introduction: The activation of adaptive immunity plays a decisive role in the pathogenesis of systemic diseases. We evaluated specific B and T lymphocyte subpopulations in ANCA-associated vasculitis/glomerulonephritis (AAV/GN), which are involved in the pathogenesis and progression of the disease and can serve as biomarkers.

Methods: Peripheral blood levels of B cells: CD19, IgD(+)CD127(-), IgD(+)CD127(+), IgD(-)CD127(+), IgD(-)CD127(-) and T cells: CD4, T-regulatory (Tregs), T-follicular (Tfol), Tfh, Tfh1, Tfh2, Tfh17, and T-follicular regulatory (Tfr) were assessed via flow cytometry in patients at AAV/GN diagnosis (Group A), in remission (Group B), and in a control group (CG), and were correlated with clinical and laboratory data. **Results:** Patients with AAV/GN (N=19, M/F=5/14, Age=68(15)) showed increased levels of Tfol (21(12.7)% vs. 8(8.3), p=0.012), Tfh1 (20.1(17.2) vs. 9(14.7), p=0.039), Tfh2 (31.4(36.9) vs. 14.6(20.9), p=0.008), and Tfr (14(12.6)% vs. 4.45(5.1)%, p=0.027) compared to the CG. Significant differences were observed among Groups A, B, and CG in the levels of CD4: 341(132), 520(359), 699(405), p=0.006; Tfh1: 20(16), 27(19), 3(11), p=0.004; and Tfh2: 30.7(36.9), 43.4(37.1), 3(14.9), p=0.001, respectively. Group A patients with alveolar hemorrhage had increased levels of T-fol: 22(3.8) vs. 12(15), p=0.02; Tfh1: 67(51) vs. 41(36), p=0.02; Tfh2: 57(34) vs. 28(28), p=0.02; and Tfr: 16(10) vs. 1.8(15), p=0.06.In patients who experienced relapse, there was also a statistically significant increase in the levels of Tfol%: 23(3.8) vs. 15.5(12.3), p=0.016; Tfr%: 17.5(10) vs. 4.9(15), p=0.016; CD19: 3.9(76) vs. 82.2(86), p=0.003; IgD+CD27-: 0.6(53) vs. 56.6(112), p=0.006; IgD+CD27+: 0.2(5.3) vs. 3.4(10), p=0.03; IgD-CD27+: 2.2(5.7) vs. 6.3(13), p=0.006; IgD-CD27-: 0.6(13) vs. 13(6), p=0.016.

Conclusions: Markers of increased disease activity in AAV/GN appear to include the peripheral Tfol, Tfh1, Tfh2, and Tfr populations. A low concentration of B lymphocytes predisposes to relapse. Specific T-lymphocyte subpopulations can be easily evaluated in peripheral blood of patients with AAV/GN. Tfh and Tfr subpopulations seem to be potentially useful biomarkers of disease activity, warranting further study into their role in pathogenesis, with a view to future therapeutic targeting. These subpopulations may also be associated with clinical factors such as disease severity, occurrence of extra-renal manifestations, and risk of relapse.

PLE08-03

Increased binding of tandem conjugates in plasma of patients with Waldenström macroglobulinemia or autoimmune diseases; an artifact with clinical significance?

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Introduction: Non-specific binding of several antibodies labeled with certain phycoerythrin (PE) tandem conjugates, was observed in rare samples, appearing as interference in dot plots. This artifact was considerably reduced when staining pre-washed samples. Several PE-Cy7-conjugates were involved. A simple flow cytometric method was developed to quantitate this interference in plasma and serum and evaluate its clinical significance.

Methods: Plasma and serum samples were studied. The entire spectrum of fluorochromes available on the Beckman-Coulter NAVIOS analyzer was evaluated in undiagnosed patients (appearing with the artifact) and in random subjects/controls. Various antibodies (CD19, CD20, CD34, CD4) conjugated with tandem fluorochromes conjugates such as PE-Cy7, PE-Cy5 and PE-Cy 5.5 were used. The final proposed method consisted of a) incubation of 20μL plasma or serum with 5μL CD20-PE-Cy7 antibody, and a control sample without antibodies, for 15 minutes at room temperature b) FSC gain assessment in the range 2-50 FS in the NAVIOS Beckman-Coulter cytometer, while Megamix-PLUS beads 0.1, 0.3, 0.5 and 0.9μ were used to assess size, c) addition of 4μL WFI and immediate kinetic 1-minute measurement, studying the percentage of fluorescence over control (%) in each fluorochrome. Reference values were established from 14 healthy blood donors. Measurements were also made on different time points: at the time of collection, the next day, 72h later. 33 patients (appearing with the artifact) were analyzed during a one-year period. Combined incubations with CD61-FITC, CD235a-PE, CD45-KO and CD10-PE-Cy7 stained platelets and microvesicles derived from red cells, white cells or platelets. Clinical and laboratory correlations were performed.

Results: In the control group, a low (%) of expression was observed (*CD20-Pe-Cy7 0.66%, 0.25-11.6%)*. Minor differences were noted between plasma and serum. In patients, an increase in CD20-PE-Cy7 fluorescence was observed. The final diagnoses were: 14 Waldenström's macroglobulinemia, 14 Systemic Lupus Erythematosus (SLE) and 5 other autoimmune diseases (Sjogren's syndrome, rheumatoid arthritis etc.). Artifact assessment proved crucial in cases with a complex initial differential diagnosis. For example, a pancytopenia case with multiple serological cross-reactivity turned out to be SLE, a suspected lymphoma case was finally diagnosed with SLE, and a suspected malaria case was ultimately diagnosed as an autoimmune disorder.

Conclusions: The identification and flow cytometric measurement of a specific interference artifact in PE tandem fluorochrome conjugates in plasma and serum, is proposed as a strong indicator that could significantly contribute to the diagnostic approach of undiagnosed cases, which may conceal a Waldenström paraproteinemia or autoimmunity.

PLE08-04

High-Dimensional Profiling of Human Microbiota via Flow Cytometry Coupled with AI Analysis

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Introduction: The microbiome is a complex, dynamic community of microorganisms that plays a vital role in human health, influencing immune function, metabolism, and disease prevention. Dysbiosis is associated with a range of diseases, highlighting the importance of maintaining a balanced microbiome. Therapeutic strategies, such as probiotics, prebiotics, and fecal microbiota transplantation, offer promising avenues for treating and preventing diseases. Continued research is essential to fully understand the microbiome's role in health and disease and to develop effective microbiome-based therapies. At present, there is no fast and cost-effective technique available for rapidly screening and studying microbiota. Spectral and imaging flow cytometry offer powerful tools for dissecting the human microbiota by enabling high-dimensional, single-cell analysis that distinguishes microbial populations based on both fluorescence profiles and morphological features. Their ability to resolve complex autofluorescence and simultaneously capture structural and functional data allows for more precise identification, phenotyping, and sorting of diverse microbial communities.

Methods: In this study, four different microorganisms commonly found in the human microbiota were selected (*Bifidobacterium*, *Lactobacillus, Helicobacter pylori* and *Escherichia coli*). A set of markers, typically used in flow cytometry to assess different microorganism features (DNA content, metabolic activity, cell viability, and mitochondrial function) was titrated and tested. The final optimized panel was then applied to the four selected microbial species. Both unstained and stained samples, analyzed either as individual pure populations or as mixed microbial communities, were examined using conventional flow cytometry, spectral flow cytometry, and imaging flow cytometry. The resulting data were analyzed using artificial intelligence algorithms with the aim of identifying individual species within complex mixtures.

Results: A flow cytometry panel was optimized to characterize microorganisms using three key markers, CFSE, DRAQ5, and Live/Dead staining selected from an initial list of ten based on their performance in distinguishing between microbial species. Each marker was titrated under assay-specific conditions, and their signal-to-noise ratios were calculated to determine optimal concentrations; a specific gating strategy was validated. Final testing on fixed samples of *Bifidobacterium* and *Lactobacillus* confirmed significant differences in marker expression ($p < 0.05$), supporting the next step of integrating AI-based analysis.

Conclusions: This study optimized a rapid, cost-effective protocol combining spectral and imaging flow cytometry with AI analysis to enable detailed phenotypic and morphological screening of human microbiota at the single-cell level. This approach offers significant advantages for research and clinical applications by providing high throughput, multiparametric data that support microbiota profiling, disease monitoring, and therapeutic evaluation.

PARALLEL SESSION PRESENTATIONS

PAR01-01

T-cell acute lymphoblastic leukemia: a flow perspective from diagnosis to minimal residual disease. Feedback of the French experience

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T-cell acute lymphoblastic leukemia (T-ALL) represent a complex and heterogeneous disease, characterized by the malignant expansion of T-cell precursors arrested at different stages of differentiation. Immunophenotyping is essential for confirming affiliation to the T-lineage and remains a cornerstone of diagnosis, following WHO classification guidelines.

Yet, despite years of effort to further stratify T-ALL based on maturational stages, these classifications have provided little clinical benefit. With new insights into the biology of both normal and malignant T-cell progenitors, it is time to revisit the clinical value of immunophenotyping for diagnosis, risk assessment, and patient follow-up.

In this presentation, we will explore how key oncogenic events reshape thymocyte development and alter immunophenotypic profiles. We will also examine emerging markers and potential avenues for a new classification approach, in light of this refined understanding of T-cell biology. Furthermore, we will discuss practical implications for diagnosis, classification, and the integration of advanced phenotyping into modern clinical workflows, especially in the era of targeted therapies. Finally, we will consider how understanding maturational dynamics can optimize post-treatment monitoring and patient care.

Our goal is to bridge biology and practice, providing the audience with an updated, evidence-based guide to immunophenotyping in T-ALL. This will open new perspectives on the upcoming challenges for improving T-ALL immunophenotyping.

PAR01-02

Cytometric issues in the diagnosis of T-lymphoproliferative disorders: T-CUS or LGLL?

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T cell lymphomas represent a heterogeneous group of aggressive hematological malignancies with different clinical presentations and molecular profiles. Accurate diagnosis of these neoplasms is crucial for appropriate patient management and therapeutic approach.

The laboratory diagnosis of T cell lymphoproliferative disorders is based on the identification of T cell populations exhibiting cytomorphological and/or immunophenotypic aberrations, often in correlation with clinical presentation and ancillary molecular studies. Definition of clonality represents the first step in the characterization of a lymphocytosis. Although molecular analyses are cornerstone in the definition of clonality, the flow cytometry analyses of the TCR Vβ repertoire can be regarded as a surrogate for molecular assessment and, more recently, the evaluation of the constant region 1 and 2 of the T cell receptor β chain (TRBC1 and TRBC2) has been proposed as an easy and reliable method for assessing Tαβ clonality.

Despite its strengths, flow cytometry faces several challenges in T cell lymphoma diagnosis. The overlapping immunophenotypes between reactive T cell proliferations and certain T cell lymphomas can lead to diagnostic ambiguity.

The immunophenotype is central also to distinguish different large granular lymphocytic leukemia (T-LGLL) subtypes. In fact, leukemic T-LGLs exhibit a post-thymic terminal effector memory phenotype (CD3+CD8+CD57+ CD45RA+CD62L-) along with a variable expression of CD16, CD56, KIRs and CD94/NKG2 receptors, indicating that these cells are late stage fully differentiated cytotoxic T lymphocytes.

Within T-LGLL, additional immunophenotypic subtypes can be identified. Based on the type of TCR, two variants (Tαβ- and Tγδ-LGLL) can be distinguished. The absence of a well-defined LGL lymphocytosis with evidence of clonality lead to a gray zone. T-cell clones of uncertain significance (T-CUS) represent a heterogeneous group of mature T-cell leukemias that do not meet criteria for established entities such as T-cell prolymphocytic leukemia (T-PLL), Sézary syndrome (SS), or T-LGLL. These cases often display aberrant immunophenotypes, with atypical co-expression or loss of CD4, CD8, CD5, and CD7, making their classification by flow cytometry alone particularly difficult.

Oveall, although flow cytometry should be complemented by molecular testing, including TCR gene rearrangement studies, cytogenetics, and next-generation sequencing, the inclusion of T-CUS further underscores the need for a multiparametric approach to improve classification accuracy and avoid misdiagnosis.

PAR02-01

The Potential of PD-1 and Siglec-1 in the Immunodiagnostic Laboratory

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Checkpoint receptors are molecules expressed on immune cell surfaces. They regulate immune responses by acting as inhibitory "brakes" that prevent tissue damage. Tumors often exploit these pathways by upregulating ligand expression to evade immune detection. This has led to the development of immune checkpoint inhibitors, which block these receptors or ligands, thereby restoring T cell activity against cancer.

In our diagnostic laboratory, we have developed and validated flow cytometric assays to quantify checkpoint receptor expression. For example, we can quantify PD-1 expression on T cells and CAR T cells as well as Siglec-1 expression on monocytes.

PD-1 is predominantly expressed on T cells and functions as an activation inhibitor when it binds to PD-L1 and PD-L2. Its expression reflects T cell exhaustion and immune status. Detecting PD-1 is important for targeting these molecules in tumor therapies and for monitoring CAR T cell persistence in patients.

Siglec-1 is predominantly expressed on monocytes and macrophages and it has emerged as an activation marker that reflects innate immune responses and monocyte activation states.

We have also examined the expression of TIM-3, TIGIT, LAG-3, and Siglec-7 on natural killer (NK) cells. We compared peripheral blood samples from healthy donors to those from patients with gastric cancer. Our study of gastric cancer patients revealed elevated LAG-3 expression and reduced Siglec-7 levels in NK cells.

Measuring these checkpoint molecules provides insight into the immune landscape of patients undergoing immunotherapy, experiencing infectious diseases, or suffering from autoimmune conditions. Targeting these checkpoint molecules may represent a promising therapeutic approach for treating various cancer types and autoimmune diseases.

PAR02-02

Blood biomarkers associated with response to anti-PD1 treatment in metastatic melanoma

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The need for reliable biomarkers in liquid biopsies to predict clinical benefit from anti-PD1 treatment in metastatic melanoma patients remains unmet.

I will first present the main parameters of response or resistance to anti-PD1 treatment previously identified in blood in different tumor contexts. Secondly, I will present the results from the longitudinal IMPROVE study (NCT02626065) aiming to identify blood immune parameters able to discriminate metastatic melanoma patients who will respond to second-line anti-PD1 treatment.

Conventional flow cytometry analysis was performed on whole blood samples collected longitudinally to assess the immune cell subsets absolute numbers, the expression of immune checkpoints or ligands on T cells and the functionality of innate immune cells and T cells. Clinical response was assessed according to Progression-Free Survival (PFS) status at one-year following initiation of anti-PD1 (responders: PFS > 1 year; non-responders: PFS ≤ 1 year).

I will present the phenotypic and functional alterations in blood immune cells detected at baseline in metastatic melanoma patients compared to healthy donors, and those associated with anti-PD1 response. I will also highlight the phenotypic modifications appearing after 3 months of anti-PD1 treatment that identified responding patients. Finally, I will present a combination of immune biomarkers accurately allowing to discriminate responding from non-responding patients in this IMPROVE cohort.

PAR03-01

NK-LGLL at the Crossroads of Cytometry and Molecular Biology: Advances in Diagnosis and Characterization

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Large granular lymphocyte leukemia (LGLL) is a rare lymphoproliferative disorder comprising two main variants: T-cell (≈85%) and natural killer (NK, ≈15%). While T-cell proliferations can usually be traced through markers of clonality, NK-cell proliferations remain more elusive. The absence of reliable clonality markers has long complicated the distinction between NK-LGLL and reactive NK-cell expansions, which are frequent in infectious, autoimmune, or inflammatory conditions. This diagnostic challenge is reflected in the latest classifications, with the 2022 WHO update adopting the term NK-LGLL, while the ICC still considers chronic lymphoproliferative disorder of NK cells (CLPD-NK) a provisional entity.
Over the past decade, significant progress has been made toward building standardized diagnostic frameworks. Integrative approaches combining immunophenotypic and molecular features have proven particularly informative. Among these, the use of an NK-clonality score has emerged as a practical tool, incorporating absolute NK cell counts, restricted Killer-cell Immunoglobulin-like Receptor (KIR) expression, CD94-NKG2A overexpression, and recurrent somatic mutations in STAT3, STAT5B, TET2, and TNFAIP3. This approach has demonstrated robust sensitivity and specificity in clinical practice, although a proportion of patients remains difficult to classify.

More recently, recurrent somatic mutations in the CCL22 gene have been identified as a promising biomarker for NK-LGLL. By impairing CCR4 internalization, CCL22 gain-of-function variants facilitate aberrant immune cell recruitment and contribute to NK-cell proliferation. These mutations are found in about 20% of NK-LGLL cases, are rare in T-LGLL, and appear mutually exclusive with STAT3 mutations, providing complementary diagnostic information. Incorporating CCL22 mutational status into diagnostic algorithms has further improved classification accuracy.
Altogether, recent advances in immunophenotypic and molecular characterization are shaping a more standardized and accurate diagnostic framework for NK-LGLL.

PAR03-02

Fitness of Immunotherapeutic NK Cells for acute myeloid leukemia: Challenges and Opportunities

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Natural killer (NK) cells exhibit potent cytotoxicity against leukemic cells, making them promising candidates for adoptive transfer immunotherapies. Clinical trials have demonstrated safety and some efficacy of NK cell therapy, but challenges remain in optimal therapy timing, donor selection, large-scale expansion, and resistence to leukemic microenvironment after adoptive transfer. Addressing these obstacles is critical for efficacy improvements and the broader clinical application of NK cell-based therapies. Our study highlights the immunosuppressive role of TGF-β in the leukemic microenvironment and its impact on NK cell-based therapies for AML. Using 2D and 3D co-culture models, we demonstrated that TGF-β, produced by both leukemic cells and mesenchymal stromal cells, impairs NK cell cytotoxicity and migration. These findings underscore the importance of overcoming TGF-β-mediated immune evasion to enhance the effectiveness of NK cell immunotherapies in AML. Furthermore, we used transcriptomic profiling and detailed functional immunophenotyping of in vitro expanded NK to define the parameters allowing predictive donor selection and successful large-scale expansion and cytotoxity. Altogether, these findings contribute to a deeper understanding of the challenges in NK cell-based immunotherapies efficacy and underscore the importance of refining strategies for their successful clinical translation.

PAR04-01

Use of carbon sources in human and murine natural killer cells: variation on a common theme

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Aim/objective: Natural killer (NK) cells display immediate effector functions after recognizing transformed or infected cells. Despite the recognition of the importance of metabolic regulators such as mTORC1 or SREBP, the environmental nutrients and metabolic requirements that sustain NK cell functions are unknown.

Methods: We addressed this gap of knowledge using primary human and mouse NK cells and characterized their metabolism using Seahorse, metabolomics (C12 and C13), total proteomics, and classic functional readouts by flow-cytometry.

Results: We show that human and mouse NK cells differ on the extracellular carbon sources required to support effector functions, signal transduction and proliferation. While human NK cells need pyruvate, mouse NK cells require glucose. Human cells are unable to generate endogenous pyruvate while murine NK cells can do so but require continuous glucose uptake. Behind this discrepancy, both cell types reduce pyruvate to regenerate glycolytic NAD^+ and oxidize it in the tricarboxylic acid (TCA) cycle to produce ATP. In addition, in both species, the TCA is in a citrate-malate configuration. Moreover, this specific metabolic wiring supports anabolic pathways producing ribose and serine to sustain proliferation. Importantly, the net output of these pathways is sensed by the production of UDP-N-Acetylglucosamine acting as a checkpoint of cellular metabolic state through O-GlcNAcylation. Supraphysiologic pyruvate concentrations increase human NK cells' effector functions. We currently pursue other strategies boosting NK cell potential.

Conclusion: Overall, this study highlights the role of carbon sources in NK cell biology, providing knowledge that could be exploited to boost NK cell potential in therapeutic settings.

PAR04-02

Flow cytometry-based immunometabolic profiling of complex samples using click chemistry (CENCAT)

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Introduction: Research in immunometabolism has demonstrated that cellular energy metabolism plays a critical role in shaping immune cell function. Extracellular flux (XF) analysis has been instrumental for immunometabolism research, but its reliance on cell purification limits its applicability in complex samples. To address this, SCENITH, a flow cytometry-based technique, was recently introduced. However, SCENITH depends on intracellular puromycin staining, a toxic antibiotic that may introduce unwanted side-effects.

Aim: To develop a flow cytometry-based method for immunometabolic profiling in complex samples using biorthogonal noncanonical amino acid tagging (BONCAT).

Methods: We developed CENCAT (Cellular Energetics through Noncanonical Amino Acid Tagging), a novel technique which utilizes click labelling of alkyne-bearing noncanonical amino acids (ncAAs) to measure protein synthesis inhibition as a proxy for metabolic activity. We applied CENCAT to human primary macrophages to assess metabolic changes upon activation with lipopolysaccharide (LPS)/interferon (IFN)γ and interleukin (IL)-4). Additionally, human peripheral blood mononuclear cells (PBMCs) and murine tissue-resident immune cells from multiple organs were analysed to evaluate metabolic profiles across different immune cell populations.

Results: CENCAT successfully reproduced known metabolic signatures of LPS/IFNγ and IL-4 macrophage activation. In PBMCs, stimulation with different activators (LPS, CpG, anti-CD3/CD28) revealed diverse metabolic rewiring across immune cell subsets. In murine tissue-resident immune cells, sample clustering was observed based on tissue origin, suggesting microenvironmental priming of immune cell metabolism.

Conclusions: CENCAT is a powerful tool for assessing immune cell metabolism in complex samples, including PBMCs and whole murine tissues.

PAR05-01

Immunophenotyping of systemic mastocytosis. Clinical and biological significance

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Systemic mastocytosis (SM) encompasses a heterogeneous group of disorders characterized by abnormal proliferation and accumulation of mast cells in one or multiple organs. Current classification relies on the extent of mast cell infiltration, the presence of organ damage, and association with non-mast cell hematologic neoplasms, leading to at least eight distinct entities. Clinical manifestations are highly variable, ranging from isolated aesthetic concern with minimal impact to severe systemic involvement compromising life expectancy in the short term.

The diagnosis of SM primarily depends on histopathological evaluation; however, laboratory investigations play a pivotal role by providing complementary morphological, phenotypic, molecular, and biochemical information essential for accurate diagnosis. Among these, flow cytometry-based immunophenotyping has emerged as a key technique for identifying mast cells and characterizing their pathological features. Despite the rarity of mast cells in bone marrow and peripheral blood, their specific immunophenotypic profile enables reliable detection. These criteria were initially established in the 1980s and have remained relatively unchanged, though their clinical utility continues to expand. Beyond diagnosis, immunophenotyping provides prognostic insights and helps unravel the biological underpinnings of the disease.

This presentation will review the current WHO classification of systemic mastocytosis and highlight the diagnostic value of immunophenotyping within this framework. We will also discuss practical considerations for optimal mast cell detection and characterization, including pre-analytical and analytical precautions to minimize diagnostic pitfalls. We will share the experience gained in France through the French National Mastocytosis Network (CEREMAST) and will endeavor to provide practical recommendations for the accurate identification and characterization of mast cells, aiming for a sensitive and reliable diagnosis of this rare condition, whose prevalence is likely still underestimated.

Ultimately, accurate immunophenotypic assessment remains essential for sensitive and specific diagnosis of systemic mastocytosis. By integrating phenotypic, molecular, and clinical data, we aim to provide evidence-based recommendations to improve diagnostic performance and patient care.

PAR05-02

BPDCN: A Diagnostic Challenge

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A multidisciplinary approach, including hematologists, dermatologists, pathologists and flow cytometric experts is required to diagnose and follow-up patients with blastic plasmacytoid dendritic cell neoplasm (BPDCN). BPDCN is a rare and aggressive CD123+ CD4+ CD56+ myeloid cancer that typically manifests initially as cutaneous lesions with or without organ involvement, including bone marrow, and subsequent leukemic spread. The prognosis of BPDCN is generally poor, if not diagnosed and treated promptly.

However, BPDCN must be differentiated from mature plasmacytoid dendritic cell (pDC) proliferation (MPDCP) and from Myeloid neoplasms with pDC differentiation. In normal bone marrow pDCs represent less than 1% of total nucleated cells, but in the setting of Acute Myeloid Leukemia (AML) or Chronic Myelomonocytic Leukemia, non-neoplastic pDC proliferations are commonly detectable. In MPDCPs, pDCs are mature cells characterized by absence or low/partial expression of CD56, unlike BPDCN, where CD56 expression is often strong and uniform. In AML with pDC differentiation, pDCs show a maturation profile from immature cells (CD34+ CD117+ CD123+-) to mature pDCs (CD34-CD117- CD123++). This maturation profile is not present in BPDCN. AML with pDC differentiation occurs in 3-5% of AML cases and shows a worst prognosis when compared to AML without pDC differentiation. In addition, the characteristic immunophenotype of BPDCN may be also found in some AML cases. Different studies showed that CD123 is expressed in 45-95% of AML cases and the CD123 overexpression is correlated with NPM1 and FLT3 mutations. In the setting of AML with plasmacytoid dendritic cell-like phenotype the expression of CD64, MPO, CD13 is of help in distinguishing it from BPDCN.

In conclusion, BPDCN represents a diagnostic challenge, requiring a multidisciplinary approach, skilled specialists and a standardized diagnostic protocol. Targeted therapies using anti-CD123 monoclonal antibodies are being developed. A gallery of representative cases, focusing on differential diagnosis will be shown.

PAR06-01

KIRs, T cell dynamics and human health

Becca Asquith [1]

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Studying human immunology is challenging, for obvious ethical reasons. We discuss how HLA class I and class II disease associations can be leveraged to learn more about our immune systems. We will focus on a family of immune receptors called KIRs (killer immunoglobulin like receptors). We combine analysis of genetic data from large patient cohorts with mechanistic mathematical modelling and assays of in vitro and in vivo T cell dynamics to gain insight into the relationship between iKIRs, T cell dynamics and human health. We suggest that KIRs enhance T cell survival and that this in turn impacts on clinical outcome in viral infection (HCV, HTLV-1, HIV-1) and autoimmunity (type I diabetes).

PAR06-02

Computational decoding of the immune repertoire

Thierry Mora [1]

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Recent advances in high-throughput sequencing of B and T cell receptor repertoires have allowed for the deep profiling of immune state and memory. However, how to extract relevant immune information from sequencing data is a major computational challenge. Here I will present different techniques, using longitudinal data, public repertoires, and network analysis to infer the infection status and HLA type of donors.

PAR07-01

New ISO guidelines for Assay Validation

Wolfgang Kern [1]

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Use of in vitro diagnostic medical devices is under control of the „Regulation (EU) 2017/746 of the European Parliament and of the Council“ (IVDR). The International Standard ISO 15189 „Medical laboratories — Requirements for quality and competence“ is guiding quality management. Many medical laboratories, and in particular those applying flow cytometry assays, are using assays not being CE cleared and thus rated as laboratory-developed tests (LDTs). While part of IVDR refers to LDTs only recently a respective guideline has been implemented, i.e. „Medical laboratories - Concepts and specifications for the design, development, implementation and use of laboratory-developed tests (ISO 5649:2024)“. The presentation will discuss LDTs in this context.

PAR07-02

Measurement Uncertainty in the ISO15189 accredited clinical flow laboratory

(1) Report from the ESCCA MU Working Group

Johansson, Ulrika, SI-HMDS, Bristol Royal Infirmary, UHBW, Bristol, UK

In ISO 15189-accredited medical laboratories, estimation and management of measurement uncertainty (MU) is a requirement. The theoretical and practical aspects of estimating MU in medical testing are still evolving; and for clinical flow cytometry assays, only a handful of publications on the subject are available. A survey sent out to ESCCA members highlighted that guidance around this subject would be useful, and that approaches were varied across centres and countries. A working group was set up to explore how MU may be implemented in flow cytometry laboratories in a way that aligns with the requirements of the ISO 15189:2022, with the view to providing helpful guidance to laboratories. The group considered the types of assays that are suited for a statistically calculated (or estimated) MU, and how to monitor that – some more detail on that will be presented in the second part of the talk, by Dr Veyrat-Masson. The working group further considered the concept of MU in context of understanding what factors in our overall processes that contribute to potential variability of the final test result, whether that result is quantitative or qualitative. In line with the risk-oriented nature of both the Laboratory Standards and Assay Validation Guidance for medical laboratories from the ISO, the working group presents a structured, risk-based methodology, that may help laboratories enhance the robustness of their assays, improve diagnostic confidence, and fulfil accreditation requirements.

(2) Applicability of the long-term uncertainty in measurement (LTUM) method

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The determination of uncertainty in measurement (UM) allows estimating the accuracy of measurements by comparing the values obtained by laboratory quantification of patient samples to reference values. UM comes from the total error of a parameter that can be subdivided in random error and systematic error. Random errors in experimental measurements are caused by unknown and unpredictable changes in the experiment. They contribute to the variability of the result, specifically affecting the precision of the result lead to precision defects. Precision in measurement is the tightness of the values obtained by repeated measurements in specified conditions. Systematic errors are recurrent errors usually caused by measuring instruments that are incorrectly calibrated and/or used. The bias quantifies this systematic error and represents the difference between the value analyzed by the laboratory and a reference value.

The Guide to the expression of UM (GUM) method (ISO/IEC GUIDE 98, 1993) is a mathematical model created for the measurement process in which the standard uncertainty of each influencing factor has to be quantified. The advantage of this method is that it allows determining the uncertainty burden of each influencing factor. Its major drawback is that it is relatively tedious and not applicable in the routine practice of a medical laboratory.
In France, the Internal Quality Control and External Quality Assessment (IQC + EQA) is the most widespread method (Martinello et al., 2020). In this method, IQC allows estimating the random error, and EQA establishes the bias and thus estimates the systematic error. The advantage of this method is its simplicity. The controls are clustered into levels, and the uncertainty values are calculated for each control level. However, this clustering can lead to inaccuracies (Bouveyron et al., 2019) in the establishment of the UM.
Finally, the Long-Term Uncertainty in Measurement (LTUM) method, described by Meijer et al. in 2002 (Meijer et al., 2002), is based only on EQA. In this method, a regression line is drawn between the laboratory values and the expected values (EQA). The random error is represented by the Long-Term Coefficient of Variation (LTCV) that reflects the point dispersion around the regression line. The systematic error is represented by the long-term bias (LTB). It reflects the deviation of the regression line compared with the identity line that can be drawn if each laboratory value corresponds to the expected value. In this method, quality controls do not need to be clustered by levels. However, it requires many values for robust evaluation. It has been estimated that with fewer than eight values, the calculated uncertainty is not relevant (Martinello et al., 2020). This presentation will demonstrate these options for UM measurements, using worked examples, and discuss the pros and cons of the methods.

PAR07-03

Critical Implications of IVDR for Innovation in Diagnostics

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The In Vitro Diagnostic Medical Devices Regulation (IVDR) that was adopted in 2017 was intended to revise the EU regulatory framework for In Vitro Diagnostics (IVDs) to enhance their safety. Nonetheless, the academic diagnostic community represented in the BioMed Alliance has in the past raised awareness of problems including delays in implementation, limited capacity of Notified Bodies, and the slow roll-out of EUDAMED. In addition, they focused on unintended consequences of the reform including high costs of certification, issues with the evaluation of orphan IVDs, research and development leaving Europe, limited transparency and predictability, restrictive requirements for In-House IVDs. Also, the IVDR has critical implications on the development and the use of in-house tests and may obstruct innovation and personalised solutions for patients. The BioMed Alliance has called for parts of the IVDR to be amended to ensure a vibrant innovative academic diagnostic sector and to propose precise medical solutions for European patients.

PAR08-01

MAIT cells in host - microbiota interactions

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Mucosal Associated Invariant T cells (MAIT) are unconventional alpha-beta T cells recognizing small metabolites derived from the microbial riboflavin synthesis pathway presented by the non-classical MHC molecule, MR1. MAIT cells have emerged as a key T cell subset because of their wide anti-microbial specificity, abundance in human blood (1-8% of T cells), liver (20-50%) and mucosal tissues (1-10%) and because of their implication in several infectious and non-infectious pathologies including inflammatory bowel disease (IBD). The role of MAIT cells (beneficial or deleterious) in IBD remains unclear. We have been interested in understanding how MAIT cell ligands produced by the microbiota influence MAIT cell biology at steady-state and upon intestinal inflammation. We found that the canonical ligand 5-OP-RU is able to cross the intestinal barrier at steady-state and to travel to distant tissues like the thymus, where it drives MAIT cell maturation and expansion. Upon thymic egress, MAIT cells seed mucosal tissues such as the colon in which they remain resident for extended periods of time. Intestinal inflammation triggers an expansion of riboflavin-producing bacteria in the gut, leading to increased production of riboflavin and MAIT ligands that activate local MAIT cells. In return, MAIT cells express tissue-repair genes and produce barrier-promoting mediators resulting in reduced intestinal pathology. Thus, MAIT cells monitor a bacterial metabolic pathway indicative of intestinal inflammation, providing host protection in return.

PAR08-02

Why monitoring gamma-delta T cells in clinical practice?

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γδ T cells represent the third lineage of lymphocytes expressing a highly variable antigen receptor generated, as for αβ T cells and B cells, through V(D)J gene rearrangement. They recognize MHC-independent stress-induced self-antigens and are thus considered an atypical T lymphocyte subset, enriched in non-lymphoid tissues, at barrier surfaces. These specific attributes that bridge innate and adaptive immunity endow them with important frontline immune surveillance functions. They have been involved in infectious diseases, and their unique capability to kill hematological and solid tumors and their natural trafficking to bone marrow and tissues, recently put them at the forefront of novel immunotherapy strategies in hematological and solid cancers. This talk aims to present clinical relevance of γδ T cells and how analyzing γδ T cells could help in patient management.

PAR09-01

Multiple Myeloma preplasmablastic progenitors are a proliferating cellular origin of disease relapse

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Multiple Myeloma (MM) is a hematological malignancy characterized by an abnormal clonal proliferation of malignant plasma cells. Recent advances in treatment with the approval of several novel agents and their combinations have significantly improved patient outcome. However, patients invariably relapse after multiple lines of treatment, with shortened intervals between relapses, and finally become resistant to all treatments, resulting in loss of clinical control over the disease. The high relapse rate in patients with MM has led to hypothesize that MM progenitors or stem cells can support the recurrence of the disease. However, the nature of MM progenitors or stem cells remains indefinite. Using flow cytometry, we investigated the presence of monoclonal preplasmablsts (PrePB), plasmablasts (PB) and plasma cells (PC) in patients with MGUS (n= 29), newly diagnosed MM (n= 90), and MM at relapse (n= 32). The median percentage of monoclonal PrePB was 4.14, 1.54 and 1.47 in MGUS, newly diagnosed MM and MM patients at relapse respectively. A median percentage of 21.29, 18.66 and 17.25 PBs was identified in MGUS patients, newly diagnosed MM and MM patients at relapse respectively. Plasma cell labeling index was measured in the different subpopulations by flow cytometry. MM PrePBs were associated with a significant higher proliferation compared to MM PB and PC. Of interest, we identified that the detection of monoclonal malignant PrePB is associated with a significant poor prognostic in a cohort of newly diagnosed MM patients treated by high dose chemotherapy and autologous stem cell transplantation and in a cohort of MM patients at relapse treated by anti-CD38 MoAb. To better characterize the malignant immature PrePB and PB subpopulations, we used next generation sequencing to analyze the paired subpopulations in different MM patients. We also analyzed RNA-sequencing data generated from normal counterparts as previously reported (Alaterre et al. Blood 2024). As previously reported in normal PrePBs, cell surface proteins targeted by immune based therapies are not expressed by monoclonal MM PrePB including CD38, BCMA, SLAMF7/CS1, TACI, SDC1 or GPRC5D. GSEA analyses revealed that malignant PrePBs are characterized by a significant enrichment in polycomb PRC2 target genes, MM genes, TP53 target genes and communication with the bone marrow microenvironment. PRC2 catalytic subunit EZH2 deposits H3K27me3, repressing Polycomb-target genes. EZH2 inhibitor induced plasma cell maturation in association with up-regulation of CD38 protein expression and re-sensitized MM cells to anti-CD38 therapy. Altogether, PrePBs eradication could represent a potential strategy for novel MM progenitor-targeted therapies.

PAR09-02

Current value and future perspectives on flow cytometry detection of circulating tumor plasma cells in plasma cell neoplasms

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Monitoring measurable residual disease (MRD) is critical for assessing treatment efficacy and predicting relapse in multiple myeloma (MM). While bone marrow (BM)-based MRD evaluation has become standard, it remains invasive and may not capture residual disease heterogeneity. Peripheral residual disease (PRD) detection from blood samples offers a less invasive alternative with the potential for dynamic disease monitoring. This presentation will review two pivotal studies advancing PRD assessment in MM through complementary methodologies.

In the first study Lasa M. et al. (JCO 2025) used next-generation flow cytometry (NGF) and mass spectrometry (MS) to detect PRD in 138 patients undergoing maintenance therapy. This approach identified PRD positivity in 11.5% of cases, which corresponded to a 13-fold higher risk of progression compared to PRD-negative patients. The integration of NGF and MS enhanced sensitivity, capturing residual malignant plasma cells and monoclonal proteins, complementary to conventional BM-based MRD assays.

Building on this, in a second study, Lasa M. et al. (Blood 2025) introduced BloodFlow technology, combining immunomagnetic enrichment with flow cytometry to evaluate PRD in 295 MM patients. BloodFlow achieved an impressive sensitivity of 6×10^{-8}, detecting PRD in 8.5% of peripheral blood samples. Notably, patients negative for PRD by both NGF/MS and BloodFlow exhibited a 97% progression-free survival rate at two years, underlining the prognostic value of blood-based PRD assessment.

Together, these studies highlight the growing role of PRD monitoring in multiple MM, offering highly sensitive and minimally invasive tools that complement BM-based MRD assessment. Incorporating PRD evaluation into routine clinical practice could support more frequent and dynamic treatment response monitoring, ultimately helping to guide personalized therapeutic decisions in MM management.

PAR10-01

At the Cross Roads of Haematology and Immunology

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At the Cross Roads of Haematology and Immunology

Close overlap between clinical disorders, diagnostic technologies and research activities has long been recognized following the emergence of clinical disciplines of haematology and immunology. In clinical practice there is consider overlap between both medical disciplines in increasing recognition of associations between autoimmune cytopaenias, EBV related haematological cancers, Hemophagocytic Lymphohistiocytic and bone marrow failure syndromes and inborn errors of immunity. Similarly insights from studies of human in born errors of immunity have revealed key proteins essential for maturation and development of innate and adaptive immune cell subsets. The increasing use of B cell and plasma cell depletion agents and extension of cell based therapies and transplantation for cancers, autoimmune disease have increased the risks of secondary immune deficiencies. This has led to more collaboration between haematology and immunology specialists. Laboratory practice has continues to evolve with advances in immunohistochemistry, genomics and flow cytometry.

There is an increasing role for flow cytometry to diagnose primary immune deficiencies and to assess immune reconstitution after treatment with B cell depleting agents or following cell based therapies and haematopoietic stem cell transplantation. Diagnostic laboratories should use expertise of colleagues in haematology-oncology in sample processing, design of flow cytometry panels, gating protocols and data analysis to diagnose primary and secondary immune deficiency syndromes. Experience derived from external quality assurance schemes to monitor CD4 T cell in HIV-1 infection should form the basis to develop harmonised protocols to diagnose primary immune deficiencies and monitor immune reconstitution after different therapeutic interventions. This presentation will use a mix of case histories and review flow cytometry approaches to highlight synergy between haematology and immunology disciplines to improve management of patients with complex immune deficiencies.

PAR10-02

Autoimmune Cytopenias and Dysregulated Immunophenotype/Inborn Errors of Immunity

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Historically, inborn errors of immunity (IEI) were primarily characterized by increased susceptibility to infections. However, they are now recognized as a broad spectrum of diseases encompassing both infectious vulnerability and various forms of immune dysregulation. Hematologic manifestations, like cytopenias and lymphoproliferative disorders (LPD), are cardinal indicators of immune dysregulation: cytopenia can reflect bone marrow failure or a peripheral autoimmune response, while LPDs represent the chronic expansion of clonal lymphoid cells, which may be benign or progress to overt lymphoid malignancies.

Multiparametric flow cytometry (MFC) is an already established screening assay in detecting and monitoring IEI. To further enhance the analytical power of MFC data, principal component analysis (PCA), a non-hypothesis-driven statistical method, is increasingly employed as an integrative tool. PCA allows for the identification of subtle patterns and variations within complex MFC datasets, facilitating the recognition of distinct IEI phenotypes that might otherwise be overlooked.

This can be dramatically relevant in the management of patients affected by hematological manifestations, whereas a minor proportion of them may be part of the kaleidoscopic IEI presentations of and deserve a germline genetic screening. For instance, cytopenic patients with clinical suspicion of IEI may present with T lymphocyte subsets significantly skewed towards the memory and effector compartments.

On the other hand, established clinical diagnoses of immunodeficiencies necessitate surveillance strategies tailored to hematological complications. In our internal experience, we analyzed the MFC data from common variable immunodeficiency (CVID)/CVID-like patients developing malignant LPD: PCA helped in revealing different clusters defined by expanded naïve CD4+ and CD8+ T-cell pools, reinforcing the theory that reduced immunologic surveillance and defective memory formation create a permissive milieu for malignant transformation.

This presentation will therefore delve into the practical clinical application of PCA on MFC data, specifically focusing on its utility for screening hematological patients who may necessitate further genetic investigation due to a suspicion of an inborn error of immunity.

POSTERS

Posters have been clustered according to the topic:

- Hematology-Oncology (HEM)
- Immunology (IMM)
- Other (OTH)

HEMATOLOGY-ONCOLOGY (HEM)

HEM-01

Evaluation of bone marrow stabilization in TransFix-treated sample for acute leukeamia diagnosis by flow cytometry immunophenotyping

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Introduction: Immunophenotyping analysis of whole blood(WB) is limited by the necessity for analysis within a short time frame after collection. TransFix proposes stabilisation for up to 14-days. We describe the effects of TransFix on three different leukaemia cases and adressing it effectiveness.

Method: We examined markers stability and recovery of fixed BMA collected in EDTA at day-0, 3, 7 and 10. Non-stabilised sample was evaluated using 7-AAD to assess on degree of nucleated cell(NC) degradation. All samples were stored at 2-8°C.

Results: Fifty-five percent viability loss of NC seen after day-3 in B-ALL. AML was least stable with 73% non-viability observed at day-3, left none as it reached day-10. T-ALL only showed 58% viability lost after day-7. The blast percentage of transfixed sample remained stable in all cases after 10 days. Fluorescence intensity(MFI) of surface markers was stable across monoclonals in all 3-cases with few were exceptionally require careful interpretation. FSC and SSC B-lymphoblast decreased with respect to unfixed sample while distinct cluster from granulocyte and lymphocyte became apparent. Significant decreased CD99 seen from day 3, which became negative as it reached day 7. Disperse pattern of skappa and slambda identified upon day-3, indicating markers instability. Complete loss of cyCD79a and nTDT was found from day-3. T-lymphoblast demonstrated increased FSC and SSC. Non-stabilised CD7 became heterogeneously reduced upon day-7. This MFI reduction was also observed on fixed T-lymphoblast, however the average loss was lower, remained at level of appropriate gating until day-10. Insensitivity for cytoplasmic stabilisation was demonstrated by marked MFI reduction(~80%) of cyCD3 at day-3. Interpretation was feasible yet challenging because of dimmer expression. Complete loss of nTDT was also observed upon day-3. Stabilised myeloblast showed increase FSC while SSC was decrease compared to non-fixed sample. Distinct cluster between the myeloblast, granulocyte and lymphocyte seen on dotplot. MFI of cyMPO was reduced, however it remained at appropriate intensity, distinguishable from negative counterpart and stable until day-10. Aberrancy of CD19, cyCD79a and CD7 was detected. CD19 and CD7 was constantly positive despite gradual MFI reduction. Expected lost cyCD79a on normal B-cell was evident, but the MFI of myeloblast was relatively stable, remained at level above the negative counterpart on dotplot. CD99 was not evaluated in T-ALL and AML cases.

Conclusion: Sample preservation provides a solution which addresses delays between sample collection and leukemic testing. Nevertheless, cautious interpretation is required for cyCD79a, cyCD3, nTDT and CD99 in case extended 72h analysis needed.

HEM-02

Exbio DryFlowEx PNH High-Sensitivity Assay: A Comparative Evaluation of BD FACSLyric and Beckman Coulter DxFLEX Cytometer Performance

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Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder caused by clonal expansion of hematopoietic stem cells lacking glycosylphosphatidylinositol (GPI) anchored proteins. Accurate detection and quantification of GPI-deficient clones in red blood cells (RBCs), neutrophils, and monocytes is essential for diagnosis and monitoring. This study evaluated and compared the analytical performance of BD FACSLyric and Beckman Coulter DxFLEX cytometers using the Exbio DryFlowEx PNH High-Sensitivity Assay, focusing on sensitivity, accuracy, reproducibility, and performance near the lower detection limits.

Methods: A parallel analysis was conducted on 10 patient samples with confirmed PNH phenotypes. Each sample was stained and analyzed using the Exbio DryFlowEx kit on both BD FACSLyric and Beckman Coulter DxFLEX cytometers, and simultaneously on an accredited clinical laboratory developed test (LDT) as reference. Specimens were processed following Exbio and ICCS guidelines, using standardized settings, compensation, and gating strategies. Analytical parameters included accuracy, precision (%CV), linear regression, and determination of the lower limit of detection (LLoD). Cut-off experiments assessed both instruments' ability to detect clone sizes near and below clinically significant thresholds. Testing was extended below Exbio's LLoD to evaluate analytical robustness above the defined clinical cut-off. The study was conducted in a CAP-accredited laboratory with Institutional Review Board approval and informed consent.

Results: BD FACSLyric showed superior accuracy across all cell populations, with RBC clone measurements closely matching the LDT (mean bias -0.52%). Beckman Coulter DxFLEX showed a consistent tendency to overestimate low-frequency RBC clones, particularly below detection and clinical significance thresholds, unlikely to affect patient management. BD FACSLyric yielded the lowest measured cutoff (0.0044%) and a well-aligned LOQ (0.012%) with its LLoD (0.005%). DxFLEX, while comparable in cutoff (0.0050%), had a higher LOQ (0.081%). Both systems performed below the 0.1% clinical threshold. FACSLyric showed tighter %CVs and better discrimination at key cut-offs. The Exbio assay showed strong concordance with the reference LDT, supporting its reproducibility and reliability.

Conclusions: The Exbio DryFlowEx PNH High-Sensitivity Assay demonstrated reproducible performance comparable to the laboratory developed test (LDT), without the associated challenges of titration, antibody selection, or conjugate variability. Its standardized design ensures consistency and ease of implementation. BD FACSLyric outperformed Beckman Coulter DxFLEX in accuracy and sensitivity, particularly below clinical decision points. These findings support the use of the assay as a reliable benchmark for evaluating both cytometry platforms.

HEM-03

FLT3-ITD positive and Philadelphia positive precursor B-ALL in a young adult female: A Rare Case Report

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Introduction: Precursor B-cell acute lymphoblastic leukemia (pB-ALL) is a malignant proliferation of immature B-cell precursors, more commonly seen in children but also affecting adolescents and adults. Diagnosis relies significantly on flow cytometry, which enables rapid lineage assignment and detection of aberrant antigen expression. Although *FLT3* internal tandem duplication (FLT3-ITD) is well documented in acute myeloid leukemia, its occurrence in B-ALL is rare and associated with adverse prognosis. We report a rare case of pB-ALL in a young adult female, initially diagnosed by flow cytometry and later found to harbor FLT3-ITD and complex cytogenetic abnormalities.

Methods: An 18-year-old female presented with anaemia, thrombocytopenia and bilateral periorbital petechial rash. Bone marrow aspiration was analyzed using flow cytometry. Further evaluation included conventional karyotyping and PCR-based molecular analysis. Ethical approval was obtained from the institutional review board, and written informed consent was secured.

Results: Bone marrow immunophenotyping by flow cytometry demonstrated positivity for CD19, CD10strong, CD20weak, CD34, HLA-DR, TdT, and negative for MPO, with aberrant expression of CD13 and CD33, confirming the diagnosis of precursor B-ALL. Cytogenetic analysis revealed a complex karyotype: 46,XX,add(3)(q25), del(7)(q22q32), add(7)(q11.2),add(9)(q22),−12,−20, add(21)(q22), der(22)t(9;22)(q34;q11.2), +mar1,+mar2[15], including the Philadelphia chromosome. Molecular testing confirmed the presence of an FLT3-ITD mutation. As flow cytometry identified B-ALL with an immature B cell profile and aberrant myeloid antigen expression, findings indicated an aggressive disease course. The patient had an inadequate response to standard induction chemotherapy and after confirmation of BCR-ABL positivity, treatment was escalated to include a tyrosine kinase inhibitor (TKI), followed by referral for allogeneic hematopoietic stem cell transplantation.

Conclusions: This case illustrates a rare occurrence of FLT3-ITD in a young adult with B-ALL, coexisting with Ph+ status and complex cytogenetic abnormalities. According to the current literature, FLT3-ITD is uncommon in B-ALL and typically is associated with a poor prognosis. This report reinforces the critical role of flow cytometry in the early diagnosis of precursor B-ALL adding to the limited number of documented cases with this mutation profile and emphasizes the importance of integrating molecular and cytogenetic findings for risk stratification and treatment planning.

HEM-04

CML with Left Shift Mimicking AML: Importance of Flow Cytometry in Avoiding Misdiagnosis

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Introduction: Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm characterized by the BCR-ABL1 fusion gene. In its advanced stages, CML may present with morphological features resembling Acute Myeloid Leukemia (AML), potentially leading to misdiagnosis and inappropriate treatment. Flow cytometry is essential in distinguishing these conditions.

Methods: A 54-year-old male presented with fatigue, weight loss, and marked leukocytosis (WBC 229,600/µL). Peripheral blood smear showed 22% blasts and a left-shifted granulocytic series. Flow cytometry was performed using a panel including CD34, CD11b, CD16, CD14, CD64, CD19, and CD3 on a Beckman Coulter DxFLEX cytometer. Molecular testing for BCR-ABL1 transcript was conducted via quantitative PCR. Cytogenetic testing was not available at the time of diagnosis.

Results: Flow cytometry analysis demonstrated:
- CD34+ true blasts: 2.2%
- Immature granulocytes (CD11b-/CD16-): 35.3%
- Promyelocytes with dim or absent CD11b/CD16: 8.3%
- Mature granulocytes expressing CD11b+/CD16+: 31.9%
- No aberrant monocytic or lymphoid marker expression

BCR-ABL1 p210 transcript was positive at 46% IS. This maturation pattern strongly supports CML in chronic or possibly accelerated phase rather than blast crisis.

Conclusion: Despite the initial morphologic suspicion of AML or blast crisis, the immunophenotypic and molecular findings supported a diagnosis of CML in an earlier phase. This case highlights the critical role of flow cytometry in accurately classifying leukemic processes, particularly when morphology alone may be misleading. Correct diagnosis has immediate implications for patient management and prognosis. This diagnostic accuracy allows for early initiation of targeted TKI therapy, which is crucial for optimal patient outcomes.

HEM-05

Accurate quantification of paroxysmal nocturnal hemoglobinuria clone in neutrophils: added value of anti-CD16 antibody in eliminating eosinophil contamination

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Introduction: International guidelines recommend reporting the total paroxysmal nocturnal hemoglobinuria (PNH) clone percentage in neutrophils with two decimal precision for high-sensitivity results. This requires a stepwise gating strategy: size/structure to select leukocytes while excluding red blood cells and debris; CD45/structure to refine leukocyte selection; and CD15/SSC to isolate highly granular CD15-strong neutrophils while excluding eosinophils with lower CD15 expression.

This study aimed to precisely evaluate neutrophil contamination by eosinophils, notably regarding glycosyl-phosphatidylinositol (GPI)-deficient cells, using an additional marker, the anti-CD16 antibody, in conjunction with the recommended gating strategy.

Methods: Patients with PNH clones were included from 2018 to 2025. Flow cytometry data were analyzed with and without the anti-CD16 (clone 3G8), which recognizes both the transmembrane (independent of GPI anchor deficiency) and GPI-anchored (affected by GPI anchor deficiency) forms of CD16.

Neutrophils were identifed as $CD45^{+}$ SSC^{high} $CD15^{high}$ with PNH neutrophils defined as $FLAER^{-/low}$ $CD24^{-/low}$ $CD16^{low}$ (due to partial expression loss related to the 3G8 clone and CD16 molecular forms), while $CD16^{-}$ cells corresponded to eosinophils and/or potential immature granulocytes.

Eosinophils were further isolated based on their higher CD45 expression and subtracted from the granulocyte population; PNH eosinophils were defined as $FLAER^{-/low}$ $CD24^{-/low}$ cells.

Results: Among 77 patients displaying a PNH clone, 35 (45%) had eosinophils ≥1% of leukocytes, indicating a non-negligible occurrence. The median PNH eosinophil clone size was significantly higher than the median PNH neutrophil clone size (5.80%, IQR25-75 [1.91-24.23] versus 0.49%, IQR25-75 [0.07-9.30]; p=0.0034).

The median eosinophil proportion among leukocytes was 4.7% (IQR25-75 [2.34-7.10]).

The median eosinophil clone-to-total granulocyte clone ratio (granulocyte clone consisting of PNH neutrophils and PNH eosinophils) was 16.60% (IQR25-75 [5.86-49.53]), reflecting significant contamination. This contamination was inversely correlated with neutrophil clone size, being more pronounced in smaller PNH neutrophil clones (PNH neutrophil clone <1%: n=20, median eosinophil/granulocyte ratio PNH clone 34.20%, IQR25-75 [15.46-80.99]; PNH neutrophil clone ≥1%: n=15, median ratio 4.41%, IQR25-75 [2.24-16.20]; p=0.0001).

Eventually, the size of the eosinophil-free PNH neutrophil clone (CD16-based strategy) was significantly smaller than the contaminated neutrophil clone (without CD16), with a median of 0.49% (IQR25-75 [0.07-9.30]) versus 0.70% (IQR25-75 [0.30-11.08]), n=35; p=0.0059.

Conclusion: Eosinophil presence significantly impacts PNH neutrophil clone size quantification, particularly in small clones <1%.

The currently recommended gating strategy is insufficient to eliminate eosinophilic contamination. We propose using the anti-CD16 antibody (3G8 clone) for eosinophil exclusion to obtain a pure neutrophil population and ensure accurate high-sensitivity PNH neutrophil clone assessment.

HEM-06

Flow cytometer DxFlex evaluation and comparison with FacsCanto II

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Introduction: The new DxFlex flow cytometer by Beckman Coulter (BC) introduces advanced features compared to our current FACSCanto instrument, including an avalanche photodiode detector, 13 fluorescent measurement channels, and a more compact design. Our objective is to assess the DxFlex's performance to determine its suitability for our clinical applications.

Material and method: 36 samples (blood, bone marrow, lymph nodes) were selected, including 4 healthy samples, 18 non-Hodgkin lymphoma (NHL) samples, and 18 samples from patients with circulating blastes (PB) or plasma cells (PP). Analyzed surface antigen included CD45, CD10, CD3, CD4, CD8, CD56, CD19, CD5, CD20, kappa, lambda, CD34 and CD38. ClearLab LS tube (BC) with the wash/lysing protocol of BC were used for the DxFlex cytometer. For the FACSCanto (BD), custom cocktails with respective wash/lysing protocol from BD were used. The % of positive cells were compared between cytometers using similar gating strategies. For all patients, the % of neutrophils (PNN), lymphocytes (Ly), monocytes, and T, B, NK ly were compared. Median fluorescence intensity (MFI) ratios were also compared (CD19, CD20, CD5, kappa, lambda on B cells, CD10 on neutrophils). For NHL patients, % of clonal B cells and their expression of CD5, Kappa/Lambda were evaluated. In PB and PP, % of CD34+ blasts and CD38++ plasma cells were analyzed. Paired Wilcoxon, Spearman correlation, Passing-Bablok, Bland-Altman tests were used.

Results: For all patients except NHL, medians were statistically different for %PNN, %LyB CD19, %LyT CD4, %LyT CD8, and %NK with no significant differences for other parameters (p-value threshold at 0.05). These differences could be attributed to varying wash/lysis protocols and reagents between suppliers. The % of monoclonal B cells, PB, and PP were statistically similar. Spearman's test showed high correlations, and Passing-Bablok and Bland-Altman tests revealed no significant bias or deviation from linearity. CD10 and lambda MFI ratios were significantly higher in DxFlex. In NHL, CD5 and lambda MFI ratios were higher in DxFlex, whereas CD20 was lower. Both cytometers produced similar results overall, with two exceptions: DxFlex detected an additional pathological B-cell population.

Conclusions: xFlex demonstrated good performance overall. All pathological B clones, blasts and plasma cells were detected.Two discordant cases were observed, one of which is discussed in Figure 4, indicating higher sensitivity for DxFlex. DxFlex's additional fluorescence channels improved sensitivity in detecting pathological cells in some cases by using a single screening LS tube.

HEM-07

Immune microenvironment markers in predicting initial therapy response in Multiple Myeloma

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Introduction: Although Multiple Myeloma (MM) originate from plasma cells, its growth and progression are heavily influenced by the immune system. Among the most affected immune cell populations are natural killer (NK) cells and monocytes, which undergo functional and phenotypic changes that contribute to disease biology and immune evasion. This study examines the potential prognostic value of early immunophenotypic markers on NK-cells, plasma cells, and monocytes.

Methods: This retrospective study included 38 patients (14 male and 24 female) newly-diagnosed with MM. The earliest treatment response to initial therapy was evaluated according to the International Myeloma Working Group criteria. Patients were classified as good (complete response/very good partial response) or poor (partial response/stable disease/progressive disease) responders. Participants' ages varied between 59 and 71 years, with a median age of 66 without differences in groups. Pre-treatment bone marrow aspirates were analyzed using flow cytometry to quantify plasma cells, monocytes, and NK cells, and to evaluate expression-intensity (MFI) of CD138, CD56, and CD38.

Results: The median percentage of plasma cells did not differ significantly between response groups: 18.20% [IQR: 12.33–37.30] in good responders and 23.30% [IQR: 6.50–40.00] in poor responders (p=0.852). The proportions of lymphocytes, monocytes, and NK-cells in the bone marrow did not differ significantly between response groups. The median MFI of CD138 plasma cells was significantly lower in poor responders groups: 8246 [IQR: 4516–18110] vs. 26107 [IQR: 13186–57518] (p = 0.0051). Analysis of monocyte subsets expressing CD56 and CD38 revealed no statistically significant differences between patients with good and poor treatment responses. However, the median percentage of CD56 monocytes was three times higher in poor responders compared to good responders: 6.3% [IQR: 0.53–10.3] vs. 2.05% [IQR: 0.00–5.53]. The median MFI of CD38 monocytes was significantly higher in patients with a poor treatment response compared to those with a good response: 4437 [IQR: 3458–7007] vs. 1859 [IQR: 1451–3126], (p = 0.0097). The proportion and MFI of NK cells in the bone marrow did not differ significantly between good and poor responders.

Conclusions: This study demonstrates that higher expression levels of CD138 on plasma cells and CD38 on monocytes are significantly associated with poor early treatment response in MM patients. These immunophenotypic features may serve as potential prognostic markers and support the role of immune biomarkers into treatment stratification. Further research is needed to confirm their utility in risk stratification.

HEM-08

Approach to T clonality using TRBC1 in Lymphoid Screening Tube (LST) by flow cytometry is a good first-line test

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Introduction: The emergence of an antibody targeting the T-cell Receptor β-chain constant domain 1 (TRBC1, clone JOVI-1) enables the detection of monotypic T-cell subsets in a manner similar to the detection of kappa and lambda immunoglobulin light chain restriction in B-cell clones. This assay relies on the restricted ("monotypic") expression of one of two mutually exclusive TRBC1 or TRBC2 within immunophenotypically distinct TCRαβ expressing T-cell subsets. We recently implemented TRBC1 to the "lymphoid screening tube" (LST) recommended by EuroFlow. This study aims to evaluate the relevance of using flow cytometry as a first-line test for T-cell clonality compared to molecular biology method.

Methods: We conducted a retrospective study at Angers University Hospital between January 1, 2024 and April 24, 2025. This study compared 2 clonality analysis techniques: the LST tube consisting of 10 antibodies together with TRBC1 for the detection of monotypic T-cell subsets, and T-cell receptor gene rearrangement of the γ-chain (TRG) by molecular biology which was investigated in another center (Rennes University Hospital).

Results: 145 samples (blood or bone marrow) were studied by molecular biology for suspicion of hematological malignancy. Of these, 101 samples were both analyzed by flow cytometry and molecular biology. 63.4% (64/101) of samples did not show any T-cell monotypic population. 19% (20/101) of patients had a monotypic T-cell population confirmed by the 2 techniques. Interestingly, flow cytometry was more sensitive than molecular biology in 9.90% (10/101) of cases. 3 samples did not show a monotypic T-cell population by flow cytometry, whereas molecular biology showed a positive peak among a polyclonal profile. In one of these cases, CMF revealed an increase in CD8+ T lymphocytes following a primary EBV infection, consistent with molecular biology results. In the other two cases, subsequent immunophenotyping did not reveal any clones either a final diagnosis of haemopathy, which could indicate a transient population detected by molecular biology. Overall, the two techniques are concordant for all studied situations.

Conclusion: TRBC1 flow cytometric immunophenotyping is a robust and fast method for identifying T-cell clonality that can be easily incorporated into routine flow cytometric practice. This method has an excellent negative predictive value and could be a good alternative to molecular biology as a first-line test.

HEM-09

A rare case of CD19-negative B-lineage acute lymphoblastic leukemia: A diagnostic, therapeutic and monitoring challenge

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Introduction: B-cell acute lymphoblastic leukemia (B-ALL) is a neoplasm of precursor lymphoid cells committed to the B-cell lineage. World Health Organization has defined essential diagnostic criteria to diagnose B-ALL, including CD19 expression. In acute leukemias of mixed or ambiguous lineage without CD19 expression other criteria (CD10, CD22, CD79a) are required but it is not clear in B-ALL. Here, we describe the case of a 55-year-old patient diagnosed with CD19-negative B-ALL.

Methods: Fresh blood cells were stained and analysed by flow cytometry using a BD Facslyric. Orientation immunophenotyping including both lymphoid and myeloid markers and specific B-ALL panel with two different CD19 antibodies (APC-H7 (BD, clone SH25C1) and BV711 (BD, clone SJ25C1) were used to characterize the blasts.

Results: A 55-year-old man underwent routine laboratory during follow-up for type 2 diabetes mellitus with no other significant medical history. Complete blood count revealed anemia (118 g/L), thrombocytopenia (65 × 10^9/L) and normal neutrophiles count (1.7 × 10^9/L). Peripheral blood smear showed circulating blasts accounting for 38% of the leukocytes. There were no biological features of disseminated intravascular coagulation (DIC) or tumor lysis syndrome. Bone marrow aspiration revealed a hypocellular marrow without megakaryocytes and massive infiltration (approximately 75%) by undifferentiated blasts, suggestive of acute undifferentiated leukemia. Flow cytometric immunophenotyping was challenging to interpret. It revealed a large blast population with decreased CD45 expression and absence of CD34. No myeloid or T-lineage markers were detected. The blasts showed intracytoplasmic expression of CD22 (weak) and CD79a (high) but the expression of CD19 was lacking with both CD19 antibody clones tested. The blasts also showed expression of CD10, CD38 and CD81, partial expression of CD58, low expression of CD20 but no expression of cIgM. The overall immunophenotypic profile was consistent with a B-ALL although all of the essential immunophenotypic criteria were not present. Karyotype study showed a low-hypodiploid B-ALL with hypodiploidy. FISH analysis for *BCR::ABL1* fusion is negative and TP53 alterations research is underway.

Conclusion: This particular case was difficult to diagnose because of the absence of CD34 and CD19, both commonly expressed in classical B-ALL. Very few CD19 negative B-ALL are reported. The lack of CD19 also raises major issues for both treatment and monitoring. Treatments such as CAR-T cells and bispecific antibodies cannot be used. Minimal Residual Disease by flow cytometry will also be challenging due to the absence of CD19, a key marker used in B-ALL panels.

HEM-10

Expression of PD-1 and PD-L1 on tumor and immune cells in DLBCL, NOS by flow cytometry

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Introduction: Diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS) is characterised by a complex tumor microenvironment in which the immune checkpoint proteins PD-1 and PD-L1 may contribute to immune evasion. The aim of this study was to investigate the expression of PD-1 and PD-L1 on lymphoma cells (LC), tumor-infiltrating immune cells (TIC) and immune cell subsets, and to compare the expression between activated B cell-like (ABC) and germinal centre B cell-like (GCB) molecular subtypes.

Methods: We analysed lymph node samples from 34 DLBCL patients diagnosed between May 2019 and August 2022. After biopsy, tissue fragments were processed into cell suspensions and analysed by multiparameter flow cytometry. The expression of PD-1 and PD-L1 was analysed for LC, TIC and immune subsets such as CD4+ and CD8+ T cells, B cells, NK cells, macrophages and monocytes. The co-expression of PD-1 and PD-L1 as well as subtype-specific differences between ABC and GCB DLBCL were also analysed.

Results: PD-1 expression was significantly higher on TIC (median 30.8%) than on LC (1.6%). Among the immune subsets, CD8+ T cells showed the highest PD-1 expression (86.9%), followed by CD4+ T cells (66.7%) and NK cells (11.5%). B cells, macrophages and monocytes showed minimal PD-1 expression. PD-L1 expression was highest on macrophages (22.3%) and monocytes (21.9%), but low on TIC (4.7%) and almost absent on LC (<1%). Coexpression of PD-1 and PD-L1 was rare, detected in only a minority of NK cells (1.17%) and absent on LC. Comparison between subtypes revealed no statistically significant differences in PD-1 or PD-L1 expression. However, modest differences were observed: PD-1 expression in T cells was slightly higher in ABC (77.1%) than in GCB (69.4%), as well as in CD8+ T cells (91.6% vs. 85.4%). NK cells showed lower PD-1 expression in ABC (9.9%) compared to GCB (13.8%). PD-L1 expression on monocytes was slightly higher in GCB (3.2%) than in ABC (0%).

Conclusions: PD-1 and PD-L1 show different expression patterns in the DLBCL microenvironment, with high PD-1 on T cells and high PD-L1 on macrophages and monocytes. The coexpression of both markers is unusual. The subtype-specific differences were minimal and not statistically significant. These results improve the understanding of immune regulation in DLBCL and could serve for future immunotherapy approaches.

HEM-11

Efficient workflow for reproducible results from a 20-color acute myeloid leukemia panel on a spectral flow cytometer

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Introduction: A single-tube, 20-color panel for acute myeloid leukemia (AML) analysis that leverages Full Spectrum Profiling™ technology was developed, enabling greater reagent and sample efficiency. To streamline the workflow for consistent results, we studied the stability of frozen antibody cocktails as well as single-color reference controls stored in SpectroFlo® software for unmixing.

Methods: To eliminate donor-to-donor variation, antibody cocktails were frozen at -20℃ every 2 weeks. The same bone marrow sample was stained in triplicate with fresh or frozen antibody cocktails comprised of the 20-color panel, acquired on a 5-laser Cytek Aurora™ system using CytekAssaySetting, and analyzed using SpectroFlo® software. The stability of the frozen antibody cocktail was assessed for up to 12 weeks. The frequencies of cell populations of interest and median fluorescent intensity (MFI) for each marker were analyzed. In addition, the stability of using single color reference controls stored in the SpectroFlo® software library for unmixing 20-color panel data was evaluated every 2 weeks for up to 12 weeks using peripheral blood samples from different donors.

Results: Comparable results were obtained in cell percentages and MFIs from frozen cocktails compared to those from fresh antibody cocktails, except for CD19 Super Bright™ 780, which exhibited more than a 30% MFI drop after 2 weeks of freezing. The 20-color panel data were correctly unmixed using the single-color reference controls in the library for at least 8 weeks.

Conclusions: Our results demonstrate that Cytek's single-tube, 20-color panel is a robust and effective tool for high-dimensional immunophenotyping in AML research, supporting streamlined experimental workflows and generating consistent results.

HEM-13

Comparison of cytology, flow cytometry, interleukin assay, and MYD88 L265P mutation detection for diagnosing primary CNS lymphomas in CSF

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Introduction: Primary Central Nervous System Lymphomas (PCNSL) account for approximately 3% of brain tumors and are mostly diffuse large B-cell lymphomas. Diagnosis typically requires brain biopsy, though it may be avoided when imaging is combined with evidence of meningeal involvement, defined by the detection of lymphoma cells in cerebrospinal fluid (CSF) using conventional cytology (CC) and flow cytometry (FCM). The presence of the MYD88 L265P mutation (detected in 50–80% of PCNSL) and elevated IL-10 can also support the diagnosis. This CytHem study compared CC, FCM, interleukin assay, and detection of the MYD88 L265P mutation in both cellular fraction and circulating cell-free DNA (cfDNA) from CSF.

Methods: Eighty patients with tumor-suggestive imaging underwent lumbar punctures (n=109; mean volume = 10 ml). CC was performed on May-Grünwald Giemsa cytospins. FCM panel (CD8/kappa, CD4/lambda, CD19, CD56, CD10, CD34, CD5, CD20, CD3, CD45) was analyzed (Navios, Beckman Coulter). IL-10 and IL-6 were measured by CBA (FACSCanto II, BD); PCNSL suggested if IL-10 > 10 pg/ml (or 4–10 pg/ml with IL-10/IL-6 > 1). MYD88 L265P mutation was assessed by ddPCR (QX200, Biorad) on cellular fraction and cfDNA.

Results: PCNSL was confirmed in 21 patients (20 brain, 1 ocular), with 37 CSF samples analysed. Fourteen had MYD88 L265P-mutated lymphoma (biopsy = 3; CSF cells = 7; both = 3; vitrectomy = 1). The control group comprised 72 CSF samples from 59 non-PCNSL patients. CC detected malignant cells in 48.6% of PCNSL samples (18/37) with 90.3% specificity. FCM detected malignant cells in 37.8% of PCNSL samples (14 /37) with 100% specificity. Interleukin assay showed 100% sensitivity (24/24 PCNSL samples) and 97.3 specificity.

The combination of CC+/FCM+ identified meningeal involvement in 57.1% of cases (12/21): 8 after the first CSF, 3 after the second, and 1 after the third. Brain biopsy (n=8) or vitrectomy (n=1) were required for the remaining patients. Among the 14 MYD88 L265P-mutated lymphomas, the mutation was detected in the cfDNA with 78.3% sensitivity (18/23 samples) and 100% specificity. Across all PCNSL cases, it was detected in the cellular fraction in 47.6% (10/21 patients) and in cfDNA in 71.4% (15/21 patients). Among patients without meningeal involvement, cfDNA analysis identified the mutation in 66.7% (6/9).

Conclusion: In patients with imaging suggestive of PCNSL and no systemic lymphoma, interleukin assays excluded PCNSL, and repeated CSF analysis avoided brain biopsy in >50% of cases. cfDNA-based MYD88 L265P detection may preclude biopsy in 85% of cases.

HEM-14

A rare case of a Persistent polyclonal B-cell lymphocytosis (PPBL) with morphological and immunophenotypic features resembling Chronic lymphocytic leukemia (CLL).

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Introduction: CLL is a monoclonal disorder characterized by progressive proliferation and accumulation of mature lymphocytes. The diagnosis of CLL requires the presence of ≥5 x 10^9/L B-lymphocytes in the peripheral blood (PB), persisting for at least 3 months. The clonality of these B-lymphocytes needs to be confirmed by demonstrating immunoglobulin light chain restriction using flow cytometry (FC).

PPBL is a rare benign disease with chronic lymphocytosis of polyclonal origin, which occurs primarily in middle-aged female smokers. These women are mostly asymptomatic with mild leukocytosis/lymphocytosis. Bilobulated, usually even binucleated B lymphocytes in the PB and a polyclonal increase of IgM in the serum are characteristic findings. Splenomegaly has been described in some cases. The immunophenotype of B lymphocytes in PPBL resembles that of splenic marginal zone lymphocytes (CD27+/CD21high/IgMhigh/CD5low/CD23low) or memory B cells, suggesting that the disease originates from the marginal zone. Consistent with the polyclonality of the disease, a normal kappa/lambda light chain ratio is observed.

Methods: We present a case of a 69 year-old female who was referred to our lab due to lymphocytosis (WBC:50 x 10^9/L, ALC: 43 x 10^9/L) persisting for 3 years without anemia and thrombocytopenia. Clinical examination along with CT scan was negative for lymphathenopathy or organomegaly.

Results: PB smear revealed small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking prominent nucleoli and partially condensed chromatin. Smudge cells were also present further supporting the diagnosis of CLL.

FC of the PB was then performed using a 4 tube-6 colour assay with the following markers: CD19/CD20/CD23/CD25/CD27/CD5/CD10/CD11c/CD38/CD43/CD79b/CD81/CD103/CD180/CD200/FMC 7/ROR1/CD45. Analysis was performed with BD FACSLyric and FACSuite software.

We identified 2 separate B-lymphocytic (CD19+) populations. The predominant one (95% of the CD19+ lymphocytes) coexpressed the surface antigen CD5 together with the B-cell antigens CD19, CD20, and CD23. The expression of CD20/FMC7/CD81/CD79b was characteristically low, which along with the expression of CD200, ROR1 and bright CD43 indicated the diagnosis of CLL. However B-cell clonality assessment demonstrated polyclonal surface and intracellular immunoglobulin light chain expression.

Interestingly, the remaining CD5 negative B-cell population showed immunoglobulin light chain restriction (lamda clonality).The 2 populations did not differ regarding the rest of the markers tested.

Conclusions: To our knowledge, this is the 3rd case of PPBL with morphological and immunophenotypic features resembling CLL described in the literature.

HEM-15

High rates of sustained MRD-negativity one-year post-ASCT in multiple myeloma patients after daratumumab-based induction quadruplets- A single center experience

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Despite the tremendous improvement in the treatment landscape of multiple myeloma (MM) patients, autologous hematopoietic cell transplantation (ASCT) remains the standard of care in the first line of treatment in young patients with adequate organ function. Quadruplets have been introduced as the mainstay of induction treatment. We studied the MRD pre and post ASCT in newly diagnosed MM patients.

Methods: MRD available data of consecutive MM patients who underwent ASCT between 2023 and 2025 in our center were analyzed. Undetectable MRD was defined as presence of less than <1x10-5 cells with next-generation flow cytometry. Analysis was performed according to EUROFLOW protocol.

Results: Our cohort consists of 45 TE-NDMM patients, F/M:18/27, of median age 57(40-70). MM type was IgG:21/IgA:11/sFLC:12/solitary plasmacytoma:1 patient. ISS and R-ISS at diagnosis was I/II/III in 11/9/9 and 11/12/6 patients respectively and not available in 8 patients. Plasmacytoma was detected in 14 patients (Osseous/Extraosseous:10/4).
Cytogenetic data were available in 37 patients. Twenty-six patients had normal karyotype and 3 hyperdiploid. One patient had del17p, 14 del1p/add1q, 6 t(11;14), 5 t(4;14) and 1 t(14;16).
Induction regimen with quadruplets was administered in 41 patients, Dara-VTD/Dara-VCD/Dara-VRD in 27/10/4 patients, respectively. The rest of them received VRD/VCD/VTD:1/2/1. A total of 8 treatment cycles were administered. All received lenalidomide maintenance following ASCT.
Median stem cell yield was $3.5x10^6$ (2.00-9.5)/kg BW. Neutrophil and platelet engraftment occurred within 11(9-13) and 14(8-26) days respectively.
Disease status pre-transplant was CR/VGPR/PR/PD in 19/14/10/1 patients. Four patients relapsed during a median follow-up of 26 (3-52) months. All had high-risk features (3 plasmacytoma, 1 del17p).
Pre-ASCT MRD data were available for 43 patients; 11(26%) had undetectable MRD.
MRD assessment was available at +1mo/+3mo/+6mo/+12mo post ASCT in 39/20/4/11 patients; MRD negativity was achieved in 69%/70%/75%/81% of them respectively.
Estimated median progression free survival (PFS) was 45.9 months and 3 year overall survival (OS) was 86.1%.

Conclusion: Daratumumab-based quadruplets are effective induction regimens. Stem cell mobilization was satisfactory. ASCT along with lenalidomide maintenance further deepened disease response achieved during induction.

HEM-16

Multiple myeloma with central nervous system involvement: Case Report

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Extramedullary involvement in Multiple Myeloma (MM) is rare, occurring in only 3-5% of patients with usually skin, nasopharynx, larynx and upper respiratory tract localizations. Central Nervous System (CNS) involvement is an uncommon complication that occurs in less 1% of patients with MM. The CNS involvement by MM is associated to a dismal prognosis with an Overall Survival (OS) of around 1.5-2 months after it is detected. The reported median age of CNS-MM involvement is between 50-60 years. CNS-MM can arise at any stage of MM, although previous studies suggest a bias towards later stages of the disease.

We report the case of a man (57 years old) with a diagnosis of Micromolecular MM with extramedullary disease involving lung nodules, hepatic and splenic parenchyma, lymph nodes and duodenal bulb (stage ISSIII). In March 2025, first line therapy with VRd schemewas started. However, after a month of therapy progressive sensorial alterations with worsening psychomotor impairments appeared. A CT-scan was performed with negative results. A spinal tap was then ordered, to collect a cerebrospinal fluid (CSF). We used the ESCCA/ISCCA protocol for the analysis of CSF by multiparametric flow-cytometry (I. Del Principe at al. 2020). We detected by flow cytometry an absolute cell concentration of 173 /mL. We then applied a high-resolution flow cytometry approach according to the EuroFlow protocol for MM measurable residual disease (MRD), including multi-epitope CD38, CD56, CD45, CD19, cytoplasmatic (cy) Kappa, cy Lambda, CD27 and CD138. Flow cytometric analysis identified a prevalence of large abnormal plasma cells (PCs) expressing CD38+, CD138+, cyLambda+, CD19-, CD56-, and CD27-. CSF cytology was performed using cytospin and panoptic staining**.** The CSF smear showed the presence of several abnormal, large and immature PCs. The diagnosis of CSF involvement in MM was achieved. The patient died shortly after the onset of MM CSF involvement, about one week later.

In this case a high-resolution flow cytometric approach to CSF analysis that followed the recent ESCCA/ISCCA guidelines and applied the EuroFlow MM-MRD staining panel was precise and accurate in rapidly detecting the malignant PCs involvement of CSF.

HEM-17

Highly sensitive monoclonal anti-TdT antibody (DAK-TDT) shows equivalent flow cytometry staining on blood and bone marrow blasts

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Introduction: Terminal deoxynucleotidyl transferase (TdT) is expressed in early lymphoid precursors and it helps distinguish immature (lymphoblastic) from mature lymphomas. In addition, TdT may be aberrantly expressed in myeloid leukemias. The current study evaluated the performance of antibody TdT/iFluor 488, clone DAK-TDT, compared to the TdT/FITC clone E17-1519. Furthermore, the results in blood and bone marrow samples with acute leukemia involvement were compared to see if there are significant differences between TdT staining of circulating and bone marrow blasts.

Methods: In the comparison between different antibodies, eleven diagnostic specimens including bone marrow, peripheral blood, and thymus were evaluated. For each sample, three tubes were prepared: one with the previously validated panel of antibodies TdT/FITC E17-1519, myeloperoxidase/PE, CD34/PE-Cy7, cCD79a/APC, cCD3/V450, CD45/V500-C, CD11b/BV605, CD19/BV786, a second tube with TdT/iFluor 488 DAK-TDT instead of TdT/FITC E17-1519, and a third tube with isotype controls in FITC and PE channels alongside the other markers. The blood versus marrow comparison included 12 patients with acute leukemia in whom both specimens contained blasts and were tested with the panel including TdT/iFluor488 DAK-TDT. Data was acquired on the BD FACSLyric instrument, and analyzed using FACSuite and/or Kaluza. A staining of at least 10% of blasts was considered positive. Correlation coefficient was calculated to assess the concordance between blood and bone marrow.

Results: Out of 10 cases, staining using TdT/FITC E17-1519 yielded positive results in 1 case, whereas TdT/iFluor 488 DAK-TDT demonstrated positive results in 4 cases. Immunohistochemistry was used to confirm TdT positivity in cases where tissue was available and supported the findings that the iFluor 488 DAK-TDT antibody shows improved sensitivity compared to the previously used reagent, with excellent specificity.

We observed full concordance of TdT positivity for circulating versus bone marrow blasts in the 12 patient samples analyzed using the TdT/iFluor 488 clone DAK-TDT, out of which 6 were positive and 6 were negative. The correlation coefficient between the bone marrow and blood was 0.986. There was no significant association between specimen source and relative percentage or MFI of TdT positive blasts.

Conclusion: Our findings indicate that the reagent TdT/iFluor 488, clone DAK-TDT antibody exhibits superior sensitivity and excellent specificty compared to TdT/FITC E17-1519. Testing of blast population in blood and in the bone marrow showed excellent accuracy and concordant results in terms of TdT expression, suggesting that repeat testing is not necessary.

HEM-18

A Masquerade B-ALL

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Introduction: In this case report we describe a 78 year old male with history of Hodgkin Lymphoma 4 years previously representing with cytopenias. Initial diagnosis was acute B-lymphoblastic leukaemia (B-ALL) however specific laboratory findings suggested presence of a masked and underlying third malignancy.

Methods: Immunophenotyping was performed on a FACS Canto II flow cytometer (Becton Dickinson) and analysed on Infinicyt™ software (Becton Dickinson). Morphological analysis was performed on bone marrow aspirate slides stained with May-Grunwald Giemsa. Genetic testing methods included Fluorescence In Situ Hybridization (FISH), single nucleotide polymorphism array (SNP-A), G banding and next generation sequencing (NGS).

Results: Immunophenotyping identified a population of CD19+ B-cell precursors accounting for 48% of TNCs with the phenotype CD45 weak, CD34-, TdT+, CD10+, CD20+, cCD79a+, mCD22+, CD38+, HLADR weak, CD66c-, NG2-, MPO-, cytCD3-, mCD3-, CD7- and SmIg-. There was also a very small population of CD34+ myeloid progenitors with aberrant CD7 expression accounting for 0.25% TNCs. Bone marrow aspirate and trephine morphology reported an excess of medium-sized lymphoid blasts.

FISH and SNP-A results were consistent with a low hypodiploid karyotype. The initial diagnosis of B-ALL with hypodiploidy was made. Subsequent G banding identified presence of a three further subclones, one 46XY, one with del5q and a third with 5q and evidence of clonal evolution with monosomy 7 and 16. The hypodiploid clone was not identified. Lymphoid NGS identified a TP53 mutation at 82% variant allele frequency.

The patient started treatment including CD19-directed therapy, following which disease response was assessed. No residual B-ALL cells were identified post cycle 1 of treatment, however immunophenotyping identified marked expansion of the initially small abnormal myeloid population at diagnosis. Morphological analysis demonstrated prominent trilineage dysplasia and 15% blasts; most consistent with myelodysplasia (MDS) post cytotoxic therapy.

Conclusion: This case demonstrates the critical importance of utilising multi-modality integrated diagnostic laboratory testing to reach a diagnosis in haematological malignancies. Immunophenotyping detected the two distinct malignant populations at diagnosis. Cyto-genomics subsequently identified these were distinct subclones, one hypodiploid B-ALL and two clones with genomic aberrations consistent with myelodysplasia post cytotoxic therapy following treatment for HL. This case also emphasises the importance of identification and subsequent measurable residual disease (MRD) monitoring of all abnormal populations by immunophenotyping, especially in the context of novel targeted therapies, where multi lineage MRD is not feasible by standard genomic methods.

HEM-19

A proposed protocol for phenotyping bone marrow resident macrophages with common antigens. Comparison with classical, intermediate and non-classical monocyte subpopulations

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Introduction: Resident macrophages (RM) in bone marrow are recognized to play a key role in hematopoiesis. Besides RM, bone marrow also includes monocyte subpopulations like those defined in blood by CD14 and CD16 expression, i.e. classical (CM), intermediate (IM) and non-classical (NC) monocytes. In this study, we introduced a protocol that seeks to discriminate and measure RM among the other monocyte populations. Another aim was to comparatively examine the expression of several commonly used monocyte markers as CD14, CD16, CD33, CD64, CD300e (IREM2), HLA-DR, CD38, CD11b, CD11c, CD36, CD86 and CD13.

Methods: 15 bone marrow samples were studied in cases with morphologically defined resident macrophages, stained by non-specific esterase, in cases of emergency hematopoiesis. 10-color protocols were used with 9-antigen common backbone: CD66pool-FITC, CD3-ECD, CD33-PC5.5, CD34-PC7, CD14-APC, CD56-A700, CD16-A750, CD64-PB and CD45-KO. Each of the following monocyte associated markers was assessed in -PE position including CD300e (IREM2), HLA-DR, CD38, CD11b, CD11c, CD36, CD86 and CD13. Gating strategy aimed to discriminate RM along with the rest monocyte subpopulations in the CD14-CD16 scattergram. So, CM, IM and NC monocytes formed the well-known arcuate pattern encompassing CD14+CD16(-) CM, CD14+CD16+ IM and CD14(-) CD16+ NC as described in blood, while RM were positioned diagonally as a comet-like cluster. Monocyte associated antigens were studied by separately gating RM as well as each one of the monocyte subsets, recording their percentages % over isotype control and the corresponding mean fluorescence histogram intensities (MFI).

Results: The median values of % and MFI were compared among RM and CM, IM, NC monocytes. We observed the following: 1) RM were CD14+CD16+ like IM but differed significantly in the expressions of the antigens CD64, CD300e(IREM2), CD11b, CD86 and CD13 which were negative or low in RM while being intense and highly expressed in IM 2) there were non-significant differences in the percentages of HLA-DR, CD38, CD11c and CD36 among RM versus the other monocytes subsets. However, fluorescence intensity differences were assessed by the median MFI values. Thus, for each of the following antigens, in the order of magnitude they were for HLA-DR: IM>RM>NC>CM, for CD38: RM>NC>IM>CM, for CD11c IM>NC>CM>RM and for CD36: CM>IM>RM>NC. Concerning the antigens expressed only in the monocyte subsets, we observed that CD300e(IREM2), CD86 and CD13 were more intense in IM versus CM and NC.

Conclusions: A routine protocol has been introduced to discriminate, measure and phenotype RM in comparison to other monocyte subsets in bone marrow.

HEM-20

EZH2 Expression and Mutational Profiles in CLL: Diagnostic Implications and Clinical Correlations

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Introduction: Chronic lymphocytic leukemia (CLL) is a genetically diverse disease with a variable clinical course. Molecular profiling, particularly the identification of recurrent mutations in genes such as *TP53*, *ATM*, *BIRC3*, *NOTCH1*, and *SF3B1*, plays a crucial role in risk stratification and therapeutic decision-making. However, existing markers are not sufficient to fully predict disease progression or treatment response. Among emerging molecular features, the epigenetic regulator *Enhancer of zeste homolog 2* (EZH2) has drawn increasing attention. Its overexpression has been associated with adverse prognosis in CLL, and pharmacologic inhibitors targeting EZH2 are already approved for other B-cell malignancies. Integrating mutational profiling with EZH2 expression analysis may improve diagnostic precision and enable more personalized treatment approaches in selected CLL subgroups.

Methods: Peripheral blood samples from 50 treatment-naïve CLL patients were collected along with detailed clinical and laboratory data, including disease stage, cytogenetics, and lymphocyte counts. Mutational profiling was performed using targeted next-generation sequencing with a custom panel covering nearly 200 oncology-related genes. *EZH2* expression was quantified using Droplet Digital PCR (ddPCR) to obtain absolute transcript levels. Integrated statistical analysis was conducted to assess associations between mutational status, *EZH2* expression, and clinical features.

Results: Somatic mutations were identified in 92% of patients (46/50), with the most frequent alterations in *ATM*, *NOTCH1*, and *POT1*, followed by *TP53* and *KMT2D*. Missense variants and C>T substitutions were predominant, with a median of two mutations per case. Significant co-occurrence and mutual exclusivity patterns were observed, reflecting the molecular heterogeneity of CLL. *SF3B1* mutations were associated with shorter time-to-first-treatment (TTFT) ($p < 0.005$). *EZH2* expression was significantly elevated in CLL patients compared to healthy controls ($p < 0.001$), and overexpression correlated with shorter TTFT ($p < 0.05$). No significant association was found between *EZH2* levels and Rai stage. Ongoing integrative analyses are exploring additional correlations between specific mutational profiles and clinical features, which may reveal further prognostic or biologically relevant patterns.

Conclusions: Our findings highlight *EZH2* overexpression as a potential prognostic marker and therapeutic target in a subset of CLL patients. In parallel, comprehensive mutational profiling revealed substantial genetic heterogeneity, reinforcing its value for improved patient stratification. The integration of molecular and clinical data may enhance risk assessment and guide more personalized therapeutic approaches. These results support further investigation into the functional relevance of specific mutations and the role of *EZH2*-driven pathways in CLL pathogenesis. This study was supported by the National Science Centre, Poland (PRELUDIUM 16, 2018/31/N/NZ5/03344).

HEM-21

Monocyte subsets and cytokine profiles as potential biomarkers in chronic graft-versus-host disease

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Introduction: Chronic graft-versus-host disease (cGVHD) is a major complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Monocytes are implicated in its pathogenesis, with inconsistent findings regarding their subsets. However, the potential role of CX3CR1/fractalkine signaling in monocyte recruitment has been highlighted. This study aims to assess the association between cGVHD, clinical features, monocyte subpopulations, and monocyte-related cytokines in peripheral blood.

Methods: We analyzed peripheral blood from 69 cGVHD patients and 32 control patients who underwent allo-HSCT but did not develop cGVHD at the University Hospital Centre Zagreb, Croatia, between 2017 and 2023. Flow cytometry was used to characterize monocyte subsets, including classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical monocytes ($CD14^{+}CD16^{++}$), and their expression of CCR2 and CX3CR1, as well as $CD14^{++}$HLA-DR^{-} monocytes. A microsphere bead array analyzed on a flow cytometer was employed to quantify 12 cytokines (IL-4, IL-2, CXCL10, IL-1β, TNF-α, MCP-1, IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, IL-8) in plasma samples.

Results: No significant differences were observed in monocyte subset percentages between cGVHD patients and controls. However, cGVHD patients had a higher absolute number of classical monocytes ($p<0,01$) and a higher percentage of $CD14^{++}$HLA-DR^{-} monocytes ($p<0,01$), along with lower HLA-DR expression on classical ($p<0,01$) and intermediate ($p<0,05$) monocytes. CCR2 and CX3CR1 expression did not differ between groups. CXCL10 ($p<0,01$) and MCP-1 ($p<0,05$) levels were significantly elevated in cGVHD patients. Elevated IL-6 ($\rho=0,391$, $p<0,01$) and IL-8 ($\rho=0,475$, $p<0,001$) levels positively correlated with global NIH cGVHD score and negatively with the Karnofsky score (IL-6: $\rho=-0,416$, $p<0,01$; IL-8: $\rho=-0,385$, $p<0,01$). Positive correlations between cytokine levels (IL-10, IL-6, IL-8, CXCL10, MCP-1) and survival were observed, despite some of these being markers of more severe disease. IL-2 was inversely correlated with genital tract involvement ($\rho=-0,323$, $p<0,05$), while IFN-γ ($\rho=-0,418$, $p<0,01$), and IL-12p70 ($\rho=-0,352$, $p<0,05$) were negatively correlated with lung involvement. IL-17A positively correlated with mouth involvement ($\rho= 0,301$, $p<0,05$), and CXCL10 showed negative correlation with joint/fascia involvement ($\rho=-0,347$, $p<0,05$) but positive correlation with liver involvement ($\rho=0,418$, $p<0,01$).

Conclusion: This study underscores the relevance of monocytes and cytokine profiles in cGVHD. Plasma levels of CXCL10 and MCP-1, along with HLA-DR expression on monocytes, may serve as useful biomarkers. The clinical heterogeneity of the patient cohort might have contributed to some of the observed patterns. Their interplay reflects immune dysregulation and warrants further investigation to improve monitoring and treatment strategies in cGVHD.

HEM-22

AICAr Promotes AML Differentiation via Nucleotide Imbalance and Replication Stress Independent of p53 Status

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Introduction: Acute myeloid leukemia (AML) is characterized by the clonal expansion of undifferentiated myeloid precursors. Although traditional chemotherapy remains the standard of care, differentiation-based strategies are emerging as promising alternatives. AICAr, an AMP analog and intermediate of purine metabolism, has been shown to promote differentiation in AML cells. However, its mechanism—particularly its biphasic effects and the contribution of nucleotide metabolism—remains incompletely understood. We hypothesized that AICAr promotes AML differentiation by inducing nucleotide imbalance and replication stress, independently of p53 status.

Methods: Human AML cell lines (U937, MOLM-13) were cultured in standard RPMI-1640 or αMEM supplemented with ribonucleosides and deoxyribonucleosides. Treatments included AICAr (low and high dose), brequinar, cytarabine (AraC), and specific inhibitors (COH29, hydroxyurea). Metabolomic profiling was performed using LC-MS. Flow cytometry was used to quantify differentiation markers (e.g., CD11b, CD64), assess cell viability, and analyze cell cycle distribution. Protein expression (RRM2, phospho-RRM2) was assessed via Western blot. RRM2 knockdown was performed using siRNA.

Results: AICAr disrupted purine metabolism in a dose-dependent manner and exhibited a biphasic effect on UMP synthase inhibition in de novo pyrimidine synthesis. A comparable biphasic effect of AICAr was also observed in the expression of differentiation markers. High doses of ribonucleosides and deoxyribonucleosides completely abolished cell cycle arrest and differentiation induced by AICAr, Brequinar, and AraC. All agents increased RRM2 expression and phosphorylation. Flow cytometry revealed that COH29 inhibited differentiation marker expression in U937 cells, whereas hydroxyurea enhanced it. RRM2 knockdown further increased differentiation without major effects on cell cycle distribution. In MOLM-13 cells, similar metabolic alterations and nucleotide shifts were observed despite different checkpoint regulation. Notably, COH29 enhanced differentiation in MOLM-13, in contrast to its inhibitory effect in U937, while hydroxyurea promoted differentiation in both models.

Conclusions: Our findings identify nucleotide imbalance as a key driver of AML differentiation and highlight metabolic regulation as a promising therapeutic avenue.

HEM-23

Comparison of cell loss using different methods for preparation of samples for immunphenotyping by flow cytometry

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Introduction: Multiparameter flow cytometry is a widely used method to assess the immunophenotypic features of cells present in peripheral blood (PB), bone marrow (BM), lymph node (LN) biopsy suspensions, bronchoalveolar lavage (BAL), and other body fluids. Minimizing cell loss during sample preparation is important to achieve highest possible sensitivity, especially with a limited amount of material, low cell count or risk of loss of malignant cells. In addition, efficient workflow and quality is essential for most laboratories. The purpose of this study was to compare the cell loss of different types of materials using manual and automatic preparation methods, combined with different wash buffers. In addition, we wanted to achieve an effective sample preparation workflow with minimal cell loss for the different materials we receive at our laboratory.

Methods: PB, BM, BAL and LN biopsy suspensions from 66 donors (normal and clinical) were processed using a stain/lyse/wash (S/L/W) sample preparation protocol. Each sample was divided in six and stained with CD45 V500-C. Then, three of the aliquots were prepared automatically on a BD FACS™ Lyse Wash Assistant (LWA) and the other three with manual L/W preparation. For both methods, each of the parallels were washed with three different wash buffers (BD® CellWASH and two in-house buffers, PBS/0,2% BSA and PBS/0,2% BSA/2 mM EDTA). All samples were resuspended in acquisition buffer, acquired on a BD FACSLyric flow cytometer and analysed using BD FACSSuite software. The number of leucocytes in the final processed sample was measured and cell loss was calculated as % difference between the start leucocyte cell count in the aliquot and in the final processed sample. To evaluate cell loss between methods, we used the manual preparation method with PBS/0,2% BSA/2 mM EDTA as reference method. Two-way ANOVA with Dunnett's multiple comparisons test was used to calculate statistical significance.

Results: For LN and BAL, the mean cell loss across samples was significantly higher in manual and automatic methods combined with BD CellWASH compared with the reference method (95% and 82% versus 45%, and 71% and 68% versus 58%, respectively). For any other sample types or methods there were no significant differences.

Conclusions: We found that cell loss depended on the type of sample material and the wash buffer employed. LWA could be used for all the tested materials when combined with the right buffer. Our results emphasize the need to validate preparation methods for every type of sample material

HEM-24

Development of a machine-learning screening model analyzing different from normal maturation patterns for MRD testing on a spectral flow cytometer

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Introduction: Measurable residual disease (MRD) testing offers valuable prognostic information for patient outcomes in acute leukemia. Challenges in interpretation, however, result in high interobserver variance in identifying the abnormal leukemic immunophenotype at an analytical sensitivity of <0.01%, particularly in diseases that lack a leukemia-associated immunophenotype (LAIP). This is further compounded by the impact of different therapeutic regimens and clonal evolution altering the leukemic immunophenotypes. MRD can also be detected utilizing the different-from-normal (DfN) approach to identify deviations from normal maturational patterns. This method poses its own set of challenges as it requires expert knowledge of both normal and abnormal hematopoiesis. To meet these challenges, we developed a highly supervised, machine learning-based DfN method to screen for MRD in acute myeloid leukemia and B-lymphoblastic leukemia.

Methods: AML samples were analyzed using a clinically validated 31-color spectral flow cytometry panel. B-ALL samples were analyzed on a 21-color spectral panel. Both panels included a viability dye. All data were acquired on the Cytek Northern Lights System. Deep learning models were trained on normal bone marrow specimens. Maturational patterns were established using high-dimensional clustering results and previously-defined maturation patterns of myelomonocytic and lymphoid maturation. A hematopathologist-informed abnormality scoring system for DfN was developed and integrated with the models. A reporting system was developed to highlight 1) the abnormality scores at each stage of maturation and 2) the most likely abnormal populations for each sample.

Results: MRD identification by the model in both AML and B-ALL samples was verified by comparison with the immunophenotypes established at the time of clinical testing. Molecular MRD results, when available, were also used. The model demonstrated sensitivity of 0.01% for a variety AML immunophenotypes, including those lacking typical LAIPs, and a sensitivity of 0.001% for B-ALL.

Conclusion: Emerging machine learning and artificial intelligence methods offer promising solutions for automating flow cytometry analysis. This screening model is a novel diagnostic aid that demonstrates high analytical sensitivity and effectively incorporates detailed hematopoietic maturational patterns. It is a valuable tool that aids a hematopathologist by providing comprehensive explanations of identified abnormal cell populations with MRD results. While many artificial intelligence analysis methods serve to follow singular abnormal immunophenotypes per patient or are "black-boxed" and do not provide explanations, this program was designed to demonstrate all suggested abnormalities for final interpretation and does not require a diagnostic immunophenotype. These programs are currently undergoing clinical validation for future integration into routine clinical practice.

HEM-25

Daratumumab: Multiple Interferences to Be Aware of in Flow Cytometry

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Introduction: Daratumumab, a therapeutic anti-CD38 IgG kappa monoclonal antibody, is increasingly used in the treatment of plasma cell myeloma but presents challenges due to its interferences in laboratory tests. Beyond the well-documented interference with CD38 detection on plasma cells, daratumumab can also cause artifacts, such as pseudo-kappa restriction on early-stage hematogones. This retrospective study analyzes patients treated with daratumumab and monitored by flow cytometry (FCM), aiming to better characterize these interferences and their duration.

Methods: We retrospectively reviewed 148 bone marrow immunophenotyping analyses performed between January 2019 and November 2022. Two groups were studied: Group D (daratumumab) included 57 samples from patients treated with standard-dose daratumumab, administered either intravenously (n = 26) or subcutaneously (n = 31); and Group C (control) comprised 91 samples from patients who had never received daratumumab. FCM was performed using a Navios cytometer (Beckman Coulter) with two antibody panels: a screening panel (CD8-FITC/kappa-FITC, CD4/lambda, CD19, CD56, CD10, CD34, CD5, CD20, CD3, CD45) and a plasma cell-specific panel (CD38, CD138, CD56, kappa, lambda, CD27, CD28, CD20, CD19, CD45). The analysis focused on light chain expression (kappa/lambda) in hematogones (CD45low, CD19+ cells) and plasma cells. The interval since the last daratumumab injection was also recorded.

Results: No light chain restriction on hematogones was found in the control group (n = 91). In Group D (n = 57), 31 analyses showed weak kappa monotypia on hematogones, 9 showed no interference, and 17 had no detectable hematogones. Interference was observed up to 162 days after the last dose (range: 2–162), regardless of route of administration. Among the 8 cases without interference, the minimum time since last dose was 158 days (range: 158–985). Regarding plasma cells, in Group D, among the 34 samples containing plasma cells, 23 showed interference: strong kappa-restricted expression (n = 13), polytypia with strong kappa expression (n = 6), and lambda-restricted expression with co-expression of kappa (n = 4). In all 23 cases, CD38 was not detected. With the screening panel, a potential overestimation of CD8+ T lymphocytes was suspected, likely due to daratumumab binding to CD38+ T lymphocytes, which are stained with the kappa-FITC reagent and thus misidentified as CD8-FITC+.

Conclusion: This study confirms several daratumumab-related interferences in immunophenotyping, lasting up to five months after treatment. Therapeutic monoclonal antibodies must be clearly indicated by clinicians, and cytometric analyses require careful review of patients' medical history and clinico-biological data to avoid diagnostic errors.

HEM-26

Usefulness of a good concordance between morphology and flow cytometry: a case of hypereosinophilia

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Introduction: we report the case of a man presenting with hypereosinophilia, the steps leading to diagnostic hypothesis, the usefulness of a shared approach involving morphology and flow cytometry.

Methods: the following analyses were performed: complete blood count, bone marrow biopsy, flow cytometry, cytogenetic and molecular tests, inflammation and autoimmunity markers, first line coagulation tests, High-Resolution Computed Tomography (HRCT), echocardiography, electrocardiogram, Bronchoalveolar lavage (BAL) analysis.

Results: patient presented complaining headache and neck pain ongoing from 3 weeks. Laboratory results documented hemoglobin of 160 g/L, platelet count of 486 x 10^9/L, leukocyte count of 58.95 x 10^9/L with eosinophils 46.57 x 10^9/L and an abnormal granulocyte scatter. Eosinophils in peripheral blood morphologically appeared atypical. Bone marrow biopsy was performed and revealed a marked granulocytic hyperplasia with dismorphic eosinophils and a small percentage of lymphoblastoid elements with vacuolated cytoplasm. Cytogenetics and molecular analysis did not reveal any anomalies of fusion genes, although the presence of cryptic and undetectable fusions is not excluded.

Other imaging and laboratory tests did not evidence any significant anomalies. The results led to hypothesize a myeloid/lymphoid syndrome with eosinophilia without organ infiltration. Treatment with prednisone was initiated, with resolution of symptoms and no changes in the blood counts. After 2 months, steroid tapering was started, and Mepolizumab was also administered, without any significant benefit. Two and a half months later the patient presented with shortness of breath, wheals, fever and asthenia. HRCT showed diffuse ground glass and consolidations. BAL analysis showed a picture of infiltration with 65% eosinophils. Abdominal CT showed a 24 cm liver and 16 cm spleen. Rheumato-immunological tests were negative. Another bone marrow examination was performed (poor sample), together with trephine biopsy. Flow cytometric analysis of BM blood was then performed and showed 60% of CD45 positive cells with an immunophenotype referable to atypical eosinophils. Moreover, the analysis showed a population, representing 7% of CD45+ events, with atypical lymphoblastic immunophenotype, in agreement with bone marrow biopsy findings. Trephine biopsy suggested a myeloid/lymphoid neoplasm with eosinophilia in blast phase, possibly associated with fusion/rearrangement of genes encoding tyrosine kinase. V(d)J clonal rearrangement was identified in IgH and TCRg. The patient developed clinical deterioration and progressive organ damage, thus was candidate to a lymphoblastic-leukemia like treatment in combination with imatinib.

Conclusions: hypereosinophilia is a complex clinical condition requiring a multidisciplinary approach. The concordance between morphological and flow cytometric data directed clinicians to a diagnostic hypothesis and subsequent treatment.

HEM-27

Sensitive Flow Cytometry - Based Peripheral Blood Screening for Pediatric Leukemia: Enhancing Early Detection and Reducing Invasive Diagnostics

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Introduction: Bone marrow evaluation remains the gold standard for leukemia diagnosis; however, it is invasive, painful, and particularly distressing for pediatric patients. A peripheral blood-based test would offer a valuable alternative for rapidly excluding leukemia, especially in children presenting with abnormal blood counts but no blasts detectable by microscopy. To address this need, we developed a multidimensional flow cytometry (FCM) assay designed to minimize unnecessary bone marrow aspirations while ensuring early and reliable detection of leukemic blasts.

Methods: Our assay comprises two pre-formulated dried-format tubes (DuraClone, Beckman Coulter Life Sciences (BECLS)), designed in collaboration with BECLS. The BTNK tube is optimized for the detection of B- and T-lymphoblastic blasts using the following markers: CD3, CD5, CD7, CD10, CD16+56, CD19, CD20, CD38 and CD45 with CD34 and CD99 as drop-in markers for enhanced blast identification. The CFU tube, originally designed for minimal residual disease detection in AML, includes: CD33, CD34, CD38, CD45, CD45RA, CD117, CD123, and HLA-DR, supplemented with drop-in markers CD10, CD99, and CD371 for refined myeloid blast identification.

Results: Using a standardized workflow for lysis, staining, and measurement using DxFlex (BECLS), LSRII (BD) or FACSymphony (BD) flow cytometers with subsequent analysis via Kaluza (BECLS) or FACSDiva (BD) software we analyzed peripheral blood samples from 2,264 pediatric patients (median age 6.35 years, range 0–17.99) without blasts visible in the microscopic analysis and therefore referred for leukemic blast detection by FCM.

Of these, 2,228 samples were FCM-negative, while 36 tested positive. Performance assessment demonstrated a sensitivity of 91.9% and a specificity of 99.9%, with two false-positive and three false-negative results. Leukemic cell percentages in positive samples ranged from 0.0024% to 4.0% (median: 0.32%). Among the 34 true-positive cases, final diagnoses included B-ALL/B-LBL (64.7%), T-ALL/T-LBL (14.7%), MPAL (2.9%), and AML/TAM/MDS-EB (17.6%). The three missed cases were later confirmed as AML or MDS-EB. Importantly, none of the 34 true-positive or three false-negative cases had detectable blasts on microscopic examination, highlighting the sensitivity of FCM in identifying minimal leukemic populations that are not morphologically apparent.

Conclusion: This study highlights the utility of our FCM panel as a frontline screening tool for pediatric blasts in peripheral blood, offering a rapid, minimally invasive alternative to bone marrow evaluation and enhancing clinical decision-making.

HEM-28

Development of a Chimeric Antigen Receptor (CAR-T) Flow Cytometry Testing External Quality Assessment (EQA) Scheme

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Introduction: Chimeric Antigen Receptor (CAR)-T therapy is becoming an important treatment for patients with certain types of haematological malignancies. Patients undergo leukapheresis to collect T cells which are then genetically engineered to express the CAR. The engineered T cells are then expanded and transfused back into the patient. Monitoring CAR-T cell expansion post infusion is useful in helping evaluate the success of the therapy and estimating systemic immune response. Testing for CAR-T cells is becoming part of the patient management process. United Kingdom National External Quality Assessment Scheme for Leucocyte Immunophenotyping (UK NEQAS LI) is developing EQA to assist laboratories.

Methods: Participants already enrolled in UK NEQAS LI schemes were surveyed to assess the need for a CAR-T EQA scheme. Based on the respondents, 6 laboratories in the United Kingdom and Republic of Ireland were selected to participate in a study. A 500ul aliquot of peripheral blood donated by a patient transfused with CAR-T cells (12 days post infusion) was shipped to each participant. Participants were required to test the sample on the date of receipt and report the percentage of T cells expressing the CAR of the total T cell population. A JotForm survey was created to receive the results from the testing, a link of which was sent to participants.

Results: 108 participants responded to the survey assessing the need for a CAR-T EQA scheme. 42 of those respondents said that they tested for CAR-T cells; 32 of those said that they would be interested in participating in an EQA scheme. Of the 6 participants selected for the study, 5 received the sample a day after dispatch and tested it the same day; 1 received the sample 3 days after dispatch and tested it then. The median percentage of T cells expressing the CAR of the total T cell population reported by participants was 8.1% (minimum value = 2.2; maximum value = 9.8). The results submitted by all the participants were as follows- 8.1, 2.2%, 7.7%, 8.2%, 8.0% and 9.8%.

Conclusions: The initial survey demonstrated that there is a need for a CAR-T EQA scheme. The results from the 6 participants demonstrated good consensus. The participant that submitted a low result received their sample later than everyone else. UK NEQAS LI has undertaken further steps to find a sustainable source of material and carried out stability studies. The intention is to expand the study to more participants.

HEM-29

An Indolent Case of gamma-delta T-cell Large Granular Lymphocytic Leukemia with Co-expression of CD56 & CD16 and STAT5B mutation

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Introduction: T-large granular lymphocytic (T-LGL) leukemia is a rare, chronic lymphoproliferative disorder characterized by the clonal expansion of cytotoxic large granular T lymphocytes. Most cases are associated with T cells expressing αβ T-cell receptors (TCRs) and a CD8-positive immunophenotype. Atypical variant expressing γδ TCR with a $CD4^-/CD8^-$ double-negative phenotype is extremely rare and remain poorly defined in the literature.; however, some reports have described shared clinical and morphological features. Here, we describe a rare case of $CD4^-/CD8^-$ γδ T-LGL leukemia with distinctive clinical and molecular characteristics, including the presence of a STAT5B mutation.

Case report: A 58-year-old woman was evaluated for persistent lymphocytosis over two years. She experienced recurrent acute pharyngitis but had neither constitutional symptoms, lymphadenopathy, nor organomegaly. Laboratory findings were a WBC count of 7,900/μL with 70.9% lymphocytes (a majority of large granular lymphocytes [23%]) and neutropenia, hemoglobin 11.3 g/dL, and platelets 204,000/ μL. Bone marrow (BM) aspirate showed 11.2% abnormal lymphocytes among total nucleated cells (51% of all lymphocytes), with characteristic morphology of large granular lymphocytes —small to intermediate in size, single nucleus, oval or irregular nuclear contours, condensed chromatin, and pale cytoplasm containing azurophilic granules. Flow cytometry of peripheral blood (PB) revealed a predominant T-cell population (56% of total cells, 74.1% of lymphocytes), expressing CD2, CD3, CD5, CD7, TCR γδ, with co-expression of CD16 and CD56, but negative for CD4 and CD8. BM biopsy demonstrated variable cellularity (<5% ~ 40%) with subtle interstitial lymphocytic infiltrates, and TIA-1 immunostaining confirmed the cytotoxic nature of the infiltrate. TCR gamma gene rearrangement revealed monoclonality and conventional karyotyping showed a normal chromosomal profile. Next-generation sequencing disclosed a likely pathogenic STAT5B mutation (c.1883C>G) and a CCND3 deletion (c.803_820del) of uncertain significance. Despite the presence of a STAT5B mutation—more often seen in aggressive αβ T-LGL leukemia—the patient remained asymptomatic without disease progression or need for treatment over a four-month follow-up period.

Conclusions: This case highlights the clinical and molecular heterogeneity of T-LGL leukemia, especially in $CD4^-/CD8^-$ γδ T-cell variants. Although STAT5B mutations are rare in this subtype, they have been associated with poorer prognosis, and targeted therapies against STAT5B alterations have been developed in typical T-LGL leukemia. The patient's indolent course and absence of autoimmune features underscore the importance of integrated immunophenotypic and molecular evaluation. Such an approach is essential for accurate diagnosis, prognostication, and management in atypical T-LGL cases.

HEM-30

Performance evaluation of the CellMek SPS Automated Sample Preparation System for Flow Cytometry

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Introduction: Flow cytometry requires meticulous manual sample preparation by skilled personnel, making the process labor-intensive and variable. The CellMek SPS (Beckman Coulter, FL, USA) is an automated sample preparation system designed to streamline and standardize flow cytometry workflows. It handles peripheral blood (PB) and bone marrow (BM) samples through automated washing, lysing, and staining, while managing reagent storage and integration with laboratory information systems (LIS) and electronic medical records (EMR).

Methods: This study evaluated the analytical performance of the CellMek SPS in comparison to conventional manual sample preparation for two key assays: lymphocyte subset analysis using PB specimens and leukemia/lymphoma immunophenotyping using BM samples. Precision, carry-over, and method comparison analyses were performed. For method comparison, 20 PB samples and 12 BM samples were analyzed. Intra-assay, inter-assay, and inter-method precision were assessed by analyzing three patient samples in triplicate over two separate days.

Results: The comparison study with manual sample preparation demonstrated high concordance. For lymphocyte subset analysis, the mean correlation coefficient (r) was 0.996 (range, 0.993–0.997), with a mean regression slope of 1.004 (range, 0.974–1.047), and intercept values ranging from –0.561 to 0.599 for CD3+ T cells, CD3+/CD4+ T cells, CD3+/CD8+ T cells, CD19+ B cells, and CD16+/CD56+ NK cells. The percent bias between results obtained using CellMek SPS and manual preparation was within ±10% for gated populations, and within acceptable limits (<±35% for rare events and <±10% for other populations) for all CD markers in leukemia/lymphoma immunophenotyping. Precision was excellent across assays, with mean intra-assay, inter-assay, and inter-method coefficients of variation (CVs) of 2.6%, 1.7%, and 1.9%, respectively, for lymphocyte subset analysis, and 1.7%, 2.3%, and 2.3%, respectively, for leukemia/lymphoma immunophenotyping. The carry-over rate was minimal, measured at –0.2%, indicating a value well below the 1.0% threshold.

Conclusion: The CellMek SPS automated sample preparation system demonstrated comparable performance to manual sample preparation for both lymphocyte subset analysis and leukemia/lymphoma immunophenotyping. The system achieved high analytical precision, minimal carry-over, and reliable results, making it well-suited for routine clinical laboratory use. It offers significant operational advantages, including reduced hands-on time and decreased risk of human error.

HEM-31

Prospective Evaluation of Alkaline Phosphatase Activity in Leukapheresis Products as an Engraftment Predictor for Multiple Myeloma and Lymphoma

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Introduction: Accurate prediction of hematopoietic transplant outcome is crucial to optimize patient outcomes and individualize treatment strategies. While $CD34^+$ cell counts are utilized routinely, they are incapable of fully defining engraftment potential, especially in poor mobilizers. In this study, our research aims prospectively to examine the application of $CD34^+$ cell counts in combination with assessing alkaline phosphatase (ALP) activity. We believe that the $APLS^+CD34^+$ cell count has great potential to predict long-term engraftment and provide a more accurate and comprehensive assessment that could help personalize transplant outcomes.

Methods: Mobilized leukopaks were recovered from two patient groups of multiple myeloma (MM), lymphoma (LY) and healthy donors (HD), that were undergoing mobilization treatment at the Blood Bank (Hospital Germans Trias i Pujol), according to ethical approval and informed consent. At the same time, analogous clinical patient information was also recovered. As per a minimal sample disturbance protocol, samples were all stained with Hoechst 33342, CD34-APC, CD45-PE-Cy7 and APLS (Alkaline Phosphatase Live Stain) in the dark for 20 minutes at room temperature. The samples were analyzed on the Attune NxT flow cytometer (Thermo Fisher).

Results: Both MM and LY patients demonstrated a significantly reduced number of $CD34^+$ cells within mobilized leukopaks (0.62 ± 0.41 in MM and 0.35 ± 0.21 in LY vs. 0.99 ± 0.47 in HD). Similarly, $APLS^{high}CD34^+$ progenitor cell counts in leukopaks were significantly lower in LY patients (13.12 ± 13.70) than in HD (42.30 ± 34.29), and in MM patients a declining trend was found. Surprisingly, patients who were treated with G-CSF + Plerixafor were seen to possess lesser $CD34^+$ counts and elevated ALP activity.

Discussion: The research presents a novel and promising technique for predicting the success of hematopoietic transplant. While $CD34^+$ cell counts are standard, they are found to be insufficient, especially in poor mobilizers, which suggests that $APLS^+CD34^+$ cell counting might yield a more precise and detailed approach to predicting long-term engraftment, potentially changing the procedure of maximizing transplant outcomes.

HEM-32

Peripheral blood flow cytometry markers to predict CMML and guide bone marrow investigation

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Introduction: Chronic myelomonocytic leukemia (CMML) remains a diagnostic challenge due to its overlap with reactive monocytosis and other myeloid neoplasms. Flow cytometric monocyte subtyping has been proposed as a screening tool, yet its practical utility remains unclear. The study aimed to identify flow cytometry markers in peripheral blood that can reliably predict CMML and guide the need for bone marrow investigation.

Methods: We prospectively analyzed 229 peripheral blood samples from patients evaluated for suspected CMML at Vilnius University Hospital Santaros Klinikos between 2019 and 2024. Flow cytometry was performed on fresh EDTA blood using CD14/CD16 for MO1 (CD14++,CD16–), MO2 (CD14++CD16+) and MO3 (CD14+,CD16++) classification, CD64 and HLA-DR (monocyte identification), and CD2, CD56 (aberrancy assessment). The final diagnosis was established by hematologists based on WHO criteria, bone marrow morphology, and molecular results. A total of 67 cases were confirmed CMML. Marker distributions were compared, and ROC curve analysis was used to identify optimal cutoffs. Logistic regression was performed to explore combinations. Ethical approval was obtained as part of Biobank research, and informed consent was secured.

Results: Among individual markers, a CD56 expression on monocytes above 13.7% showed 59.7% sensitivity and 78.3% specificity for CMML. CD2 expression above 3.0% had limited predictive value. MO3 percentage was significantly lower in CMML, but not sufficient as a standalone marker. Using combined cutoffs of MO1 ≥ 79.7%, MO2 ≤ 24.3%, MO3 ≤ 2.18%, and CD56 ≥ 4.28%, a rule-in strategy achieved 59.7% sensitivity and 85.7% specificity. No tested combination achieved sufficient sensitivity for reliable CMML rule-out. Logistic regression supported the relevance of CD56 and MO3 as the strongest individual predictors.

Conclusions: Peripheral blood flow cytometry, particularly the combination of monocyte subset distribution and aberrant CD56 expression, can support a rule-in strategy for CMML diagnosis. A defined marker profile (MO1↑, MO2↓, MO3↓, CD56↑) may reduce diagnostic delays and prioritize patients for bone marrow evaluation. However, absence of these features does not reliably exclude CMML. These findings support the use of targeted flow cytometry as a triage tool but emphasize the need for comprehensive diagnostic confirmation including bone marrow and molecular data.

HEM-33

Development of a high-sensitivity flow cytometric method to detect circulating tumor cells in monoclonal gammopathy using minimal blood volume

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Introduction: Detection of circulating tumor cells (CTCs) by flow cytometry at the time of monoclonal gammopathy diagnosis has shown prognostic significance. This study aimed to develop a simple, routine method for CTC detection and quantification, which was then applied to a 50-patient cohort.

Methods: Analyses were performed using residual volume of whole blood collected for the complete blood count on the day of monoclonal gammopathy diagnosis. Plasma cell immunophenotyping was conducted following bulk lysis, using a panel of antibodies (anti-CD45, CD38, CD138, CD19, CD28, CD56, CD81, CD200, CD27, CD117, cytoplasmic Kappa and cytoplasmic Lambda). A total of 200,000 leukocytes were analyzed, with a quantification threshold of 0.01% (Garcés, JCO, 2022). Three different permeabilization techniques, Dako (Agilent®), PerFix-HF, and PerFix-AGB (BC®), were compared using ten samples with varying plasma cell percentages. CTCs were subsequently assessed in blood samples from patients diagnosed with gammopathy. Patients were then classified into two groups: those with detectable CTCs (CTC+) and those with undetectable CTCs (CTCi). Clinical and biological characteristics of both groups were then compared.

Results: All three permeabilization techniques showed acceptable performance, though some variations in MFI was observed; most notably, PerFix tended to yield lower CD45 expression in plasma cells, complicating cytometric profile interpretation. Consequently, Dako was selected for subsequent analyses. From September to December 2024, 50 patients were analyzed: 30 (60%) with multiple myeloma, 7 (14%) with indolent myeloma and 13 (26%) with MGUS, (M/F ratio = 0.9; median age 69 years [60–75]). The median blood volume used was 308 µL [242–400]. CTCs were detectable at a 0.01% threshold in 20 patients (40%), the majority of whom (19/20, or 95%) had multiple myeloma. Hemoglobin levels were significantly lower in the CTC+ group (97 [93–108], vs 115 [106–128], $p<0.001$), while β2-microglobulin were higher (4.1 [3.5–6.2], vs 2.7 [2.2–3.8], $p<0.001$) compared to the CTCi group. Bone marrow plasma cell percentages, assessed by both morphology and flow cytometry, were also higher in the CTC+ group (41% [32–61], vs 9% [3–14], $p<0.001$ and 12% [5–32] vs 2% [1–4], $p<0.001$, respectively). No significant differences in ISS scores or Prognostic Index were observed, likely due to the limited number of patients in our study.

Conclusion: This study demonstrates the technical feasibility of implementing CTC detection by flow cytometry in a diagnostic laboratory using a limited volume of peripheral blood, while reliably maintaining a quantification threshold of 0.01%.

HEM-34

Clonality assessment of T Cells by flow cytometry

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Introduction: Diagnosis of mature T cell neoplasms is challenging. T cell receptor (TCR) Vβ antibodies were the gold standard to assess T cell clonality by flow cytometry. This approach, however, lacks sensitivity and is costly and time consuming. More recently, antibodies to TRBC1 and TRBC2, which target mutually exclusive isoforms of the constant β domain of the TCR, have been introduced as an alternative means of T cell clonality assessment. This project aims to compare TCR Vβ versus TRBC1, and TRBC1 alone versus TRBC1 combined with TRBC2 (TRBC1+2).

Methods: We present two cohorts of patients with prospectively collected data on specimens referred for clinical flow cytometry. In cohort 1, specimens were simultaneously analysed with TCR Vβ and TRBC1 antibodies. In cohort 2, specimens were simultaneously analysed with TRBC1 alone and with TRBC1+2. Suspicious populations were identified with routine markers, gated, and assessed by the Vβ and TRBC antibodies.

Results: In cohort 1, 89 specimens were analysed for clonal T cell populations. Compared to Vβ analysis, clonality assessment using TRBC1 had a sensitivity of 93% and a specificity of 91% for monoclonal T cell populations. This deceased specificity is, in part, explained by the Vβ panel's ability to better identify oligoclonal populations. In cohort 2, 149 specimens were analysed, of which 20 cases received a final diagnosis of T cell neoplasm. TRBC1 alone and TRBC1+2 detected an abnormal population in all 20 cases. 7 cases were TRBC1 positive in both assays. Of the 13 cases that were TRBC1 negative by TRBC1 alone, 10 were TRBC2 positive, and the other 3 were negative for both TRBC1 and TRBC2. TRBC1 alone reported a T cell clone of uncertain significance (T-CUS) in a further 47 samples. However in 8 of these samples, criteria for monoclonality were not satisfied in TRBC1+2 analysis.

Conclusion: There was a high degree of correlation between TCR Vβ and TRBC1 alone. The combination of TRBC1 and TRBC2 provided increased confidence in clonality reporting compared to TRBC1 alone. Furthermore, the TRBC antibodies are quicker, simpler and cheaper than the TCR Vβ panel.

HEM-35

How does flow cytometry of cerebrospinal fluid and peripheral blood help diagnose primary CNS lymphoma

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Introduction: Primary central nervous system lymphoma (PCNSL) is a rare and aggressive subtype of diffuse large B-cell lymphoma that develops in immunologically privileged areas as the central nervous system. It most commonly affects individuals over the age of 60. Rapid and accurate interpretation of flow cytometry results from cerebrospinal fluid (CSF-FCM) and peripheral blood (PB-FCM) is essential for accurate diagnosis and effective treatment. Diagnostic errors may lead to withholding therapy and worsening prognosis. This case report illustrates the role of CSF-FCM and PB-FCM in confirming the diagnosis of PCNSL.

Methods: A 69-year-old woman with suspected lymphoma underwent flow cytometric analysis of cerebrospinal fluid and peripheral blood. The goal was to detect a pathological B-cell population in the CSF and to simultaneously rule out a clonal population in peripheral blood, which would help distinguish primary from secondary CNS lymphoma (SCNSL) originating from systemic disease.

Results: CSF-FCM revealed a clonal B-cell population expressing CD19, CD20, CD22, IgM, and HLA-DR, without light chain restriction (kappa/lambda) and lacking CD23 expression. The cells demonstrated markedly increased FSC and SSC parameters, higher than those observed in T cell lymphoma. PB-FCM showed no pathological lymphoid populations. Myeloid lineages were normally represented, with preserved granularity and a low percentage of myeloblasts (<0.1%). Relative neutrophilia was noted. The immunophenotype was consistent with atypical B-cell lymphoproliferation, suggestive of PCNSL. The combined use of CSF cytology and flow cytometry significantly increases diagnostic sensitivity (73% compared to 32% for cytology alone). Bone marrow biopsy was not performed, in line with current evidence questioning its relevance in PCNSL diagnostics. The CSF sample was free of blood contamination, supporting the reliability of the result.

Conclusions: This case emphasizes the diagnostic importance of cerebrospinal fluid flow cytometry in suspected primary central nervous system lymphoma. In this patient, the test proved essential for establishing a correct diagnosis and initiating treatment. It is worth noting that untreated PCNSL carries a very poor prognosis, with a median survival of approximately 3.3 months. In contrast, standard treatment with the MATRix regimen followed by consolidation (autologous stem cell transplant or whole-brain radiotherapy) can result in a 7-year overall survival rate of around 70% in patients with good performance status. Although often overlooked, peripheral blood flow cytometry serves as a useful complementary tool in differentiating PCNSL from SCNSL—detection of the same B-cell clone in blood would indicate secondary involvement and necessitate a different diagnostic and therapeutic approach.

HEM-36

Bone marrow microenvironment and monocyte phenotype in patients with plasma cell neoplasms

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Introduction: The bone marrow microenvironment plays a pivotal role in the progression and pathogenesis of Multiple Myeloma (MM), significantly contributing to clinical manifestations such as anemia and bone lesions. Among the various cellular and molecular players shaping this environment, monocytes are particularly important, not only for sustaining inflammation but also for their role in bone destruction via differentiation into osteoclasts. Monocytes can be categorized into classical (cMo), intermediate (iMo), and non-classical (ncMo) subsets. Non-classical monocytes, corresponding to M2 macrophages, are involved in anti-inflammatory and pro-angiogenic processes, and also serve as precursors for osteoclastic cells. Additionally, the phenotype of memory B cells in the bone marrow microenvironment is believed to influence MM progression through mechanisms such as self-renewal, resistance to therapy, and potentially contributing to the origin of myeloma cells.

Methods: Multiparameter flow cytometry (MFC) was utilized to analyze 55 bone marrow samples collected from patients diagnosed with plasma cell neoplasms at onset. The analysis focused on clonality markers (kappa and lambda), expression of CD19 and CD56 on plasma cells, and quantification of post-germinal center/memory CD19+/CD27+ B lymphocytes. Monocyte subtypes were distinguished based on phenotype: cMo (CD14+CD16-), ncMo (CD16+CD14low), and iMo (intermediate phenotype).

Linear regression models were employed to examine the relationships between the percentage of plasma cells (PLs) and the aforementioned bone marrow cell populations.

Results: A significant relative reduction in classical monocytes (cMo) (p-value 0.003), along with an increase in intermediate and non-classical monocytes (iMo and ncMo) (p-values 0.034 and 0.022, respectively), was observed in parallel with higher percentages of plasma cells. There was also a correlation between a relative increase in CD19-/CD56- plasma cells (p-value 0.05) and the total plasma cell burden, though no association was identified between monocyte and plasma cell phenotypes. An increasing trend in post-germinal center/memory CD19+/CD27+ B lymphocytes was noted (p-value 0.052) when compared to plasma cell percentages.

Conclusions: Our study demonstrates a shift in monocyte phenotype within the bone marrow of patients with plasma cell neoplasms, characterized by reduced classical monocytes and increased intermediate/non-classical subsets as plasma cell involvement intensifies. While no direct association was found between monocyte and plasma cell phenotypes, a significant link was identified between CD19-/CD56- plasma cells and overall plasma cell quantity. These results suggest that monocyte alterations may influence the bone marrow microenvironment and disease progression in MM. Further research is needed to clarify the clinical implications of these findings.

HEM-37

Incidental finding of asymptomatic Hairy Cell Leukaemia in context of CLL

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Introduction: Hairy cell leukaemia (HCL) is a rare, indolent B-cell malignancy diagnosed through a combination of clinical, morphological, and molecular assessments. Flow cytometry typically confirms expression of CD123, CD11c, CD25, and CD103. Most patients are symptomatic at diagnosis with cytopenias, infections or bleeding, or symptomatic splenomegaly, while disease burden in blood is often low due to marrow fibrosis and cytopenias. (Grever *et al*, 2017).

Case Report: Diagnosed low-count CLL elsewhere in 2020, a 61-year-old man arrived at our centre for watch-and-wait management. In January 2024, the lymphocytosis doubled. Remaining counts were normal, except the persistently reduced red cell count, present since 2016 (ranging from 4.0-4.3 over the years); Hb was normal. Flow cytometry confirmed that the increased lymphocytosis was indeed due to the CLL. The absolute CLL clone count was 15.8 x 10^9/L, blood film morphology was typical for CLL. Flow additionally detected a discrete Hairy cell phenotypic clone (19^+, 20^{++}, 103^+, $11c^+$, 25^+, 123^+, 200^+) at 0.05x10^9/L, or 0.2% of WBC. The patient did not have any palpable spleen, and no suspicious hairy cells could be seen after extensive screening of the blood film. Genetics showed IG-mutated profile. No lesions or variants; including absence of BRAFV600E: possibly related to sensitivity of assay used. The lymphocyte count appeared stable; the patient asymptomatic: a marrow was deemed unnecessary. Repeat flow after 2 and 5 months showed the HCL clone persisting. 17 months later, the patient remains asymptomatic and without cytopenias. Watchful waiting continues.

Conclusion: Circulating disease <5% of WBC has been reported in 40% of a single centre HCL cohort (Cornfield *et al*, 2001) all however, were symptomatic. We speculate that detecting asymptomatic HCL may become more frequent if 13+ colour upfront B-cell panels that includes HCL-detecting antigens are used. Flow is widely used in B-NHL work-up and there is a known association of HCL with other neoplasms, including B-NHL (Au *et al,* 1998; Valvano *et al*, 2022).

To the best of our knowledge, there is no larger study on average time to treatment for incidentally diagnosed, asymptomatic patients, or whether a co-existing malignancy may influence this. Troussard *et al* (2022) state that about 10% of asymptomatic newly diagnosed HCL patients may stay on watch and wait for "several years". Whether the future will see increased identification of asymptomatic individuals with low level, long-term stable HCL-phenotypic clones, "HCL-like MBL", remains to be seen.

HEM-38

Evaluating Beads Performance on the CellMek SPS Instrument

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Introduction: CellMek SPS instrument is an automated sample preparation system for for in vitro diagnostic use in flow cytometry labs. It processes whole blood (WB) and other single-cell specimens for downstream analysis. CellMek SPS instrument's application range has expanded with onboard beads. This study is to assess bead performance on CellMek SPS with a representative sample preparation workflow.

Methods: WB samples were obtained from normal and clinical donors and processed using a stain-lyse /fix workflow with a 4-color antibody panel (CD45-FITC, CD56-RD1, CD19-ECD, and CD3- PC5). Samples were prepared on CellMek SPS and compared to a manually/TQ-Prep-prepared matched-donor using equivalent workflow and reagents. All samples were acquired on a Navios EX flow cytometer and analyzed using Kaluza software.

Results: The results demonstrate that the manual and CellMek SPS-processed samples are highly comparable in % recruitment of the positive populations and absolute counts. A comprehensive Measurement Procedure Comparison and Bias Estimation revealed minimal bias. For absolute counts, the total bias was within ±40 cells/µL for ≤ 300 cells/µL, and within ±13% for > 300 cells/µL. % positive sub-populations measurement also showed excellent agreement, with a total bias within 1.5 percentage points for populations ≤ 40% and within 2.5 percentage points for populations > 40%. A strong correlation was observed between CellMek SPS and manually prepared datasets across all % positive and Absolute counts, confirming its reliability.

Conclusion: The CellMek SPS instrument demonstrated bead sample preparation performance comparable to manual methods, delivering consistent results despite common variability

HEM-39

In Vitro Evaluation of the Anticancer Activity of Selected Phytocannabinoids in Human Lung Cancer Cells

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Introduction: Lung cancer is the second most frequently diagnosed cancer and the leading cause of cancer-related mortality worldwide. Phytocannabinoids, which are natural compounds derived from Cannabis sativa L., are the subject of intense scientific research. An increasing body of evidence suggests that these compounds have therapeutic potential in the treatment of various types of cancer.

The aim of this study was to evaluate the anticancer potential of chemicals extracted from hemp (Cannabis sativa L.). CBD (cannabidiol), CBT (cannabitriol), CBG (cannabigerol) and CBGA (cannabigerolic acid) on the A549 lung cancer cell line.

Methods: Cytotoxic effects were assessed using MTT assays and three-dimensional (3D) spheroid culture models. Apoptosis induction was analysed via Annexin V/PI staining, followed by flow cytometry.

Results: All of the cannabinoids that were tested exhibited significant, dose-dependent cytotoxicity in A549 cells, with IC_{50} values ranging from 1.62 to 3.97 µg/mL. CBD was the most effective at reducing cell viability. CBG and CBGA induced the highest levels of apoptosis at 6 µg/mL, while the remaining compounds displayed comparable pro-apoptotic effects. Necrotic cell death was generally low across all treatments; however, CBT induced necrosis in approximately 5–6% of cells. These findings were corroborated in 3D spheroid models, where CBG and CBGA further enhanced apoptosis and CBT exhibited increased necrotic activity.

Conclusions: These results highlight the therapeutic potential of CBD, CBT, CBG and CBGA in inhibiting the proliferation of lung cancer cells and inducing apoptosis. The results support the need for further preclinical studies and mechanistic investigations to evaluate the potential of phytocannabinoids as anti-cancer agents.

HEM-40

The anti-cancer mechanism of the aqueous extract from the fruit of Punica granatum L. on breast cancer cell lines.

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Introduction: This study aimed to evaluate the biological activity of aqueous pomegranate peel extract using two breast cancer cell lines: MCF-7 (luminal, ER+) and MDA-MB-231 (triple-negative, TNBC).

Methods: The study aimed to determine the effect of the extract on cell viability using the MTT method, the level of reactive oxygen species (ROS) using the DCFDA assay and whether apoptosis was induced using flow cytometry. The extract was tested at five concentrations: 12.5, 25, 50, 75 and 100 µg/ml. Concentrations of 25, 50 and 100 µg/ml were used in the apoptosis analysis.

Results: In the MCF-7 cell line, the extract exhibited a clear, concentration-dependent cytotoxic effect. The greatest decrease in viability was observed at 100 µg/ml and the IC_{50} value was approximately 98.71 µg/ml. In the case of the MDA-MB-231 line, however, the effect was much weaker and a significant reduction in viability only appeared at the highest doses. It was not possible to determine the IC_{50} in the tested range. MCF-7 cells showed a linear, statistically significant decrease in the level of reactive oxygen species with increasing extract concentration in the ROS analysis: a 32% reduction at 100 µg/ml compared to the control. In MDA-MB-231 cells, the effect was less pronounced, only becoming significant at higher concentrations. The extract induced apoptosis in both cell lines, but the effect was stronger in MCF-7 cells. The highest proportion of cells in the early phase of apoptosis was observed in this line at the lowest concentration of 25 µg/ml (62.34%). As the concentration increased, a transition to the later phase of apoptosis was observed, with up to 41% of cells in late apoptosis at 100 µg/ml. In the MDA-MB-231 cells, the proportion of apoptotic cells increased with concentration, reaching 26.94% in the early phase and 30.48% in the late phase at 100 µg/ml. No significant necrotic changes were observed in either line.

Conclusions: This study highlights that the MCF-7 breast cancer cell line is markedly more sensitive to pomegranate peel extract than the MDA-MB-231 cell line. The findings confirm the extract's strong pro-apoptotic properties, particularly in hormone receptor-positive cells.

The results suggest that the effectiveness of the extract depends not only on its concentration, but also on the characteristics and oxidative stress response of the cancer cells. Overall, the results point to pomegranate peel extract as a promising natural compound that could support conventional anti-cancer therapies.

HEM-41

Utility of Flow Cytometric Immunophenotyping and TRAP Cytochemical Staining in Distinguishing Hairy Cell Leukemia from Other Splenic B-cell Leukemias/Lymphomas

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Among B-cell neoplasms, hairy cell leukemia (HCL) and other splenic B-cell leukemias/lymphomas show significant morphologic and immunophenotypic overlap. CD103 and CD11c expression are well-established characteristics of HCL but may be present in some cases of splenic B-cell lymphomas/leukemias (SBCL), posing a diagnostic challenge. While definitive diagnosis of these entities requires tissue evaluation, our aim was to investigate which parameters testable on peripheral blood or bone marrow aspirate specimens are most useful in distinguishing HCL from SBCL with CD103 and CD11c expression.

We systematically reviewed the flow cytometric immunophenotyping (FCI) histograms for cases with CD103+CD11c+, that were diagnosed as HCL (n=37) and SBCL (n=7) based on clinical, histopathological, immunohistochemical, and/or molecular findings. FCI parameters reviewed include the expression intensity of CD19, CD20, CD22, and CD200 (compared to normal B cells), and the location of the neoplastic cell population in the monocyte gate on CD45/side-scatter plots (referred to as “monocyte gate”). Given its historical significance to HCL and ability to be performed on aspirate specimens, we also reviewed the results of TRAP cytochemical staining (performed on 23 HCL and 4 SBCL cases). Fisher’s Exact test was used for statistical analysis.

The frequency of various parameters seen in HCL and SBCL is shown in Table 1. Bright CD200 and bright CD22 were observed in 94% and 92% of HCL, respectively, significantly higher than in SBCL (17%, 43%, $p<0.05$ for both). Monocyte gate and TRAP positivity were seen more often in HCL than SBCL, although they did not reach statistical significance. Bright CD19 and bright CD20 were equally observed in more than half of HCL and SBCL cases.

We demonstrated that in distinguishing HCL from SBCL for CD103+CD11c+ cases, TRAP cytochemical staining was inferior to FCI. Thus, TRAP cytochemical staining could be obsoleted due to its difficulty and subjectivity in interpretation and logistical challenges to the laboratory. Of FCI parameters, bright CD19 and bright CD20 carried limited value in HCL and SBCL distinction. Bright CD200 and bright CD22 expression were significantly more associated with HCL than SBCL cases. Given these characteristics are not specific to HCL, additional morphological, immunohistochemical, or molecular studies of tissue specimens are required for a definitive diagnosis.

HEM-42

Minimal expression of CD300e (IREM-2) in Paroxysmal Nocturnal Hemoglobinuria (PNH) monocyte clones - A proposed novel GPI-anchored receptor in monocytes

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Introduction: CD300e (also known as IREM-2) is a cell surface immune activating receptor of the Ig-superfamily that is selectively expressed by monocytes and myeloid dendritic cells. In normal individuals it has been proved to be compatible with monocyte differentiation. The important role of CD300e in MDS progression was also revealed, which indicated that high expression of CD300e might be a favorable prognostic marker for MDS. The initial aim of this study was to assess the significance of CD300e expression in PNH monocytes.

Methods: The expression of CD300e in monocytes of patients with PNH was studied, comparing the intensity of CD300e expression between normal monocytes and pathological monocytes belonging to the PNH-clone. In 7 patients in whom the presence of a PNH clone was initially documented through CD55, CD59 and FLAER, immunophenotypic separation of normal from pathological monocytes was followed and the difference in expression of CD300e in the different monocyte populations was examined. The protocol used was CD11c(FITC) CD300e(PE) CD14(ECD) CD33(PC5.5), CD34(PC7), CD117(APC) CD123(A700) CD38(A700) CD36(PB) CD45(KO), Total monocytes were defined by CD36/CD33 gating.

Results: In 7 patients with documented presence of PNH clone, a difference in CD300e expression was observed between normal monocytes and pathological monocytes belonging to the PNH clone. In normal monocytes, an increased expression intensity of CD300e was found with an average percentage of 84.05% as well as an increased mean fluorescence intensity (MFI) with a median value of 4.1. On the contrary, in the pathological monocytes of the PNH-clone there is an absence or minimal expression of CD300e with a median percentage of 0% as well as a significantly reduced MFI with a median value of 0.3. The expression of Glycosylphosphatidylinositol-anchored proteins (GPI-AP) like CD14 and CD16 were absent in monocytes associated with the PNH as expected. The repeated finding of CD300e absence in all patients with PNH monocyte clones substantiates the identification of CD300e as a novel GPI-AP related molecule.

Conclusions: To our knowledge this is the first study that CD300e (IREM-2) is identified with negative expression in PNH monocyte clones in cases of diagnosed PNH, contrary to the clearly positive CD300e (IREM-2) expression in the normal monocytes of the same patients. Thus, clear evidence is provided that CD300e (IREM2) may be a Glycosylphosphatidylinositol-anchored protein to be included in the diagnostic assessment of monocytes in PNH.

HEM-43

Comparative persistence and phenotypic characterization of autologous vs allogeneic dual-targeting CAR-T-cells (CD19/CD22) following allo-HSCT in pediatric ALL

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Introduction: Monitoring the persistence of CAR-T-cells in peripheral blood (PB) is crucial for evaluating therapy efficacy alongside clinical data and minimal residual disease (MRD) assessment. The persistence of CAR-T-cells can be influenced by various factors, including features of the clinical protocols that involve the use of immunosuppressive and cytotoxic agents. We investigated the persistence of anti-CD19- and anti-CD22-CAR-T-cells in pediatric ALL patients under two protocols: Group1 (allogeneic CAR-T with allo-HSCT) and Group2 (autologous CAR-T) using an antibody panel developed in-house.

Methods: Group1 comprised 12 patients; Group2, nine patients. In Group1, CAR-T infusion occurred on days +5 to +6 after allo-HSCT. CAR-T cells were produced via lentiviral transduction in G-Rex®10M bioreactors from T-cells sorted by MACSQuant Tyto. Group1 patients received one infusion of donor-derived anti-CD19 and anti-CD22-CAR-T (1×10^5 CAR+ cells/kg each). Group2 patients received two infusions of autologous CAR-T: first 1×10^5 CAR+ cells/kg, and second 9×10^5 CAR+ cells/kg after 13–16 days. Persistence was monitored by flow cytometry on days 7, 14, 21, 28, 60, 90, 120, 180, and 420 after infusion using an in-house antibody panel: CD4, CD8, FITC-Labeled-CD19-Protein, PE-Labeled-Siglec-2/CD22-Protein, TIGIT, 7AAD, CD197, CD3, CD45RA, CD57. Analysis was performed only if a sufficient number of CD3+ lymphocytes (>5×10^4 cells/mL) was present in PB, which was determined using the StainExpress Immune Cell Composition Cocktail.

Results: Both CAR-T types persisted in 11/12 Group1 patients (92%) and in 6/9 Group2 patients (66%). One Group1 patient showed only anti-CD19 persistence; two Group2 patients showed only anti-CD22 persistence. Peak CAR-T persistence occurred on day 14 in both groups. Median peak counts in Group1 were: anti-CD22-CAR-T - 9×10^4 cells/mL (64% of CD3+), anti-CD19-CAR-T - 5×10^3 cells/mL (3% of CD3+). In Group2: anti-CD22 - 1×10^5 cells/mL (37% of CD3+), anti-CD19 - 1×10^4 cells/mL (1% of CD3+). A predominance of the CD8+CAR+ lymphocyte population over CD4+CAR+ was observed, and in Group1 in CAR-T-22 it was statistically significantly higher than in Group2 ($p=0.0259$). No other statistically significant differences were found between the Groups. On day 14, CD4+ and CD8+ CAR-T-cells in both Groups were mainly effector-memory T-cells (CD3+CD197–CD45RA–) and expressed the exhaustion markers TIGIT and CD57.

Conclusions: This study demonstrated that the developed panel can be used to monitor the persistence of anti-CD19- and anti-CD22-CAR-T-cells in patients' PB, which, together with clinical data, will help to assess the effectiveness of the therapy. The panel proved applicable to both treatment protocols.

HEM-44

Could IKZF1 gene polymorphism and IKAROS transcription factor increase risk of metabolic disorders in young ALL survivors after therapy?

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Introduction: Acute lymphoblastic leukaemia (ALL) is the most common systemic cancer in children. Advances in treatment protocols have improved survival but also led to late complications, including metabolic syndrome. Genetic predisposition may contribute, with the IKZF1 gene and IKAROS transcription factor considered potential factors. The study aimed to assess their role in the risk of late metabolic consequences in ALL survivors.

Material and Methods: The study included 95 patients aged 5–30 years (median 12.9) who had completed treatment for ALL at least one year earlier, and 86 healthy controls aged 4.6–29.8 years (median 15.1). Patients were treated according to either the ALL-IC 2002 or ALL-IC 2009 protocols. Both groups were tested for IKZF1 gene polymorphism and IKAROS transcription, as well as fasting glucose, insulin, and lipid profile (cholesterol, LDL, HDL, triglycerides) as indicators of metabolic syndrome. Data were analysed using EPIINFO 7.2.3.1 and Statistica 13.3. The study was approved by the Bioethics Committee of Wroclaw Medical University (KB No. 98/2019).

Results: Metabolic disorders were found in 40/68 patients and 18/43 controls. Full criteria for metabolic syndrome (three to five features) were met in five patients and two controls. Among treatment groups, metabolic abnormalities were seen in 23/48 patients treated with the ALL-IC 2002 protocol, compared to five/28 in the ALL-IC 2009 group. Metabolic syndrome was diagnosed in four patients from the 2002 protocol group but in none from the 2009 group. Elevated LDL cholesterol, as well as lower HDL, higher triglycerides, glucose, and insulin, occurred more often in the 2002 protocol group. These patients had received higher cumulative doses of glucocorticoids. Genetic analysis showed no IKZF1 polymorphisms or IKAROS transcription abnormalities in either patients or controls.

Conclusions:

1. IKZF1 polymorphism and IKAROS transcription factor were not associated with metabolic abnormalities in patients or controls.
2. Survivors of childhood ALL are at increased risk of metabolic disorders, including metabolic syndrome, after therapy.
3. The ALL-IC 2002 protocol, involving higher glucocorticoid doses, was linked to greater metabolic risk compared to the 2009 protocol.
4. The findings highlight the need for larger studies to confirm these observations.

IMM-01

BTK expression analysis by flow cytometry (FC) for diagnostic confirmation of X-linked agammaglobulinemia (XLA): a single-center experience

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Introduction: Despite the FC value for diagnostic immunophenotyping in patients with suspected XLA, systematic descriptions of its routine use are scarce. We aimed to validate FC-based BTK expression analysis as a central diagnostic approach for XLA, supplemented by B-cell subset immunophenotyping in peripheral blood (PB) and bone marrow (BM).

Methods: We studied 58 PB samples from XLA patients (diagnosed according to ESID criteria), divided as follows: Group 1- pathogenic *BTK* variants (n=40); Group 2- likely pathogenic (n=12) and variants of uncertain significance (VUS) (n=6). Controls were healthy donors (HD, n=25). BTK protein expression in monocytes ($CD45^+CD14^+$) was assessed by FC (CytoFLEX, CytExpert 2.3). The staining index (SI) was calculated as (MFI-ISO HD)/(MFI-ISO patient). B-cell precursor subpopulations in BM aspirates were gated from $CD79a^+$ cells: Pro-B ($CD19^-CD34^+CD22^+$), Pre-BI ($CD19^+CD10^+CD34^+CD20^-$), Pre-BII ($CD19^+CD10^+CD34^-CD20^{dim}$), Immature B ($CD19^+CD10^{dim}CD34^-CD20^{high}$), Mature B ($CD19^+CD34^-CD10^-CD20^+$) (PMID:16237084) in 11 patients and 20 HD. Absolute B-cell counts in PB were available for 48 patients. B-cell composition was assessed for 8 samples with >10 cells/µl using DURAClone IM B Cell (Beckman Coulter).

Results: ROC analysis using Group 1 (SI median=3.6, range 0.9-9) and HD (SI median=1.1, range 1-1.7) defined an SI threshold >1.79 (AUC=0.973; sensitivity 95%, specificity 100%). Two patients had normal expression (SI=0.93, 1.45). Applying the cutoff to Group 2 identified 94% of patients, excluding one borderline case (SI=1.7). All BM B-lymphocyte precursor populations in patients, except Pro-B, differed significantly from controls: Pre-BI (Patients: median=79.1%, range 8-95.4; HD: 12.1%, 5.7-24.4; p<0.0001), Pre-BII (Patients: 16.6%, 1.3-85; HD: 67.3%, 30.8-81.9; p=0.0002), Immature B (Patients: 2.4%, 0.2-6.8; HD: 19.4%, 8.9-41.5; p<0.0001). All patients except two (pathogenic variant (SI=0.93) and VUS (SI=3.92)) showed Pre-BI lymphocyte prevalence with arrested development. PB B-cell median was 0 cells/µl (range 0-65) (n=48). Four patients had predominantly transitional B cells ($CD19^+IgM^+CD38^+$ - median=71%, range 62-88); four others had predominantly naïve B cells (IgD+IgM+CD27- - median=58.35%, range 57-74.6).

Conclusions: Applying the SI >1.79 threshold confirmed diagnosis in 55/58 patients (95%). We aim to integrate BTK expression analysis, along with complementary immunophenotyping methods presented in this study, into routine diagnostic workflows to enhance diagnostic accuracy and support variant classification in clinical settings.

IMM-02

Extracorporal Photophoresis (ECP) and Chimeric Antigen Receptor (CAR) T-Treatment – The Influence of Immunomodulation on Cytotoxicity in Vitro

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Introduction: Chimeric Antigen Receptor (CAR) T-Cell Therapy has revolutionized treatment of patients with B-cell non-Hodgkin lymphoma (NHL). However, CAR T-treatment is associated with severe inflammatory toxicities, like cytokine release syndrome or neurotoxicity. Immunomodulatory interventions like extracorporal photopheresis (ECP) could be of use to reduce the incidence of inflammatory adverse events of CAR T. Here, we explored the effects of ECP treatment *in vitro* on T-cell proliferation, cytokine production and cytotoxicity.

Methods: We performed a series of *in vitro* experiments using mononuclear cells (MNC) of peripheral blood (PB) from N=11 patients undergoing treatment with anti-CD19 CAR T-cells for B-NHL (tisagenlecleucel and axicabtagene-ciloleucel). PBMNC samples were collected 12-14 days and 30-34 days after reinfusion of CAR T cells. The samples were diluted with NaCl to a hematocrit of 2 % in ECP treatment bags (ECP+). Following the addition of 340 ng/ml 8-MOP, an exposure to ultraviolet A light at 2 J/cm^2 (Macogenic G2 device) was performed. Untreated aliquots served as controls (ECP-). PBMNC were further isolated using density gradient centrifugation and incubated in cell culture medium in 48-well plates for 48 hours (37°C / 5 % CO_2). ECP+ and ECP- PBMNC were evaluated for T-cell proliferation by *STAT5*-phosphorylation assay; cytokine production of CCL2, IFN-gamma, IL-6, TNF-alpha, IL-1alpha, IL-1beta, anti-inflammatory cytokines IL-10, growth factors IL-2, IL-15, GM-CSF, IFN-alpha2, IFN-beta per Human XL Cytokine Luminex Assay and *in vitro* cytotoxicity against CD19+ Raji cells.

Results: The overall proliferation rate of T cells on days 12-14 after the treatment with CAR T cells was lower in the ECP+ group (median: 8.36%, [-2.57-39.89%]) than in the ECP- group (median: 23.93% [2.23-66.51%]; p=0.13). We observed a trend towards lower production of both pro- and anti-inflammatory cytokines in ECP+ samples at both time-points, especially at days 33-34 after CAR T-cell infusion. However, we detected no difference in *in vitro* cytotoxicity against CD19+ Raji cells between the ECP+ (median: 14.10% [-28.40-55.70%]) and ECP- groups (median: 19.20% [-25.80-64.80%]; p=0.43).

Discussion: Our experiments indicate that treatment with ECP may reduce T-cell proliferation and cytokine production without affecting cytotoxicity *in vitro* using a discontinued system. Our data suggest that ECP could be an alternative for treatment of inflammatory complications after CAR T-treatment. Both validation data in continuous commercial systems and clinical implementation through controlled trials are needed to test this hypothesis.

IMM-03

Sex Hormone-Mediated Modulation of Immune Checkpoints in Pregnancy and Recurrent Pregnancy Loss

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Introduction: Sex hormones (SH) are known to influence immune responses, particularly in the context of autoimmune disorders and vaccine efficacy. However, their role in modulating the expression of immune checkpoints (ICPs) remains insufficiently understood. Current literature primarily focuses on the indirect effects of SH in the context of cancer immunotherapy involving ICP blockade. Our previous findings demonstrated differential ICP expression patterns in immune cells of pregnant women and those experiencing recurrent pregnancy loss (RPL). Given that successful implantation and maintenance of pregnancy rely on finely tuned immune and hormonal interactions, the present study investigates the impact of SH on the expression of ICPs on T, NK, and NKT cells from pregnant women and patients with RPL.

Materials and Methods: The study cohort consisted of 20 women with physiological pregnancies (11th–13th gestational week) and 20 women diagnosed with RPL. Peripheral blood samples (10 ml) were collected, and peripheral blood mononuclear cells (PBMCs) were isolated and cultured for 48 hours with progesterone (250 and 500 ng/ml), β-estradiol (250 and 750 pg/ml), or dihydrotestosterone (DHT; 250 and 500 pg/ml). Following incubation, the expression of ICPs (PD-1, TIM-3, LAG-3, TIGIT, and VISTA) on T cell subsets (Th, Tc, Treg, NKT) and NK cells was assessed via flow cytometry.

Results: Progesterone and β-estradiol significantly upregulated TIM-3 and PD-1 expression on Th and Tc cells. DHT similarly increased PD-1 expression but led to a pronounced reduction in TIGIT expression, particularly in NKT cells from RPL patients. All hormones enhanced VISTA expression on cytotoxic T cells, with DHT further increasing VISTA levels on Th cells. Progesterone also elevated the proportion of VISTA-positive NK cells.DHT consistently downregulated TIGIT across all lymphocyte subsets. A comparable suppressive effect was noted for TIM-3 on NK cells. DHT also significantly increased LAG-3 expression on T cells and Tregs. In contrast, TIM-3 expression in lymphocytes from pregnant women remained unchanged post-stimulation. Nevertheless, LAG-3 and VISTA were upregulated in both T and NK cells across all hormone conditions and concentrations.

Conclusion: Sex hormones exert distinct and cell-specific regulatory effects on ICP expression in immune cells of pregnant women and those with RPL. These findings offer novel insights into the immunomodulatory role of SH and suggest potential therapeutic applications involving hormonal or ICP-targeted strategies in the management of recurrent pregnancy loss.

IMM-04

Flow Cytometry Application for Early Diagnosis of X-Linked Hyper-IgM Syndrome (X-HIGM) in Pediatric Patients

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X-HIGM is a rare inborn error of immunity caused by pathogenic variants of *CD40LG* gene. This results in defective CD40L protein expression and function, leading to profound impairments in humoral and cellular immunity. This study evaluates flow cytometry (FC) as a rapid and precise complementary tool for early X-HIGM diagnosis in pediatric patients through CD40L expression analysis and extended immunophenotyping of T and B-cells.

The study was conducted in our Center from 2020 to 2025 and included 9 patients with impaired CD40L expression and retrospectively confirmed X-HIGM by genetic tests and clinical manifestations. Intra-laboratory reference intervals for CD40L expression were obtained from blood samples of healthy individuals (n=57). Induced CD40L expression following phorbol-12-myristate-13-acetate and ionomycin stimulation was assessed by FC (CytoFlex, Beckman Coulter) and reported as percentage of CD40L-positive CD3+CD8- cells (median with range). Peripheral blood B and T cells maturation patterns were assessed by FC using DURAClone tubes, and compared with those of healthy children in the corresponded age group. Statistical analysis was performed by GraphPad PRSIM 8.0.1.

Five of the identified *CD40LG* variants were previously described as pathogenic, while 4 novel variants were classified as likely pathogenic. Healthy donors demonstrated the median CD40L expression of 90.6% (81.7-94.4), while all X-HIGM patients showed severely reduced expression (0.1%, 0-0.8%; $p<0.0001$). One exception was observed: a sample with a likely pathogenic *CD40LG* variant (*c.375T>A, hemi*) exhibited a normal percentage of CD40L-expressing cells (92.5%), but with a 10-fold lower median fluorescence intensity (MFI) compared to healthy controls. Analysis of B cells maturation pattern revealed a significant shift towards the immature phenotype: the key deviations were detected in percentage of naive (IgD+ IgM+ CD27-) B cells (z-score=3.4 (1.4-3.6)), and, accordingly, a decrease in switched (IgD- IgM- CD27+) B memory cells (z-score=-2.8 (-3.6 - -2.0)). Similar trend was observed in T cells: among both CD4+ and CD8+ subpopulations, an increased percentage of naive (CD197+CD45RA+) T cells (z-score=1.24) in combination with a gradient decrease in memory subpopulations (central, effector and TEMRA median z-score was respectively -0.71, -0.90 and -0.96). Noteworthy, sample with the novel *c.375T>A* variant did not demonstrate significant deviations in T and B-lymphocytes maturation.

FC evaluation of induced CD40L expression provides rapid, reliable X-HIGM diagnosis method and facilitates the interpretation of uncertain *CD40LG* variants. This validates comprehensive immunophenotypic and functional approach as an essential component of modern diagnostics for patients with suspected inborn errors of immunity, particularly in atypical or diagnostically challenging cases.

IMM-05

The ability of the flow cytometric test to detect platelet-activating HIT antibodies

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Introduction: Heparin-induced thrombocytopenia (HIT) is a potentially life-threatening complication of heparin therapy, caused by antibodies targeting platelet factor 4 (PF4)/heparin complexes. Screening tests such as PF4-dependent enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay are highly sensitive, enabling reliable exclusion of HIT when negative. However, positive results must be confirmed with functional platelet activation assays. With this study, we aim to evaluate the ability of a flow cytometric assay to detect platelet-activating HIT antibodies in comparison with ELISA and lateral flow immunoassay.

Methods: Serum samples from two patients undergoing routine HIT diagnostics and one external HIT control were serially diluted (from 1:2 up to 1:2048). All samples were tested in parallel using a commercially available ELISA (PF4), a lateral flow immunoassay, and an in-house flow cytometric platelet activation assay. In the flow cytometric assay, patient sera were incubated with donor-derived platelets in the presence of both pharmacological and excess heparin concentrations. Platelet activation was assessed by detecting CD62P (P-selectin) expression on CD61-positive platelets.

Results: At lower dilutions, all three assays consistently detected HIT antibodies. The lateral flow immunoassay demonstrated slightly higher sensitivity than ELISA. At higher dilutions, the flow cytometric assay detected antibodies in agreement with ELISA.

Conclusions: Preliminary results suggest that the flow cytometric platelet activation assay can detect platelet-activating HIT antibodies at dilutions comparable to those detected by ELISA and lateral flow immunoassay in the tested samples. Although this suggests comparable analytical sensitivity under controlled conditions, further testing on a larger cohort is required for more precise conclusions.

IMM-06

Flow cytometry-based analysis of Water Buffalo colostrum and milk EVs: phenotypes, uptake, and immunomodulatory effects on macrophages

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Introduction: Extracellular vesicles (EVs) have garnered attention for their ability to transport bioactive molecules and mediate intercellular communication (1). However, their qualitative and quantitative analysis in colostrum (colo) and milk is challenged by the presence of fat globules and casein micelles, which contribute to the complexity and heterogeneity of these biological fluids (2). This study aims to evaluate phenotypes and subcellular effects of colostrum and milk EVs from water buffalo, on both no ultracentrifuged whey samples and EV pellets obtained through ultracentrifugation, by conventional Flow Cytometry (FC). Acidification and EDTA treatment were employed for reducing casein micelles, a significant obstacle in EV isolation; indeed, subcellular immunomodulation effects in water buffalo macrophages were investigated.

Methods: No ultracentrifuged whey samples were analyzed post-collection, adhering to MISEV2023 guidelines. Phenotypic EV characterization is based on staining with anti-CD63, -CD81, and -CD9 mAbs. Ultracentrifuged EVs from colo and 15- and 60-day milk (dm) were counted by NTA, labelled PKH67 and then added to BoMac for uptake studies at 4 and 24 hours. Indeed, water buffaloes' PBMCs were employed to evaluate phagocytosis modulation.

Results: Preliminary experiments highlight that both colo and milk samples contain EVs with diameters ranging from ~70 nm to ~350 nm (FACSCanto acquisition) and with diameters ranging from ~85 nm to ~300 nm (Cytoflex acquisition). Colo samples are enriched in <200 nm Small EVs. Large EVs (200–350 nm) appear more abundant in 15 dm. Furthermore, colostrum samples exhibit a higher EVs content, with a greater proportion of $CD63^+$ and $CD9^+$ vesicles than those from 15- and 60-dm. Uptake experiments revealed increased internalization of colostrum EVs at both 4h and 24h in BoMac cells, promoting cell re-adhesion and highlighting major EV persistence. An increase in phagocytic activity was noted in *ex vivo* buffalo macrophages, assessed by pHrodo Green Zymosan uptake, after pretreatment with 60-dm EVs. Additionally, CD14 surface density was higher in macrophages treated with either colo or 60-dm EVs.

Conclusions: These results confirm that Conventional Flow Cytometry is suitable for rapid and affordable EV detection, size and phenotype in colo and milk samples. Tetraspanins can be employed to highlight samples heterogeneity. Finally, our preliminary data reveal 60-dm EVs as the most effective in boosting phagocytosis.

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IMM-07

Cold Agglutinin Phenomenon and Aberrant B-cell Population Reveal Waldenström Macroglobulinemia: An Integrated Diagnostic Case

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Introduction: Waldenström macroglobulinemia (WM) is a rare lymphoplasmacytic lymphoma characterized by bone marrow infiltration and monoclonal IgM production. The diagnosis often requires integration of hematologic, immunologic, and flow cytometric findings. We describe a case where a multimodal diagnostic approach—including CBC anomaly, cold agglutinin interference, free light chain analysis, peripheral blood smear, and flow cytometry—led to the final diagnosis of WM.

Methods: A 70-year-old male presented with normocytic anemia. CBC revealed a discrepancy between hemoglobin (11 g/dL) and hematocrit (16%), suggestive of red cell agglutination. After warming the sample, hematocrit corrected to 31%, supporting cold agglutinin activity. WBC was 3.59×10^9/L, with absolute neutrophils 1.52×10^9/L and lymphocytes 1.51×10^9/L. Manual smear showed RBC agglutination, abnormal lymphoid cells, and immature monocytoid cells. DAT was positive. Serum free light chain assay revealed markedly elevated kappa (12,778 mg/L) with suppressed lambda (5.82 mg/L). Flow cytometry was performed on peripheral blood and bone marrow.

Results: Peripheral blood FACS identified a monoclonal kappa-restricted B-cell population constituting 84% of total leukocytes. Immunophenotype: CD19+, CD20+, CD5−, CD38−, CD10−, CD23+, CD200+, CD79b+, CD43−, CD22+, CD71+ (partial), CD11c+. Additionally, 0.88% monoclonal plasma cells were detected: intracellular cy-kappa+, CD38+, CD19+, CD27+, CD200+, CD48+, CD138+. Similar findings were confirmed in the bone marrow, with 76.8% pathological B cells and ~1% plasma cells.The findings supported a diagnosis of lymphoplasmacytic lymphoma. Considering the marked IgM paraprotein (by implication), cold agglutinin activity, light chain excess, and immunophenotype, Waldenström macroglobulinemia was diagnosed.

Conclusions: This case highlights the power of integrated diagnostics—including flow cytometry, manual microscopy, cold agglutinin recognition, and serum free light chains—for accurate identification of rare hematologic neoplasms. Early suspicion and appropriate sample handling were key to revealing cold agglutinin-mediated artifacts and guiding further workup toward a conclusive diagnosis of Waldenström macroglobulinemia.

IMM-08

Diagnostic Challenges in Severe Combined Immunodeficiency (SCID): Flow Cytometry Study of patients with Typical and Atypical Phenotypes

Elvira Musaeva [1], Viktoria Vedmedskaia [1], Tatiana Sozonova [1], Oyuna Lodoeva [1], Mariia Fadeeva [1], Ekaterina Malakhova [1], Elena Kulakovskaya [1], Andrey Russiyanov [1], Elizaveta Samykina [1], Varvara Brilliantova [1], Maxim Aleksenko [1], Elena Raykina [1], Andrey Marakhonov [2], Natalia Kuzmenko [1], Anna Mukhina [1], Yulia Rodina [1], Michael Maschan [1], Anna Shcherbina [1], Dmitry Pershin [1]

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Introduction: SCID is a group of genetically determined disorders, the main characteristic is the absence of autologous T-lymphocytes. However, in some cases, T-cells can be detected by FC ranging from low to values within the age-related reference range. This study retrospectively analyzes data from a large cohort of patients with genetically confirmed SCID to inform and improve diagnostic laboratory algorithms, with particular emphasis on atypical forms.

Methods: The study included 58 samples from patients with SCID diagnosis, for which immunological evaluation was performed at the Dmitry Rogachev National Research Center by FC using DURAclone BASIC/T tubes (Beckman Coulter). Genetic variants in the following genes *IL2RG*(n-27), *DCLRE1C*(n-11), *RAG1*(n-5), *ADA*(n-5), *JAK3*(n-3), *RAG2*(n-2), *AK2*(n-2), *IL7RA*(n-2), *CD3E*(n-1) were detected. Transplacental maternal lymphocytes engraftment (TME) was assessed by PCR if T-cell count >50 cells/µl. The cohort was divided into typical and atypical (Omenn syndrome included) groups based on PIDTC 2022 SCID Definitions. The typical group included patients with T^- phenotype (n-41, 70.7%) and T^+ with TME detected (T^+TME^+, n-9, 15.5%), the atypical group included patients' samples with T+ phenotype and TME excluded (T^+TME^-, n-8, 13.8%). T^+TME^- group included genetic variants in *IL2RG*(n-3), *RAG1*(n-3), *ADA*(n-2).

Results: Median absolute T-cell count in T^- group was 4 cells/µl (range 0-49), T^+TME^+ – 281 cells/µl (54-10500), T^+TME^- – 316 cells/µl (84-4130). CD4:CD8 ratio exhibited high variation and was more pronounced in T^+TME^+(median 1,5; range 0.1-60) and T^+TME^- (6; 0.1-37) compared to T^- group (0.8; 0,1-14). Naive T-cells (CD45RA+CD197+) were not detected in any T^- and T^+TME^+ patients. In T^+TME^- group, Naive T-cells were detected in 2 patients (11% and 26% in CD4+; 74% and 66% in CD8+) with genetic variants in *IL2RG* and *ADA*. Exhaustion marker CD279 (PD-1) expression was high in all patients: 95% (40-100%) CD4+ and 90% (44-99%) CD8+ in the T^+TME^+group, 67% (31-97%) CD4+ and 39% (22-71%) CD8+ in the T^+TME^- group.

Conclusions: The diagnosis of SCID remains challenging: T-cell counts can be elevated in some patients, potentially masking the underlying immunodeficiency, but detailed FC evaluation can reveal abnormalities consistent with SCID diagnosis. In our study, a T^+ phenotype was observed in both typical (TME^+) and atypical groups, with the latter showing detectable Naive subsets in rare cases. However, low Naive CD4+, deviated CD4:CD8 ratio, and elevated PD-1 expression were consistent findings. Therefore, comprehensive FC analysis is essential for timely SCID diagnosis and early HSCT, particularly in atypical cases, as clinical presentation may be delayed.

IMM-09

Improved In Vitro Expansion of IL-12/15/18 Induced Memory-Like NK Cells and their Immunophenotypic Landscape

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Natural killer (NK) cells are fundamental components of the innate immune system, essential for defending the host against virus-infected cells and for tumor surveillance. *In vitro*, exposure to interleukins IL-12/15/18 can induce a memory-like phenotype in NK cells (Cytokine-Induced Memory-Like, CIML), characterized by increased survival and enhanced cytotoxicity. Circulating NK cells from healthy donors (n=15) were stimulated in vitro with IL-12/15/18 to generate memory-like NK cells, or with IL-15 alone as controls. Both populations were expanded for 7 days. Phenotypes were assessed at Day 0 (pre-stimulation), Day 1 (16 h post-stimulation), and Day 7 (post-expansion). CIML NK cell function was evaluated by measuring intracellular IFN-γ, TNF-α, and CD107a expression (degranulation) in response to K562 and 721.221 tumor cell lines.

Peripheral blood NK cells were isolated with 91.81% ± 5.70% purity and cultured with IL-12, IL-15, and IL-18, resulting in 97.50% ± 1.43% purity and a cell density of 9.9×10^5, compared to 91.80% ± 3.71% and 6.08×10^5 in controls at day 7 ($p=0.0103$). Compared to controls, CIML NK cells showed a shift towards the $CD56^{bright}$ phenotype ($p<0.0001$), along with decreased expression of LAG-3, TIGIT, and TIM-3 ($p<0.01$), while PD-1 remained elevated ($p<0.05$). Expression of activating receptors NKp30, NKp46, and NKG2D was significantly higher ($p<0.001$). A greater proportion of CD25+ CD69+ NKG2D+ NKp30+ NKp46+ cells was observed in CIML NK cells ($p<0.0001$), indicating higher activation. Dimensionality reduction confirmed enrichment of $CD56^{bright}$ NK cells with low inhibitory receptor expression. Functionally, CIML NK cells exhibited increased degranulation (CD107+) and cytokine production (IFN-γ, TNF-α) in response to K562 and 721.221 leukemia cells ($p<0.0001$).

This study presents an optimized, accessible, and minimally invasive protocol for generating and expanding CIML NK cells from peripheral blood, yielding a highly purified and functional population. It also provides novel insights into their immunophenotypic profile, revealing distinct patterns of inhibitory and activating receptor expression linked to enhanced effector function. These findings highlight the therapeutic potential of CIML NK cells in antitumor immunotherapy and underscore the value of detailed phenotypic characterization for identifying highly cytotoxic subpopulations—crucial for developing more effective and targeted strategies.

IMM-10

Metabolic and inflammatory stimuli drive divergent monocyte responses in diabetic bone marrow: focus on CX3CR1 and SLAN monocyte subsets

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Introduction: Type 2 diabetes (T2D) is associated with chronic low-grade inflammation and dysregulated innate immunity, yet little is known about how the bone marrow reflects these immune alterations.
Non-classical $CD16^+$ monocytes are known to be associated with inflammatory states and chronic immune activation. Among these, $SLAN^+CD16^+HLA\text{-}DR^+$ cells represent a distinct pro-inflammatory subset with monocitic-like features. CX3CR1, a key chemokine receptor involved in monocyte adhesion, migration and in ischemic conditions, is highly expressed in non-classical monocytes and could play a role in recruitment into inflamed tissues. In this study, we explored specific expression of bone marrow-derived monocyte subsets (BMMCs), namely $SLAN^+CD16^+HLA\text{-}DR^+$ and $CX3CR1^+SLAN^+CD16^+HLA\text{-}DR^+$ populations, in diabetic foot.

Methods: BMMC were obtained from 8 T2D patients (D, age 66±4) and 4 non-diabetic individuals (C, age 72±9) and were processed to isolate mononuclear cells. Samples were cultured for 8 days under various conditions: no treatment (NT8), high glucose concentration (30 mM), TNF-α (10ng/ml), L-lactate (20mM), or LPS (100ug/ml). Flow cytometry was performed using a multi-parameter panel including SLAN (APC-A), CD16 (PE-Cy7-A), HLA-DR (V450-A), and CX3CR1 (PE-A). Gating strategies were applied to identify $SLAN^+CD16^+HLA\text{-}DR^+$ monocytes and their $CX3CR1^+$ subsets. Frequencies were compared between conditions and between groups.

Results: $SLAN^+CD16^+HLA\text{-}DR^+$ cells were more abundant in D across all treatments compared to C. Notably, D showed strong increases under glucose (p-value=0,0301) lactate (p-value=0,0157) and LPS (p-value=0,0332) exposure, when compared to C.
At baseline, $CX3CR1^+SLAN^+CD16^+HLA\text{-}DR^+$ BMMCs were more expressed in C than in D (p-value=0,0057). C cells exhibited a sharp decrease upon all stimulations, including complete loss of expression after lactate. In contrast, D BMMCs showed increase CX3CR1 expression in response to lactate (p-value=0,0342) and TNF-α, but decrease levels under LPS (p-value=0,0401).

Conclusions: These findings highlight a disease-dependent inflammatory shift in BMMCs, particularly under metabolic stress resembling ischemic conditions.
The increased abundance of $SLAN^+CD16^+HLA\text{-}DR^+$ cells in diabetics and their heightened responsiveness to metabolic (lactate) and inflammatory (LPS) stimuli suggest an altered myeloid landscape in diabetic bone marrow.
Interestingly, the expression of CX3CR1was strongly upregulated in diabetic patients compared to controls under condition mimicking tissue ischemia, such as high-lactate exposure.
Moreover, in diabetics, migration could be driven by inflammatory cues, such as TNF-α exposure.

The data support CX3CR1 as a potential marker of monocyte dysregulation in T2D and warrant further investigation into therapeutic modulation of this axis.

IMM-11

Detection of Rare and Low-expressing CAR-T cells with BD® 1-step CAR Detection Reagent

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Introduction: Flow cytometric identification and analysis of CAR-expressing cells is applied in many stages of early drug discovery and translational research. However, surface CAR protein expression can vary widely, making accurate identification and flow cytometric analysis of CAR+ cells difficult.

Methods: BD® has developed 1-step reagents for resolving CAR-expressing cells using flow cytometry that are compatible with multiple fluorochrome options to accommodate varying panel needs and instrument constraints.

Results: here, we show that our CAR Detection Reagents specifically stain CAR-expressing CHO, Jurkat CAR-T, and primary human CAR-T cells. In addition to *in vitro* cell culture settings, our CAR Detection Reagents performed well on lysed whole blood and in simple multi-color panels and showed compatibility with intracellular staining workflows. CAR-T cells added to donor PBMC were detected by this reagent at <0.1% proportions, showing potential value in research studies on CAR-T persistence. Importantly, background staining was minimal on CAR-negative blood cell populations, and CAR^{lo} cells could be resolved from negative cells.

Conclusions: overall, our data indicate that these new reagents address the challenges of CAR+ cell detection and will empower CAR cell research.

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IMM-12

Downregulation of pan-T-cell antigen CD7 in children during the acute phase of EBV infection: Preliminary findings

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Introduction: Downregulation of pan–T-cell antigens (CD2,CD3,CD5,CD7) is a criterion in the immunophenotypic diagnosis of T-cell lymphoproliferative disorders. However, aberrant expression of these markers has also been reported in infections, including infectious mononucleosis. Despite the efficiency of flow cytometry (FC) analysis to identify aberrant antigen expression, limited data exist regarding the immunophenotypic features in the course of lymphotropic viral infections, such as Epstein–Barr virus (EBV). Hence, this preliminary study aimed to investigate the immunophenotypic features of peripheral blood lymphocytes in children with acute versus past EBV infection, focusing on the expression of pan-T-cell antigens CD5 and CD7.

Methods: In the study we included 13 children with serologically confirmed acute EBV infection (EBV-IgM+), that were hospitalized at our department for diagnostic evaluation of lymphoproliferation, transaminitis and/or cytopenias, during the previous two years. In all patients we performed a comprehensive immunophenotypic analysis of peripheral lymphocytes, focusing on T-cell subsets and the expression of pan-T-cell markers CD5/CD7. In 8 patients, who presented with lymphoproliferation, we also evaluated T-cell clonality by anti-TRBC1 antibody-based FC analysis. As a comparison group, we studied the immunophenotypic profile of 5 children with past EBV infection, that were referred to our department due to persistent lymphoproliferation and/or immune dysregulation, with or without persistent EBV-IgM positivity.

Results: Compared to age-matched reference values, all patients with acute EBV infection displayed reduced decreased CD4+/CD8+T-cell ratio (0.29±0.21), and elevated HLADR+CD3+T-cells (64.0%±20.3%), predominantly CD8+ (78.8%±18.0%). A pronounced shift from naive CD45RA+CD45RO–CD27+CCR7+CD8+T-cells (7.92%±7.38%) to effector memory CD45RA-CD45RO+CD27-CCR7-CD8+T-cells (T_{EM}:77.0%±11.2%) was observed. While all T-cell subsets retained normal CD5 expression, CD7 expression was markedly decreased in total CD3+T-cells (24.6%±18.2%), especially in the CD8+ subpopulation (16.1%±13.3%). No atypical lymphoid cells or monoclonal T-cell populations were detected (TRBC1+ out of: αβCD3+T-cells=35.3%±7.7%; αβCD4+T-cells=47.0%±6.8%; αβCD8+T-cells=38.6%±7.2%).

In children with past EBV infection, we observed:

a) normalization of CD4+/CD8+T-cell ratio (1.66±0.27; p<0.001), and HLADR+CD3+T-cell frequencies (2.74%±0.77%; p<0.001), as well as restoration of CD7 expression on all T-cell subsets (CD7+CD3+:70.7%±9.45%; p=0.008; CD7+CD4+:87.8%±2.8%; p<0.001; CD7+CD8+:85.5%±6.95%; p=0.007);

b) restoration of naive CD8+T-cells (47.0%±13.65%;p=0.004) and CD8+T_{EM} (28.96%±14.75%;p<0.001), with expansion of terminal effector memory CD45RA+CD45RO+CD27-CCR7-CD8+T-cells (T_{EMRA}: 11.26%±4.09% vs 2.03%±1.52%; p<0.001).

Conclusions: Acute EBV infection in children is a benign condition associated with transient downregulation of pan-T-cell antigen CD7, particularly in CD8+T-cells. Prospective immunophenotyping of children with history of EBV infection may provide valuable insights into the immunopathogenesis of its rare progression to persistent benign or malignant EBV-associated lymphoproliferative disease.

IMM-13

T follicular lymphocytes enhance B-lymphocytes' activation in patients with systemic lupus erythematosus

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Introduction: SystemicLupus Erythematosus (SLE) is characterized by activation of the adaptive immune system. T follicular helper (TFH) lymphocytes specialize in the activation and differentiation of high-affinity B cells within germinal centers. As their role in the periphery remains unclear, the aim of this study is to investigate the relationship between TFH cells and peripheral B cells in patients with SLE.

Methods: In 63 patients with SLE and 48 healthy controls, subpopulations of B and TFH lymphocytes in blood serum were identified using flow cytometry, based on surface marker expression:

B cells: IgD+CD27-, IgD+CD27+, IgD-CD27+, IgD-CD27-

TFH cells (CD4+CD45RA-CXCR5+): TFH1 (CXCR3+CCR6-), TFH2 (CXCR3-CCR6-), TFH17 (CXCR3-CCR6+), TFR (CD25+CD127-FOXP3+), and ICOS+ TFH

Results: In the patient group (Males/Females = 5/58, Age = 39±13.5 years, lupus nephritis = 50/63), reductions were observed in total lymphocytes [1537 (871) vs. 2000 (1000)/μL, p<0.001], B cells [78.5 (144) vs. 186 (100)/μL, p<0.001], and their subpopulations:
IgD+CD27-: 39 (101) vs. 107 (76)/μL, p<0.001

IgD+CD27+: 4.3 (9.1) vs. 17 (27)/μL, p<0.001

IgD-CD27+: 15.7 (18) vs. 26 (44)/μL, p<0.001

IgD-CD27-: 10.7 (20) vs. 19 (23)/μL, p=0.003
compared to controls.

Additionally, patients with SLE showed a proportional predominance of TFH1 and ICOS+ TFH cells (p=0.005 and p=0.02, respectively).
Disease activity was assessed using the **SLEDAI-2K** score; 32 patients were classified as having active disease and 31 as inactive. **Only in inactive patients** (SLEDAI-2K ≤ 4) were correlations observed between TFH cells and CD19, IgD+CD27-, IgD+CD27+, IgD-CD27+, and IgD-CD27- cells (r=0.6, p=0.04; r=0.6, p=0.04; r=0.6, p=0.02; r=0.7, p=0.003, respectively).
Furthermore, **IgD-CD27+ cells** were associated with TFH1, TFH2, and TFH17 cells (r=0.7, p=0.01; r=0.7, p=0.01; r=0.6, p=0.02, respectively).

Conclusions: Immunological disturbances are evident even in patients with inactive SLE, involving redistribution of B-cell subpopulations as well as interactions with peripheral TFH cells, which may act as a "memory" compartment sustaining B-cell activity.

IMM-14

The potential protective role of peripheral T follicular helper (Tfh) cells in Systemic Lupus Erythematosus (SLE) myocardial involvement

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Introduction: Myocardial involvement in Systemic Lupus Erythematosus (SLE) is likely a consequence of immune-mediated mechanisms. This study evaluated the effect of peripheral T follicular helper (TFH) lymphocytes on myocardial involvement in SLE.

Methods: End-diastolic (EDV) and end-systolic (ESV) volume indexes of the left (LV) and right ventricles (RV), myocardial mass (MM), and ejection fraction (EF) were assessed using Cardiac Magnetic Resonance Imaging (MRI) in patients with SLE. At the same time, peripheral blood samples were analyzed via flow cytometry for the following TFH subtypes:TFH (CD4+CD45RA−CXCR5+), TFH1 (CXCR3+CCR6−), TFH2 (CXCR3−CCR6−), TFH17 (CXCR3−CCR6+), TFR regulatory cells (CD25+CD127−FOXP3+), and ICOS+ TFH cells.

Results: A total of 32 SLE patients were studied [Males/Females: 4/28, Age: 36±10.8 years, 25/32 with Lupus Nephritis (LN)].
Increased values were observed for: LV-EDV index: 89±21 ml/m² (normal: 78±8.7 ml/m²), RV-EDV index: 95±17 ml/m² (normal: 78±9 ml/m²), LV-ESV: 35±11 ml/m² (normal: 26±4.7 ml/m²).
Patients with LN had significantly elevated values compared to those without LN: LV-EDV index: 80.6±18 vs. 59±21 ml/m², LV-EDV: 151±50 vs. 106±27 ml, p=0.05, LV-ESV index: 31±8 vs. 23±8 ml/m², LV-MM: 95±36 vs. 74±13 ml/m², p=NS.
Compared to healthy controls, SLE patients had significantly increased levels of: TFH1: 40.3 (41.8)% vs. 4 (3.7)%, p=0.005, ICOS+ TFH: 0.2 (0.5)% vs. 0.1 (0.3)%, p=0.03, TFH1/TFR ratio: 20.3 (120) vs. 4.5 (4.8), p=0.02, TFH1/TFH2 ratio: 1 (0.8) vs. 1 (0.2), p=0.008.
Significant negative correlations were observed between: CD4+ cells and LV-EDV, LV-ESV index, LV-ESV: r = -0.7, p = 0.005; r = -0.7, p = 0.005; r = -0.6, p = 0.03, respectively, LV-MM: r = -0.6, p = 0.04, TFH1, TFH2, and TFR with LV-EDV: r = -0.6, p = 0.02; r = -0.5, p = 0.03; r = -0.6, p = 0.05, respectively, and with LV-ESV: r = -0.6, p = 0.04; r = -0.6, p = 0.03; r = -0.6, p = 0.03, respectively.

Conclusions: Cardiac MRI can detect myocardial involvement in SLE, which appears more pronounced in cases with lupus nephritis. Peripheral CD4+ and TFH cell subpopulations may exert a protective role against the development of cardiomyopathy.

IMM-15

Development of a streamlined, miniaturized and multi-allergen basophil activation test (SMMABAT) and early evaluation in the clinical research settings

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Introduction: After demonstrating the feasibility of developing a streamlined, miniaturized and multi-allergen basophil activation test (SMMABAT) strategy, a pilot study of this technology was implemented in three different clinical centers. As the role of basophil activation tests (BAT) in food allergy testing is expected to increase, major challenges have yet to be tackled to enable its widespread use in clinical settings. To help address current limitations, a 96-well plate-based approach relying on the use of dry, room temperature and ready-to-use reagents has been developed to potentially provide ease of implementation, high throughput capabilities and depth of characterization.

Methods: Whole blood samples were collected from subjects aged 1 to 36 years (n=81) and processed within 4 hours of venipuncture with 96-well plate-based prototype reagents that enabled the realization of 87 different test conditions with less than 2 mL of whole blood (20 microliters per well). Besides negative and positive controls, samples were tested against 11 allergenic extracts using 7 concentrations (from 10000 to 0,01 ng/mL) and against 7 individual components at 1000 ng/mL.

Results: In total, 891 dose responses were generated. Significant correlations (p<0,0001) were observed between SMMABAT reactivities and the allergic (n=245) or tolerant (n=465) clinical status to each tested allergen. Considering all allergens tested (peanut, hazelnut, cashew, pistachio, almond, walnut, pecan, sesame, Brazil nut, milk and egg), most informative doses were found to be ranging from 10 ng/mL to 1000 ng/mL with a positive predictive value (PPV) of 88,3% at 10 ng/mL and a negative predictive value (NPV) of 87 % at 1000 ng/mL. In comparison, on a subset of 162 allergic and 89 tolerant statuses for which titers were available, specific IgE (sIgE) to raw extracts exhibited a PPV of 64% and an NPV of 92%. Allergic patients undergoing allergen immunotherapy exhibited significantly lower SMMABAT reactivities (p<0,0001) than allergic patients without treatment while the levels of sIgEs were not significantly different.

Conclusions: SMMABAT can be implemented in clinical research settings to complement classical sIgE testing and provide insights usually provided by more classical BAT. It may thus contribute to enhanced management of allergic patients, especially those at risk of multi-food allergies.

IMM-16

Impact of Blood Specimen age on Cell Reactivity and Possible Strategies to Implement Basophil Activation Testing in Multi-Centric Studies

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Introduction: Based on the use of whole blood, the Basophil Activation Testing (BAT) is a flow cytometry based functional approach that enables the *ex vivo* characterization of basophil reactivity against specific allergenic molecules and/or raw extracts. The main focus now revolves around standardizing and democratizing this tool. After having recently developed a simplified and streamlined approach to BAT, with dry and room temperature stable reagents and an optimized workflow, herein, we wanted to characterize and further compare two possible strategies for implementing BAT in multi-centric studies. The main question was whether it was preferable to store blood before or after sample stimulation and processing.

Methods: Fresh heparin and EDTA whole blood samples were collected and processed in parallel following two workflows: “collect, store, process & analyze” or “collect, process, store & analyze”. Storage times between 0 and 7 days at temperatures of 18-25°C or 2-8°C were considered. Basophil reactivity to increasing doses of anti-IgEs ranging from 0.01 to 100 ng/test was assessed using dry and ready-to-use BAT reagents consisting of 5 markers, including CD45 and CD294 as gating markers, CD3 to exclude CD294+ T cells, and CD203c and CD63 as activation markers.

Results: The “collect, store, process & analyze” workflow showed that blood can be kept at least 2 days at 18-25°C or 2-8°C before further processing without impact on basophil reactivity. A careful analysis of the basophil activation phenotype revealed that significant upregulation of CD203c and slight CD63 decrease occurred after 2 days of storage. The “collect, process, store & analyze” workflow demonstrated that blood can be processed and then kept at least 5 days at 18-25°C or 2-8°C before being analyzed with no significant impact on CD203c or CD63 expressions, thereby enabling the accurate characterization of basophil reactivity to increasing doses of anti-IgEs.

Conclusions: These results demonstrate that various strategies can be implemented to integrate BAT in multi-centric studies. The “collect, store, process & analyze” workflow remains a simplified logistical approach but depending on the time available between clinical centers and reference laboratories.

The “collect, process, store & analyze” workflow, made possible by the format of the new BAT, can thus constitute a workflow improvement to provide significant flexibility without impact on the level of basophil reactivity detected.

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IMM-17

Fungal infection does not impair nCD64 and mCD169 biomarkers detected by flow cytometry induced during co-infection with bacteria and viruses

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Introduction: 13,500,000 people are currently suffering from common life-threatening fungal infection, and over 1.6 million die annually of related complications. Laboratory tools to complete the diagnosis of fungal diseases appear somewhat insufficiently accurate. Recently, the input of the flow cytometry was explored to distinguish between bacterial and viral diseases. A new rapid technique, based on the expression levels of the CD64 marker at the surface of neutrophils (nCD64), and CD169 marker on monocytes (mCD169, a type I interferon-inducible receptor) has demonstrated high diagnostic accuracy. However, nothing is known about the potential of these biomarkers during fungal infection. Herein, we prospectively tested these biomarkers in the blood of patients that were diagnosed with fungal diseases.

Methods: Whole blood sample was collected in EDTA tube ≤ 3 days from the diagnosis date of fungal infection and processed with the IOTest Myeloid Activation® antibody cocktail (Beckman Coulter, Marseille, France). Results were expressed as the ratio of signal intensity detected on neutrophils (nCD64) or monocytes (mCD169) over the signals measured on lymphocytes. An index > 3.0 was considered positive, similarly to previous reports.

Results: The cohort (n=49) included 43 patients with Candida infection (including 15 caused by C. albicans and nine by C. parapsilosis), as well as five cases of aspergillosis (all due to A. fumigatus species) and one of cryptococcosis. Noteworthy, 29 patients suffered from concomitant proven or probable co-infection, 25 due to bacteria and four to viruses. 25 exhibited high level of nCD64, 13 showed elevated CD169.

Conclusions: This is the very first time nCD64 and mCD169 are evaluated on a quite large cohort of patients suffering from fungal infection. The data obtained herein in real-life conditions confirmed the previous ones that nCD64 and mCD169 are reliable biomarkers to detect bacterial and viral diseases, respectively. the main finding of this study highlights that, by contrast, fungal infections did not induce increased expression of nCD64 and mCD169, including candidiasis, aspergillosis, or cryptococcosis, representing the most common fungal infection encountered in routine practice (~70% of cases). Moreover, it also demonstrated that fungal infection does not alter the bacterial- or virus-signature observed during co-infection. Of course, this finding should encourage to discover additional biomarker(s) specific to fungal disease in order to enrich the panel. Also this study also suggests that specific search for fungal cause should be considered when patients exhibit clinical signs of infection and both nCD64 and mCD169 signals are not detected.

IMM-18

Optimization of a T, ILCs, MAIT and NK cells multicolor panel with CytoFLEX mosaic Spectral Detection Module

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The application of spectral flow cytometry to the analysis of immune cell subsets offers a powerful approach to studying the complexity of immune responses. This study presents the optimization of a spectral flow cytometry panel designed to comprehensively characterize T cells, Innate Lymphoid Cells (ILCs), Mucosal-Associated Invariant T (MAIT) cells, and Natural Killer (NK) cells and its analysis using the new CytoFLEX mosaic Spectral Detection Module, which provides spectral capabilities for our CytoFLEX flow cytometer. The panel was meticulously designed to include markers that define each of these key immune cell populations, enabling simultaneous identification of multiple subsets with minimal spectral overlap. By leveraging the unique advantages of spectral flow cytometry, which allows for the detection of a higher number of parameters in a single sample, this panel provides a detailed, multi-dimensional analysis of immune cell differentiation, activation, and function. The optimization process involved selecting appropriate fluorochromes, designing optimal antibody combinations, and ensuring precise unmixing. The enhanced resolution and sensitivity of the spectral flow cytometer enabled the detection of rare or subtle immune subsets that are difficult to identify with conventional flow cytometry techniques. This optimized panel offers significant advantages for studying the roles of T cells, ILCs, MAIT cells, and NK cells in immune responses, autoimmune diseases, cancer immunotherapy, and infectious diseases. The integration of spectral flow cytometry in immunological studies paves the way for more detailed and accurate immune profiling, ultimately contributing to a better understanding of immune regulation and therapeutic development.

IMM-19

Immune cell profiling and natural killer (NK) cell cytotoxicity assessment by flow cytometry (FC) in patients with NBAS-associated syndrome (NBASS)

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Introduction: NBASS is a clinically heterogeneous disorder featuring short stature, optic atrophy, Pelger-Huët anomaly, osteogenesis imperfecta, immunodeficiency, and liver disease. The homozygous *"Yakut"* mutation (*c.5741G>A*), common in Russia, causes milder SOPH syndrome, but its immunophenotype is unclear. This study summarizes extended immunophenotyping and NK-cell function in a genotype-stratified Russian NBASS cohort.

Methods: We performed extended immunophenotyping of 22 samples from patients with genetically confirmed NBASS (median age=9 years) and compared lymphocyte subpopulations with healthy controls (internal reference values from 151 healthy children (1 month-16 years)) using age-adjusted linear regression models. The results were presented as β coefficients and statistical significance.

The results are presented as β-coefficients reflecting the degree of deviation from controls: β>10 (severe), 7-10 (marked), 3-7 (moderate), and 0-3 (mild).Genetic subgroups: homozygous c.5741G>A (n=13) – **group1** vs compound-heterozygous other *NBAS* variants (n=9) – **group2**.Extended immunophenotyping (23 subsets) was performed using DuraClone IM Basic/T cell/B cell (Beckman Coulter, USA). The subsequent analysis is based on the percentage.NK-cell degranulation was assessed in 12 of 22 patients by flow cytometric measurement of CD107a surface expression following co-culture with K562 target cells and IL-2/PHA stimulation.

Results: All patients showed lymphopenia (β=-20.3, p<0.001) with decreased T- and B-cells, while NK-cell counts remained normal. B-cell compartment analysis revealed genotype-dependent differences: **group2** showed severe B-cell deficiency (β=-13.33, p<0.001) while **group1** displayed moderate reduction (β=-5.61, p<0.001). Detailed subset analysis identified decreased naïve B-cells in **group2** (CD19+IgD+IgM+CD27-, β=-8.3, p=0.012) and elevated transitional B-cells (CD19+IgM+CD38+, **group2** β=+3.4, **group1** β=+2.3, p=0.01) in patients.T-cell profiling uncovered significant CD4+ lymphopenia, more pronounced in **group2** (β=-25.6, p<0.001) than **group1** (β=-16.9, p<0.001). CD8+ cells were expanded only in **group2** (β=+10.7, p<0.001). Both genotypes showed elevated senescent CD57+ populations in CD4+ and CD8+ subsets β = 4, β = 10, p>0,05). Notably, PD-1 expression (CD279) was increased on CD4+ cells (**group2** β=+10, **group1** β=+5, p<0.001).NK-cell cytotoxicity remained within normal range (Me=36.7% vs >18%) for both groups (n=11). The sole exception was one compound heterozygous patient (c.2535G>T, c.969G>T) showing decrease NK activity (12.9%) that normalized after IL-2/PHA stimulation.

Conclusion: NBASS exhibits distinct B/T-cell defects with intact NK function. CD8+ expansion and CD4+ PD-1+ accumulation suggest chronic immune activation. Homozygous "Yakut" mutation correlates with milder immunophenotype, highlighting clinical heterogeneity in NBASS, warranting further study.

OTHER (OTH)

OTH-01

Optimization of a flow cytometry-based Receptor Occupancy (RO) assay for the pharmacodynamic analysis of a bispecific antibody biotherapeutic

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Introduction: Precision for Medicine (PfM) developed and validated a 15-color Receptor Occupancy (RO) assay to support a sponsor's clinical trial focusing on a first-in-class bispecific antibody targeting two immune checkpoints.

Methods: RO assays aim at quantifying the binding of biotherapeutics to their specific target on the surface of cells. RO is a combination of three basic formats: free receptor measurement, total receptor measurement, or direct assessment of bound receptor. Since the staining is at single cell level, the RO is key to determine the saturation and the half maximal effective concentration (EC50) of the drug on the target cell population.

Results: In this panel, free receptors are evaluated by using the fluorescent conjugated parental drugs while the total drug bound is detected using an anti-idiotype antibody recognizing the unconjugated drug. The present assay was optimized to accurately quantify receptor occupancy of two differentially and independently expressed immune checkpoints targeted by the bispecific drug on diverse cell populations within a defined sensitivity and linearity range. Verified transfer of the RO assay from fresh to cryopreserved mononuclear cell samples allows to test samples in batch, analyzing e.g. several visits of the same patient simultaneously during the course of the clinical study. Moreover, the use of molecules of equivalent soluble fluorochrome (MESF) beads enables the standardized quantitation of the free and total receptors on the cell surfaces.

Conclusions: This study described PfM approaches to support sponsor with novel compounds to achieve clinical trial success and, ultimately, to more quickly bring effective therapies to the market.

OTH-02

Effects on immunomodulation and atrial remodeling of Bacterial Membrane Vesicles from Escherichia coli and Klebsiella pneumoniae: Implications for Atrial Fibrillation

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Introduction: Atrial fibrillation (AF) is increasingly recognized as a condition associated with systemic and local inflammation. Intestinal dysbiosis, characterized by Gram-negative bacterial overgrowth and elevated systemic lipopolysaccharide (LPS) levels, has been linked to AF, contributing to chronic inflammation via increased gut permeability and immunity activation. Bacterial membrane vesicles (MVs) have emerged as potent modulators of host immune responses. This study investigates the biological and immunological effects of MVs derived from *Escherichia coli* and *Klebsiella pneumoniae*, bacteria associated with dysbiosis in AF.

Methods: Human macrophages, under basal and pro-inflammatory (LPS-stimulated) conditions, were incubated with MVs, isolated from *E. coli* and *K. pneumoniae* reference strains. Immunophenotyping was performed by flow cytometry, analyzing CD14, CD80, CD163, CD11b, CD172a, CD146, and CD142. Human primary atrial fibroblasts were also incubated with MVs to assess effects on proliferation, cytotoxicity (LDH release), redox signaling (NRF2 immunofluorescence), collagen deposition (Sirius Red staining), and vesicle uptake (Operetta CLS).

Results: Under basal conditions, high MVs concentrations from both species induce a strong pro-inflammatory response, comparable to LPS. In a pro-inflammatory environment, MVs display an additive effect compared to LPS at higher doses, while lower concentrations have limited impact. MV-treated macrophages show increased expression of M1 markers, especially CD80 and CD14, and decreased CD11b. In atrial fibroblasts, *E. coli* MVs significantly decrease proliferation after 6 hours without increasing LDH release, suggesting growth arrest rather than cytotoxicity. NRF2 localization differs between species, while *E. coli* MVs promote nuclear translocation, *K. pneumoniae* MVs lead to cytosolic NRF2 downregulation. The Sirius Red staining shows that *E. coli* MVs reduce collagen content with membrane-associated localization, whereas *K. pneumoniae* MVs induce perinuclear accumulation. MV uptake was time- and dose-dependent, with *E. coli* MVs internalized more efficiently.

Conclusion: MVs from *E. coli* and *K. pneumoniae* elicit distinct biological responses relevant to inflammation and atrial remodeling. These findings suggest that bacterial MVs contribute to the inflammatory and structural alterations underlying AF, emphasizing the role of human microbiome-derived signals in cardiac disease pathophysiology.

This work was supported by EU–NextGenerationEU–M4–C2–Investment 1.5, building local R&D leaders as THE–Tuscany Health Ecosystem–Prj “Microvescicole batteriche, implicazioni nella fibrillazione atriale-MICROFIBRA”–CUP: B83C22003920001–ECS00000017.

OTH-03

Beyond Doublets: Identifying Single Cells with Flow Cytometry Imaging

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Introduction: Accurate characterization of a sample by flow cytometry relies on distinguishing true single cells from non-single-cell events, such as doublets or aggregates. This distinction is typically achieved by evaluating the forward light scatter pulse's area or width versus its height. However, their distributions for single cells, doublets, and other events often overlap. This overlap raises concerns that standard gating methods may inadvertently exclude true single cells while failing to remove doublets that fall within the "singlet gate”. In this study, we used imaging flow cytometry to better characterize events conventionally gated as doublets.

Methods: Bone marrow and peripheral blood samples, the EGFP-K562 cell line, and fluorescent microbeads were analyzed. Samples were acquired using the Invitrogen™ Attune™ CytPix™ flow cytometer (Thermo Fisher) without washing steps. Automated image analysis translated event features into morphometric parameters, which were combined with standard fluorescence and scatter parameters. A standard "doublet gate" was established based on FSC, SSC and fluorescence (DNA content). Circularity versus major diameter plots were used to further characterize events within the doublet region.

Results: Three distinct populations were identified within the "doublet gate" based on circularity and major diameter: (1) events with high circularity and low diameter, were single cells (mean percentage ± SD in 10 bone marrow samples = 37.44% ± 19.59% and can be found up to 50%); (2) events with intermediate circularity and high diameter (doublets); and (3) events with low circularity and high diameter (coincident events). These findings were independent of the gating method used (light scatter or fluorescence). Microbeads were less likely to appear in the doublet gate and, when they did, less likely to be singlets. In contrast, when spiking K562 in peripheral blood samples, the single-cell K562 blasts were more likely to appear in the doublet gate, indicating that large cells are more likely to fall within the doublet compartment.

Conclusion: Accurate identification of singlets and removal of false-positive results are crucial for both flow cytometry analysis and cell sorting. The doublet compartment is heterogeneous, and standard doublet discrimination strategies can exclude single cells. Pathological, larger, activated, or proliferating cells are more likely to be misclassified as doublets, potentially leading to the underestimation or exclusion of key cell populations. Care must be put into the routine doublet gating step, and we propose defining the singlet gate in the last steps of the analysis when working with larger or activated cells of interest.

OTH-04

Reducing Operator-Dependent Variability in Immunophenotyping Through Automated Antibody Panel Preparation

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Manual antibody cocktail preparation for high-dimensional flow cytometry introduces variability and consumes valuable hands-on time, limiting reproducibility across experiments and users. In this study, we evaluated an automated workflow for antibody mastermix preparation using Curiox C-Free™ Pluto workstation, a programmable benchtop liquid handler, benchmarking its performance against traditional manual methods across 10- and 24-color panels. Whole blood samples from five to six healthy donors were stained using cocktails prepared either manually or by the automated system, and processed via both centrifuge-based and centrifuge-less sample preparation protocols.

Across all tested conditions, staining quality as measured by Stain Index (SI) was comparable or superior with automated prep. Key markers across lymphocyte and myeloid subsets showed tight clustering of fluorescence intensity, and inter-donor consistency was preserved. Importantly, coefficient of variation in replicate cocktail preps was consistently below 5%, and SI variation across donors remained within acceptable limits for immunophenotyping studies. The integration of fixed-volume scripting and real-time liquid handling validation contributed to the robustness of the process.

In addition, the automated prep significantly reduced overall turnaround time—from over 24 minutes manually to approximately 16 minutes hands-off workflow —while eliminating user variability and pipetting fatigue.

The workflow also facilitated the preparation of FMOs and single-color controls with no additional pipetting steps, and enabled reproducible scaling of panels using pre-validated CSV templates. Combined with centrifuge-free processing, this approach supports fully automated immunostaining pipelines with minimal user intervention. Our findings demonstrate that automated antibody mastermix preparation is not only feasible but advantageous for high-throughput, high-dimensional cytometry workflows where consistency, traceability, and speed are critical.

OTH-05

Impact of blood anticoagulants and stabilization on monocyte subset proportions: a comparative analysis in human, bovine, and water buffalo species

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Introduction: Monocyte subsets play a crucial role in regulating immune responses and inflammation and are used as biomarkers in human and veterinary medicine. In clinical and research settings, delayed sample processing is common and often requires stabilizing reagents. Yet, the effects of different anticoagulants and stabilizers on monocyte subset proportions remain underexplored. Standardizing pre-analytical variables, starting with anticoagulant selection, is essential to ensure accuracy and reproducibility in monocyte phenotyping across species. This study aimed to evaluate the potential negative effects of different anticoagulants and stabilizers in human, bovine (Bos taurus), and water buffalo (Bubalus bubalis) blood samples.

Methods: Peripheral blood from 4 cattle, 4 buffaloes (via the jugular vein), and 10 healthy human donors was collected into tubes containing K2EDTA, lithium heparin, and Cyto-Chex® BCT tubes (Streck). Samples were processed within 4 hours of collection, and species-specific multicolor flow cytometry panels were used to identify classical (cMo), intermediate (intMo), and non-classical (ncMo) monocytes, defined by their differential expression of CD14 and CD16. Veterinary samples were acquired using a CytoFLEX flow cytometer and analyzed with CytExpert software (Beckman Coulter), whereas human samples were acquired on a FACSLyric (BD Biosciences) and analyzed with FACS Suite™software. Data were compared using Wilcoxon test, (Jamovi software). p-values <0.05 were considered statistically significant.

Results: Significant differences were observed in all 3 monocyte subsets when comparing EDTA and heparin samples in each species, with the exception for bovine ncMo. Although the use of the stabilizer didn't result in significant differences in proportions of cMo and ncMo in buffalo and human samples, it markedly reduced CD14 and CD16 labelling, respectively. This negatively affected subset gating, thus discouraging the use of the stabilizer for monocyte characterization.

Conclusions: Although this is a preliminary study, our findings highlight the importance of anticoagulant or stabilizer choice for reliable monocyte subset analysis in veterinary species. Heparin showed the lowest variability and may be considered the anticoagulant of choice for monocyte phenotyping in cattle and buffalo. Cyto-Chex® showed negative effects in all species studied.

Further research with larger sample sizes is needed to validate these observations and to evaluate K2EDTA or heparin's suitability for delayed monocyte subset analysis, particularly when immediate processing isn't feasible. In human studies, K2EDTA remains the standard anticoagulant; however, heparin may represent a promising alternative for extended storage, requiring further investigation.

OTH-06

Nanoplastic Contamination in Common Beverages and Infant Food: The Role of Packaging

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Introduction: Nanoplastics (NPs) are a significant environmental pollutant increasingly recognized as a global health threat. NP bioaccumulation has been documented in human tissues, including blood and placenta, and in vivo studies have shown its adverse effects on the digestive, respiratory, reproductive, neurological, and cardiovascular systems. Considering ingestion as a significant entry pathway for NPs into the human organism, this research explores NP contamination in commercial waters, soft drinks, and infant formula, products with widespread consumption.

Methods: Thirteen samples, including commercial waters (n=8), infant follow-on formula (n=1), lyophilized cereal porridge (n=1), and three soft drinks in aluminum, plastic, and crystal containers, were analyzed for NP content. Sixteen samples of pyrogen-free water were included as negative controls. Lyophilized follow-on formula and cereal porridge samples underwent organic matter digestion using 1% potassium hydroxide (KOH) at 60°C in a dry block for 10 days. A 20 µL aliquot of each digested sample and soft drink was diluted in 1ml pyrogen-free water and subsequently stained with 2µL of Nile Red, achieving a final concentration of 0.2 µg/mL. The staining process was conducted for 15 minutes at room temperature in the absence of light. Commercial waters and pyrogen-free water (1 mL) were stained with Nile Red. NP accumulation was assessed in triplicate. Submicron microspheres were used for flow cytometry calibration, and all samples were analyzed using the Attune™ NxT flow cytometer. Sample preparation was conducted under controlled conditions within a Biosafety Level 2+ (BSL2+) environment.

Results: Pyrogen-free water was analyzed to establish the negative control for NP concentration, yielding 5.03 ± 2.06 events/µL. Compared to pyrogen-free water, infant formula and cereal porridge showed very low NP concentrations, with values of 10.27 ± 6.85 and 6.78 ± 2.27 events/µL, respectively, following triplicate analyses of six samples. NPs were detected at significantly elevated concentrations ($p < 0.0001$) in 5 of the 8 plastic-bottled commercial water samples analyzed in triplicate, with a range of 5.93 to 124.9 events/µL.

Conclusion: Nanoplastics, as ubiquitous contaminants, can be ingested by organisms through food and drink. Potential NP contamination in commercial water can arise from factors such as source water contamination, filtration, and packaging. The presence of very low NP concentrations in infant food suggests effective stringent quality control. Finally, NP presence in soft drinks was not affected by container type. While soft drink beverages have higher NP levels than water, container type had no effect on NP presence in soft drinks.

OTH-07

Plant-Derived Extracellular Vesicles: Isolation, Characterization, and Innovative Applications in Nanomedicine

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Extracellular vesicles (EVs) are tiny, non-living, and non-replicating membranous particles, naturally secreted by both plant and animal cells. They carry a wide range of bioactive molecules, including miRNAs, lipids, proteins, and small biomolecules, they are secreted both in physiological and pathological conditions, transport bioactive molecules with relevant functions and modulate intercellular crosstalk processes. Plants especially fruits, vegetables, and their by-products produce plant-derived extracellular vesicles (PDEVs), which have attracted significant interest for their potential uses in the fields of food, nutrition, and health.

This study aimed to isolate PDEVs derived from grapes (GDNVs) and assess their potential for therapeutic use. The GDNVs were extracted from fresh grapefruits through the processes of ultracentrifugation, tangential flow filtration (TFF) and fluorescence activated cell sorting. GDNVs were analyzed using various techniques, including nano-flow cytometry, nanoparticle tracking analysis (NTA), zeta potential measurement, atomic force microscopy (AFM), transmission electron microscopy (TEM), proteomics and raman spectroscopy (RS).

Conventional and nano-flow cytometry was used to quantify GDNVs and assess their phenotypic characteristics. EVs isolated from grape juice exhibited sizes consistent with small extracellular vesicles (< 200 nm), as confirmed by NTA, TEM and AFM. When comparing isolation methods, TFF produced a significantly higher yield approximately ten times more EVs than ultracentrifugation. After isolation, GDNVs were further analyzed using flow cytometry. Their stability was then tested by storing them at different temperatures (2°C, -20°C, and -80°C) to evaluate how temperature affects their shape and concentration. Our results indicate that GDNVs have promising qualities, including good stability across storage conditions, which supports their potential use in therapeutic delivery. Overall, this study offers valuable insights into the processes of isolating, analyzing, and storing GDNVs, as well as their future applications in medicine. Their versatility makes them valuable in drug delivery, cosmetics, and nutrition, highlighting the need for further research to unlock their full potential in healthcare