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ABSTRACT BOOK

Baltic Flow Cytometry Society

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ORAL ABSTRACTS

Session 1:
Flow Cytometry in Basic and Applied Sciences: Cell analysis

Cytomics as a Driver of Advancing Scientific Discovery

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Cytomics, the study of cellular systems at the single cell level, uses cytometry as a central tool to analyze the molecular architecture and function of cells. Cytomics has transformed biological and biomedical research by enabling multi-parametric, high-throughput analysis of single cells.

Cytomics has enabled important scientific discoveries, such as the Human Genome Project, in which chromosome sorting based on cytometry played a central role in large-scale genome sequencing. In cancer research, cytomics has improved the understanding of tumor heterogeneity and apoptosis, while in regenerative medicine it has facilitated the isolation and characterization of hematopoietic stem cells. The development of monoclonal antibodies using flow cytometry has led to a major breakthrough in diagnostics, therapy and vaccine development and has shaped the foundations of modern medicine.

Beyond biomedical science, cytomics has also had a significant impact in non-traditional fields. In marine biology, it enables the study of plankton diversity and its role in global biogeochemical cycles. In environmental sciences, cytometry is used to analyze microbial populations, monitor pollutants and assess the impact of microplastics and nanoparticles on ecosystems, providing important insights into environmental sustainability.

Recent technological advances, such as spectral and image cytometry and AI-assisted data analysis, have further expanded the scope of cytomics. By integrating genomics, proteomics and metabolomics, these innovations are enabling new breakthroughs in precision medicine, biotechnology and ecological research.

This talk will highlight the groundbreaking discoveries made possible by cytomics and emphasize its versatility and central role in addressing complex scientific challenges across multiple disciplines.

Setting-up a Flow Core facility – do we need to reinvent the wheel?

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Flow cytometry, as a research technique, has been supporting modern science for over four decades. Since its early commercial beginnings in the 1970s, the technology has come a long way. The complexity of the systems has increased over time, along with improvements in user-friendliness.

Today, the majority of systems are user-friendly devices, allowing almost anyone to operate them after a short introduction. However, it still takes an experienced cytometrist to identify errors, troubleshoot issues, and optimize the scientific process for greater efficiency and accuracy.

The best place to find someone dedicated to this specialized technique is a flow cytometry core facility, where experts deal daily with a wide variety of samples, technical issues, and challenges in sample preparation. That's just one of the reasons why flow cytometry core facilities play a crucial role in academia and beyond.

Let's discuss some of the other reasons, problems, and challenges that facility staff and managers face in our part of Europe. And most importantly—is there a shortage of flow cytometry facilities in Eastern Europe?

I'd love to invite you to this interactive talk, where we can explore these questions together and try to find some answers.

A 4 Decade Cytomic Odyssey: From Flow to Capillary then Image Cytometry, with a little passage through space… to end up in perlo™

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Introduction: Conventional anticancer therapies primarily target rapidly proliferating cancer cells but fail to prevent resistance, metastasis, and tumor relapse due to the persistence of cancer stem cells (CSCs). These highly tumorigenic cells drive metastasis and recurrence, making them key targets for complete malignancy eradication. CSCs are a major cause of chemotherapy failure and multidrug resistance.
Methods: Our in perlo™ liquid pearl culture system is unique in that the only extracellular matrix present is the one secreted by the cells themselves, eliminating the need for artificial matrices (animal-derived components, batch variability). Our human cell-line organoids, derived from various cellular phenotypes, exist in liquid suspension, enabling rapid diffusion of added molecules, whether hydrophilic, lipophilic, or protein-based.
Results: We will present findings on different human cancer cell phenotypes treated with drugs such as Gemcitabine. Healthy HaCaT keratinocytes and cancerous HeLa epithelial cells were co-cultured despite originating from distinct media conditions. The application of potent and selective anticancer molecules demonstrated the suitability of the in perlo™ system for high-content screening (HCS) in co-culture environments.
Conclusions: After 40 years of cytomic research on CSCs, the journey continues—paving the way for novel strategies in cancer treatment.

Best practices in autofluorescence extraction: Real examples from the core facility

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Introduction: Autofluorescence (AF) extraction is a key advantage in spectral flow cytometry, aimed at annotation of the enhanced background fluorescence caused by cellular components such as lipofuscin, flavins, porphyrins as well as extracellular matrix components. Analysis of complex samples, like tissues, tumors, nervous system isolates, and hematopoietic organs with varying autofluorescence patterns may be challenging. Endogenous fluorochromes emit fluorescence signals that overlap with the emission spectra of the fluorochromes used for specific labeling, leading to spectral overlap and inadequate separation of colors. Here we show examples of successful autofluorescence extraction, resulting in appropriate resolution and accurate measurements.
Methods: For the identification of alveolar macrophages and other types of immune cells in dissociated murine lung tissue we employed a 5-laser Cytek Aurora spectral flow cytometry analyzer and for sorting of myloid cells from murine brain isolates we used a spectral sorter, the 5-laser Cytek Aurora CS. Full spectrum profiling of unstained samples served as a tool for annotation and redistribution of highly autofluorescent populations hidden within a heterogeneous mixture of cells. We compared three different approaches to extract autofluorescence: 1) default unmixing in SpectroFlo® without autofluorescence extraction using the Ordinary Least Squares (OLS) algorithm; 2) default unmixing with single autofluorescence extraction; 3) unmixing with extraction of multiple autofluorescence patterns. The selected autofluorescence tags (AF tags) were then used as reference controls along with the single-stained controls. After unmixing, we compared all strategies and selected AF tags indispensable for adequate identification of immune cells and sorting of myeloid cells based on marker selection.
Results: We found that in case of complex samples with multiple autofluorescence patterns, an approach with several AF tags extraction is essential for achieving a reliable gating strategy and separation of events. Autofluorescence extraction strategy can also improve purity of cell sorting, ultimately enhancing quality of downstream applications.
Conclusions: We propose that careful autofluorescence extraction before making a final gating strategy both for analysis and sorting should be taken into account, particularly when separation of cell populations within heterogeneous samples and cell mixtures relies on dim markers or fluorochromes emitting in the areas of highest autofluorescence.

Session 2:
Translational and Clinical Flow Cytometry

Human Pressure as a Driver of Plastic Pollution: A Global Crisis Perspective

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The impact of plastics on human health is increasingly being investigated at all levels. We have developed a novel method to detect nanoplastics by flow cytometry. This technique allows the rapid and accurate identification of these particles in blood samples and other tissues and body fluids, a crucial step in understanding their accumulation in the body.

We have demonstrated the presence of nanoplastics in the blood of all individuals tested, including newborns and healthy adults. This finding indicates widespread exposure to nanoplastics and suggests that these particles can enter the bloodstream by various routes, such as inhalation or ingestion."

This talk highlights the potential health risks associated with exposure to nanoplastics as a global threat. These particles can interact with cells and tissues, which could lead to adverse effects. The adaptation of a reliable method for detecting nanoplastics will facilitate research into the long-term health consequences of this exposure. This work aims to lay the groundwork for developing strategies to mitigate the potential risks associated with plastic pollution and how humans are altering ecosystems.

Spectral flow cytometry revisited: statistical foundations, optimal panel design, and why having more channels is better… but not always

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Spectral flow cytometry, pioneered by Robinson, Rajwa, Grégori, and colleagues in the early 2000s, fundamentally changed how we interrogate complex cell populations. Its early reception was marked by skepticism, given that deconvolving broad-spectrum fluorescence signals ran counter to the conventional cytometry method of detecting narrow emission bands, where a single detector is assigned to each fluorochrome. Over time, however, the commercialization of Purdue's intellectual property, along with advancements in hardware and data analysis, has solidified spectral cytometry as a transformative tool in fields such as immunology, oncology, and more.

A fundamental facilitator of this technique is spectral unmixing, which computationally disaggregates observed photon counts into contributions from individual fluorophores. Central to this procedure is accurate noise modeling. While compensation implicitly relied on Gaussian assumptions, the Poisson or Pólya distribution, along with other overdispersion models, more effectively encapsulates variability in photon counts. These noise models inform estimate methodologies, particularly the Cramér-Rao lower bound (CRLB), which provides a fundamental statistical limit governing the precision with which fluorophore abundances can be discerned from noisy observations.

Additionally, the selection of fluorophores (endmembers) that comprise the mixing matrix is essential for effective unmixing. It is important to achieve minimal overlap between spectral signatures to ensure a well-conditioned matrix, often evaluated using metrics such as cosine distance between columns (which assesses orthogonality) and the condition number (which reflects sensitivity to minor errors in measurements). While expanding the spectral detection range by adding more channels can theoretically improve resolution and differentiate closely related signals, it may also lead to numerical instabilities if the end-member spectra lack sufficient distinction.

To address these challenges, researchers increasingly apply optimal experimental design principles. In particular, D-optimality seeks to maximize the determinant of the Fisher information matrix, thereby minimizing the joint uncertainty of estimated parameters. Other design criteria, like A-, E-, or G-optimality, can likewise optimize different aspects of estimation accuracy and robustness. Balancing these criteria against practical considerations—laser availability, signal intensities, photostability, and sample constraints—helps identify a design that respects the CRLB's fundamental limits and maintains a well-conditioned mixing matrix. This presentation will trace the historical roots of spectral flow cytometry, examine the statistical foundations of noise modeling and unmixing, and illustrate how optimal design strategies—guided by orthogonality measures, condition numbers, and Cramér-Rao limits—can maximize the power of the technique.

The effects of chronic stress on the ageing immune cell profile in 'dirty' mice

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Chronic stress is known to accelerate ageing and impair immune function, yet its specific impact on T cells in the context of natural microbial exposure remains unclear. In this study, we subjected 13–14-month-old 'dirty' mice (generated by transferring fomite bedding from zoo mice to standard pathogen-free mice) to 21 days of chronic variable stress. We comprehensively analyzed multiple T cell subsets (CD4+, CD8+, naïve, memory, Th1, Th2, Th17, Treg, Tfh, and TRM cells) across various lymphoid and peripheral tissues. Our findings reveal that chronic stress induces a redistribution of T cells, with numbers decreasing in the thymus and inguinal lymph nodes and increasing in the bone marrow; however, splenic T cell numbers as well as T cell subsets remain largely unchanged. Single-cell RNA sequencing (scRNA-seq) of splenic T cells corroborated the robust subset proportions but showed significant upregulation of homeostatic genes, including Il7r and several Hsp-related genes, which likely act to maintain proteostasis. To evaluate functional consequences, we vaccinated mice with a mRNA COVID-19 vaccine and subjected them to chronic stress. Re-stimulation of isolated splenocytes with spike protein and Brefeldin A, revealing an elevated production of pro-inflammatory cytokines (IFN-γ and TNF-α) in the stressed group. Collectively, these results suggest that, in aged dirty mice, chronic stress triggers transcriptomic adaptations that sustain splenic T cell homeostasis, potentially via enhanced proteostasis, even as T cell numbers shift in other tissues. These insights into the interplay between stress, ageing, and immune function may bring forward strategies to mitigate immune dysregulation in chronically stressed, ageing populations.

Identification of human CD1c+CD14^{+}CD163^{+} type III dendritic cells

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Dendritic cells (DCs) act as sentinels of the immune system as they possess the superior ability to prime and activate T cells. In humans, the diversity, ontogeny and functional capabilities of DC subsets are not fully understood. Historically, DCs have been divided in plasmacytoid DCs and classical DCs (cDCs), where cDCs are further separated in cDC1 and cDC2. However, recent findings have shown further heterogeneity within the DC population with the addition of DC3. We have reinforced these finding by identifying circulating CD88^{-}CD1c^{+}CD14$^{+/-}$ CD163^{+} DC3 as an immediate precursor of inflammatory CD88^{-}CD14^{+}CD1c^{+}CD163^{+}FcεRI^{+} DC. *In vitro*, in contrast to cDC1 and cDC2, growth factor GM-CSF and not FLT3L promotes the expansion of DC3. DC3 also develop via a specific hematopoietic pathway activated by GM-CSF, independent from the cDC-restricted (CDP) and monocyte-restricted progenitors (cMoP). Furthermore, we show that DC3-commited progenitor exists in the CLEC12A^{+} fraction of granulocyte, monocyte and DC progenitor (GMDP). *In vivo*, DC3 can infiltrate humanized mouse B16 lung tumours expressing GM-CSF and human luminal breast cancer primary tumours. Furthermore, DC3 show a superior ability to induce the proliferation of CD8^{+}CD103^{+} T cells, termed tissue-resident memory T cells (TRM), and the DC3 infiltration correlates positively with TRM in luminal breast cancer patient samples. Collectively, this data defines DC3 as plastic but specialised DC subset with a distinct origin, developmental requirements and specific functional capabilities.

UMAP, tSNE or Autoencoder: what is the best in silico technique to interpret in vivo CAR-T and CAR-CIK experimental data?

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Introduction: Dimensionality reduction (DR) is one of the crucial steps in multi-parameter data analysis that enables scientists to discover underlying patterns and simplify complex datasets for further studies. Thus, it is important to choose the appropriate DR technique that considers the specific peculiarities of a dataset, as well as accurately represents the relationships within its structures, both global and local. This study provides a comparative analysis of three popular dimensionality reduction algorithms – UMAP, t-SNE, and Autoencoders While UMAP and t-SNE are widely used for medical biology translational research, Autoencoder (a multi-layered self-learning neural network) excels at single-cell RNA-seq data analysis. The aim of the study was to evaluate the performance of the reviewed DR techniques on a FACS data, considering various configurations such as the number of events in a dataset, neighboring sets parameter (UMAP), number of iterations (t-SNE).
Methods: The research was conducted on a cell population of xenograft tumors from NSG mice, which were non-treated and treated with CD19 chimeric antigen receptor (CAR) modified T cells or CD19 CAR cytokine-induced killer (CIK) cells in different time points. The objectives were to compare the algorithms based on the defined criteria (structures preservation, computational efficiency, runtime, interpretability) and provide best use-cases for each technique. The following tools were used during the study: FlowJo v10.10, Python 3.13, Notepad.
Results: The results of our study showed that UMAP outperforms t-SNE in terms of runtime and scalability while preserving local structures well, with decent global structure preservation with a room for improvements. t-SNE showed a great preservation of local relationships, yet it suffers from a higher computational cost and poor global structures interpretation. Autoencoder requires further learning for making a stronger conclusion for FACS data use-case. Overall, UMAP is recommended for large biological datasets; t-SNE can be used to identify rare or anomalous patterns in medical data; Autoencoder remains to be a subject of further studies.

Session 3:
Flow for Newbies

A newbie's guide to flow cytometry

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Flow cytometry is a powerful analytical technique widely utilized in diverse fields such as immunology, haematology and cell biology. This presentation will provide an overview of the fundamental principles of flow cytometry, including the physics of light scattering and fluorescence, which form the basis of cell characterization and quantification. We will begin by discussing the components of a flow cytometer, highlighting key elements such as fluidics, optics, and electronics. The mechanism of sample preparation and the importance of appropriate fluorescent labelling techniques will be examined to ensure accurate and reproducible results. Attendees will gain insight into various data acquisition methods and how multiparametric analysis enhances our understanding of complex biological systems. The presentation will also cover the applications of flow cytometry in clinical and research settings, emphasizing its role in immunophenotyping, cell cycle analysis, and apoptosis detection. Furthermore, the challenges in data interpretation and the importance of employing robust gating strategies will be outlined, highlighting best practices for data analysis using modern software. This introduction aims to outline the main principles for conducting a successful flow cytometry experiment, starting from complex panel design all the way through to appropriate data interpretation – everything a "newbie" might need to know for a great start at flow cytometry.

Tips and Tricks for a sample flow

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In flow cytometry, if something can go wrong, it often will. So how can you minimize errors and ensure the best possible results? This talk will provide practical, hands-on strategies to optimize both sample preparation—such as cell isolation and staining—and instrument settings, including quality control and voltage/gain adjustments. By refining these key steps, you can reduce variability, enhance sensitivity, and improve the accuracy and reproducibility of your assays. Join us to learn how small optimizations can lead to big improvements in your flow cytometry workflow!"

Session 4:
Flow Cytometry in Basic Science: Nanoworld and microworld

Welcome to the Micro and Nano World: Expanding the Limits of Flow Cytometry

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Originally designed for mammalian cells, flow cytometry has continuously evolved to accommodate a wider range of applications, including the analysis of non-mammalian cells, plant cells, and even nanoparticles. With advancements in sensitivity and precision, modern flow cytometers can now detect increasingly smaller particles, from bacteria to extracellular vesicles and even polystyrene particles as small as 40 nm. However, working at the nano scale presents unique challenges, requiring meticulous sample preparation and optimized workflows. In this talk, we will explore essential tips and techniques for accurately identifying and characterizing extracellular vesicles, nanomaterials, and microorganisms such as bacteria and yeast.

Extracellular Vesicles: Don't Let Pitfalls Derail Your Research!

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Extracellular Vesicles (EVs) are a heterogeneous group of membranous vesicles released by all cell types. They vary in size, biogenesis, release mechanisms, and cargo (mRNAs, miRNAs, lncRNAs, proteins). Their presence has been confirmed in multiple biofluids, such as blood, urine, saliva, and cerebrospinal fluid, highlighting their involvement in various physiological and pathological processes. One of the most widely used methods for EVs analysis is flow cytometry**,** which enables multiparametric, single-vesicle-level analysis. However, due to the small size of EVs, proper experimental setup and controls are crucial for obtaining reliable results.

To evaluate recent advancements and current challenges in this field, a literature search was conducted using the PubMed database, focusing on publications from 2024-2025**.** The search terms *"Extracellular Vesicle" AND "Flow Cytometry"* were used, with *NOT "review"* applied to exclude review articles. The selected studies were analyzed based on their reporting of calibration procedures, control implementation, and result presentation.

Among the 274 articles identified, 23% did not perform direct EV analysis via flow cytometry despite utilizing the technique. 14% employed indirect analysis by immobilizing EVs on beads. Only 22% adhered to MIFlowCyt-EV recommendations for proper study reporting. The most frequently omitted controls included detergent/lysed controls and dye/antibody aggregate controls as they were performed in 26% of studies. Additionally, 54% of studies lacked appropriate sizing calibration, often misinterpreting bead sizes as equivalent to EV sizes, and only 28.6% reported sample dilution controls.

Despite efforts from international organizations such as ISAC and ISEV to standardize experimental procedures, significant inconsistencies remain in machine setup, control implementation, and result validation. These findings highlight the urgent need for increased awareness and stricter adherence to standardized protocols to improve data quality, reproducibility, and comparability in EVs' flow cytometry studies. Future efforts should prioritize enhanced training, reporting standards, and the development of automated tools to improve calibration and control procedures. By adopting more rigorous experimental practices, the field can achieve greater consistency and generate more meaningful insights into EVs' biology and their potential clinical applications.

Application of flow cytometry in pharmaceutical quality control: validation protocol for probiotic analysis

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Flow cytometry is a promising tool in pharmaceutical quality control, providing rapid and accurate assessment of cellular properties. This study explores its application in the analysis of probiotics, focusing on validating flow cytometry as an alternative to traditional plate counting methods. Probiotics are integral to many pharmaceutical and dietary products, and ensuring their quality requires precise and efficient quantification of viable bacteria. The validation protocol developed in this study compares flow cytometry with plate counting to evaluate its accuracy, specificity, repeatability, and robustness. The number of probiotic bacteria were determined in accordance with Protocol B of ISO 19344|IDF 232 (2015) using dual fluorescent staining (propidium iodide and SYTO9). Flow cytometry effectively distinguishes live, dead, and damaged bacterial cells, offering insights into probiotic viability. Sensitivity tests confirm its ability to detect low bacterial concentrations, while reproducibility studies highlight consistency across operators and instruments.

This approach significantly reduces analysis time compared to plate counting, enabling real-time quality control and faster decision-making. Additionally, flow cytometry's precision ensures compliance with regulatory standards and enhances batch-to-batch consistency. Notably, FCM showcased advantages in assessing physiological states, estimating metabolic activity, and saving time and costs. Nevertheless, our findings highlight the importance of recognizing the complementary nature of FCM and PC. Both methods offer unique insights into viability and the presence of VBNC bacteria, necessitating their combined or tailored utilization for robust microbiological assessments in the probiotic industry and pharmaceutical quality control. This study underscores the critical need for comprehensive evaluation methodologies to ensure the efficacy and safety of probiotic products.

Our analyses show flow cytometry as a powerful method for quality control in probiotic manufacturing, setting the stage for its broader adoption in the pharmaceutical industry.

Flow Cytometry Integration in Extracellular Vesicle-Based Biomarker Discovery for Multiple Myeloma

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Introduction: Multiple myeloma (MM) is an incurable hematologic malignancy characterised by malignant plasma cell expansion in the bone marrow (BM). Current MM diagnosis and monitoring rely on invasive BM biopsies and M-protein quantification, which lack sensitivity for early detection and disease staging. Extracellular vesicles (EVs) offer a promising alternative as liquid biopsy biomarkers, carrying proteins and miRNAs that reflect disease status. This study integrates flow cytometry into the biomarker discovery pipeline, enhancing EV quantification, characterisation and facilitating assay development for clinical translation.
Methods: Peripheral blood (PB) samples were collected from patients with MM (n=23), monoclonal gammopathy of undetermined significance (MGUS) (n=4), and healthy controls (n=10) at Mater Misericordiae University Hospital. Plasma was separated using Lymphoprep gradient centrifugation, followed by platelet and debris removal. EVs were isolated via density gradient ultracentrifugation and characterised per MISEV2018 guidelines using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blotting. Flow cytometry was used to quantify EVs on a Beckman Coulter CytoFLEX LX and S, with EV size gating (80–500 nm) established using Apogee beads and red and violet side scatter (SSC). EV concentrations were comparable across peripheral blood (PB) samples from MM, MGUS, and normal controls.
Results: Flow cytometry-based EV quantification provided a crucial reference for subsequent multi-omic analyses, including mass spectrometry for proteomics and RNA sequencing for transcriptomics. Differential expression analysis identified significantly dysregulated proteins and miRNAs, forming a biomarker panel for MM disease staging.
Conclusions: Since MM EV biomarker signatures comprise both internal and external EV proteins, flow cytometry will be further utilised to develop an assay for detecting external MM EV biomarkers. This approach advances the clinical translation of EV-based liquid biopsies, supporting early MM detection, disease monitoring, and therapeutic decision-making.

Flow Cytometry Sorting of Extracellular Vesicles

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Extracellular vesicles (EVs) are nano- and micro-sized membranous particles (30–5000 nm in diameter) released by cells, playing a major role in cellular communication via horizontal transfer of functional cargo. EVs are highly abundant and stable in biofluids such as blood, urine, and saliva, and they reflect the characteristics of their cell of origin, making them an easily accessible source of information. Thus, EVs have immense potential for diagnosis and treatment of a wide variety of diseases, for example, as non-invasive biomarkers for cancer diagnosis, prognosis, and longitudinal monitoring.
Cancer-derived EVs (cEVs) constitute up to 10% of total EVs in patient plasma, representing less than 0.01% of the total blood volume. Isolation of specific populations of cEVs is required for accurate biomarker discovery and clinical translation, however, this requires specialised and standardised methodologies. Flow cytometry has emerged as a promising tool for EV characterization. Moreover, flow cytometry sorting offers the opportunity to isolate EV subtypes, including cEVs, based on surface antigen expression for downstream characterization. However, the process is challenging due to variables such as sorting time, sample dilution, and purity of the sorted EVs.
In this study, we address some of the challenges of cytometry based EV detection and sorting. This includes instrument calibration using polystyrene beads, EV surface antigen staining and detection as well as EV subtype sorting based on scatter and surface antigen expression.

Analysis of Human Cartilage and Synovium Explant Extracellular Vesicles: Targeting Cellular Senescence

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Introduction: Cellular senescence is a critical factor contributing to osteoarthritis (OA) progression, leading to impaired tissue repair and chronic inflammation. Connexin 43 (Cx43) has been identified as a key regulator of senescence, potentially influencing OA development. Targeting Cx43 with specific peptide inhibitors, such as TUB1, may help prolong cellular function and mitigate senescence-related damage. This study investigates the effects of TUB1 on extracellular vesicles (EVs) derived from human cartilage and synovium tissue explants, aiming to assess its therapeutic potential in OA management.

Methods: Cartilage and synovium tissues were obtained from hospitals following surgical joint replacement procedures in patients with knee OA, and cultured in vitro. Explants were treated with the Cx43 inhibitor TUB1, followed by analysis of glycosaminoglycan (GAG) deposition using the Blyscan dye reagent kit. Cytokine secretion was assessed via multiplex analysis, and cellular proliferation was measured using the CCK-8 kit. Additionally, EVs were isolated and characterized for Cx43 expression to determine the effects of TUB1 treatment on their molecular composition.

Results: TUB1 treatment led to attenuation in cell senescence, as demonstrated by β-galactosidase activity in cell cultures and flow cytometry. A decrease in GAG release into the culture medium, and improved extracellular matrix content as demonstrated by safranin O staining in histology, indicates protective effects on cartilage matrix metabolism. Furthermore, cytokine secretion was altered in response to TUB1 treatment, suggesting immunomodulatory effects. Notably, Cx43 expression in EVs was modulated, demonstrating that Cx43 inhibition influences EV composition. These findings highlight the potential role of TUB1 in altering cellular communication in the joint and extracellular vesicle-mediated signaling in OA.

Conclusion: This study, conducted within the collaborative framework of the Horizon EU TWINFLAG project involving IMC, UCD, and UVigo, provides novel insights into the role of Cx43 in cartilage and synovium-derived EVs. The results suggest that TUB1 may be a promising candidate for future applications aimed at inhibiting cellular senescence in OA patients.

Session 5:
Clinical Flow Cytometry

Identification of mutation-independent BRCA2 protein deficiency by flow cytometry expands diagnostics of patients with pancreatic cancers for personalized therapy by PARP1 inhibitors

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Introduction: Recently, therapies involving poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP1) inhibitors have been approved for metastatic BRCA1/2-mutated pancreatic ductal adenocarcinoma (PDAC). The current scheme of identification of patients with BRCA deficiency relies on genetic screening. Here, we tested the hypothesis that pancreatic tumors have a broader spectrum of BRCAness than can be identified solely by gene mutations. We focused on BRCA2 deficiency, which is predominant in pancreatic ductal adenocarcinoma (PDAC).
Methods: Pancreatic cancer cell lines (wt or *BRCA2* mutated) were used to set up a protocol to verify antibody specificity and detect BRCA2 protein levels by flow cytometry. Post-surgery pancreatic tumor samples were assessed by spectral cytometry with unsupervised analysis to identify BRCA2-deficient clusters together with the expression of stemness and metastasis markers. Personalized tumor signatures specified BRCAness phenotype and cancer state to increase the accuracy of selection for therapy with PARP1 inhibitors (PARPis). In parallel, *BRCA2* mutations were identified by NGS analysis.
Results: We developed a cytometric panel to assess BRCA2 levels associated with sensitivity to PARPis (olaparib and talazoparib). Analysis of BRCA2 protein levels in patients' samples showed high diversity. Unsupervised cluster analysis identified BRCA2-deficient clusters, together with metastasis and stemness markers, which indicated advanced tumors with dismal prognoses. Cluster composition confirmed the high heterogeneity of pancreatic tumors. In parallel, NGS did not recognize the BRCA2/1 mutations in any of the analyzed tumors. Therefore, based on the current selection, these patients would be excluded from PARPis therapy. Finally, analysis of each tumor personalized signatures of tumor cell subsets potentially sensitive to PARPis were demonstrated.
Conclusions: We found that BRCA2 protein deficiency (BRCAness) is detected with metastasis/stemness markers in pancreatic tumors also in individuals lacking BRCA2 mutations. Our findings show that integrating the flow cytometry-based BRCA2 protein assessment with genetic screening is important to improve the effective selection of PDAC patients for therapy with PARPis. This might also be relevant for other BRCA-deficient tumors.

Determining peripheral blood T-cell phenotype distributions using conventional flowcytometry analysis versus cluster analysis software

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Introduction: Conventional flow cytometry-based immunophenotyping relies on subdividing white blood cells into distinct subpopulations using two-dimensional plots, typically categorized as 'positive' or 'negative.' In some cases, intermediate levels such as 'negative,' 'low,' and 'high' are designated. These divisions must be consistently applied across all samples by defining 'regions' or 'quadrants,' which are then combined into logical 'gates' to establish a standardized 'gating strategy' for the cohort. However, manual gating often requires slight adjustments for each sample to accommodate biological variations in marker expression. Furthermore, as manual gating selects cells based on only two dimensions at a time, relevant information may be lost. Additionally, conventional gating risks double-counting and misassigning cells, potentially impacting final results, therapeutic decisions, and research directions. In contrast, emerging clustering algorithms eliminate the need for population classification based solely on two-dimensional projections. Unlike human analysis, these algorithms process multiple dimensions simultaneously, enabling the identification of cell clusters in an n-dimensional space. By grouping cells based on their full expression profiles, clustering minimizes cellular loss and misassignment, providing a more comprehensive and unbiased representation of cell phenotypes.
In this study, we compare peripheral blood T-cell populations identified through conventional gating with those generated by an automated clustering algorithm using cloud-based software.
Methods: Our cohort consists of 40 individuals with early arthritis, evenly divided between cytomegalovirus (CMV)-seropositive and CMV-seronegative individuals. We assess T-cell populations derived from both approaches, using a phenotyping panel that includes CD3, CD4, CD8, and seven additional markers (CCR7, CD27, CD28, CD45RA, CD57, GPR56, and PD1). Given the well-documented influence of CMV serostatus on T-cell subset distribution, we specifically examine its impact across both population-based and cluster-based analyses.
Results and Conclusions: Our exploratory analysis reveals both differences and similarities between T-cell populations identified through conventional gating and automated clustering. These findings underscore the potential of clustering algorithms in research settings, offering an alternative, unbiased approach to cell population characterization.

Flow Cytometry and Solid Organ Transplantation: A Perfect Match?

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Flow cytometry is increasingly used in clinical immunology, particularly in crossmatch reactions between organ donors and recipients. Beyond this, it has also been applied to detecting donor-directed antibodies, identifying HLA-specific B cells, assessing immune responses to viral infections, evaluating the efficacy of immunosuppressive therapy, and predicting allograft rejection or acceptance. In the field of transplantation, crossmatch assays between donors and recipients remain a cornerstone of compatibility testing. Many laboratories still rely on the complement-dependent cytotoxicity (CDC) assay to assess graft acceptance. This method evaluates the cytotoxic effect of a patient's serum on donor leukocytes in the presence of commercially prepared complement, with viability assessed microscopically using viability dyes. While straightforward in interpretation, the CDC assay has notable limitations. Traditionally, the presence of cytotoxic antibodies was believed to predict graft rejection, whereas their absence suggested a favourable outcome. However, not all antibodies capable of inducing graft rejection are detectable by CDC, and not all detected antibodies are clinically relevant. This results in poor specificity, leading to a high rate of false-positive reactions. The evolution of flow cytometry in transplantation has overcome many limitations of the complement-dependent procedure. The enhanced sensitivity compensates for low titer antibodies. Developments in the flow cytometry procedure have improved the specificity as well. For instance, by using IgG specific probes, false positive reactions attributable to non-specific IgM class auto- and alloantibodies have essentially been eliminated. Still the need of viable donor cells, especially from deceased organ donors remains as a limitation of the method. In light of this, developments in cell-free solid phase assays now offer a feasible solution in post-transplant antibody monitoring. Flow cytometry has advanced immunophenotyping, enabling detailed characterization of immune subsets linked to graft tolerance or rejection. Initially focused on T cells, research now includes NK cells, dendritic cells, regulatory T cells, myeloid-derived suppressor cells, and regulatory macrophages. Further improvements in immunophenotyping are essential in transplantation field, as leukocyte subset changes can be subtle.

Potential use of naphthyridine derivatives in cancer treatment

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Introduction: The increasing incidence of cancer diseases and developing resistance to standard chemotherapy regimens remains a current problem, and the search for new anticancer drugs is still an important goal of research around the world. Naphthyridines are heterocyclic compounds with documented anticancer activity. In previous studies, naphthyridines had shown significant cytotoxic effects on a wide range of cancer cell lines in vitro, based on multidirectional mechanisms - inhibition of topoisomerase II, intercalation into DNA, induction of apoptosis, and interference with pro-cytotoxic pathways.
Aim: The study's purpose was to determine the chemopreventive activity of three naphthyridine derivatives—Naph-1, Naph-2, and Naph-3—comprising cell viability suppression, determine the required concentration, and induce the formation of reactive oxygen species.
Methods: A study of the chemopreventive activity of naphthyridines has been conducted on MDAH-2774, SKOV-3, MCF-7, A549 cancer cell lines, and CHO-K1 nontumoral cells. The cytotoxic activity was assessed using the Presto Blue assay after 72 hours of cell incubation with tested compounds (at the 5 different concentrations), with IC50 (half-maximal inhibitory concentration) values determined. The effect on the level of intracellular reactive oxygen species was tested with the DCF-DA assay.
Results: Compounds Naph-1 and Naph-3 exerted significant cytotoxic effects on ovarian cancer (MDAH-2774), breast cancer (MCF-7), and cisplatin-resistant ovarian cancer (SKOV-3) cell lines. The strongest effect was observed for Naph-3 on the MDAH-2774 cancer cells with an IC50 value of 36.05 μM. The effect on A549 lung cancer cells was noticeable only for Naph-3 at a high concentration of 200 μM. Naph-2 showed the lowest potential to reduce cancer cell viability. No compound affected the generation of reactive oxygen species.
Conclusions: Further detailed studies should be conducted on the effects of Naph-1 and Naph-3 on the viability and apoptosis of the MDAH-2774 cells to determine their relevance in the therapy of ovarian cancer.

Blood-Based Flow Cytometry for Immunological Insights in Female Infertility and Diagnostic App Development

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Introduction: Infertility remains a complex challenge, affecting up to 17,5 % of couples worldwide, with unexplained infertility accounting for up to 30% of cases in women. Increasing evidence suggests the role of immune dysregulation in reproductive dysfunction, necessitating novel diagnostic and therapeutic approaches. This study aimed to develop an AI-driven app for infertility diagnostics based on flow cytometry results of blood serum and assess its clinical applicability.
Methods: Peripheral blood was collected from 3 groups: women with unexplained infertility (GR1M), women with defined non-immunological infertility (GR2M), and healthy controls (GR3M). Multi-parameter flow cytometry was employed to analyze immune cell phenotypes, focusing on markers helper and cytotoxic T-lymphocytes, NK and NKT cells, monocytes, B-lymphocytes and granulocytes: neutrophils and eosinophils. Diagnostic algorithms were constructed using AI-based modeling, integrating up-sampled data of blood cytometry.
Results: In total, 43 women were included in the study: GR1M (N=20), GR2M (N=6), GR3M (N=17). Cytometric analysis of blood biomarkers was performed and the data was used to train the infertility diagnostic tool. While standard statistical methods did not reveal any significant differences between the groups based on blood biomarkers, the AI-based diagnostic model struggled with accuracy due to limited patient recruitment. To solve this, the diagnostic model was additionally trained with up-sampled dataset of 1,500 samples (500 per group). Finally, the AI-based infertility diagnostic model achieved an accuracy of 95%, with helper and cytotoxic T-lymphocytes, B-lymphocytes, NKT cells identified as key predictors of infertility.
Conclusions: This study demonstrates the potential of flow cytometry in elucidating immune status underlying unexplained infertility. The integration of AI with cytometric data offers a promising pathway for personalized diagnostics in infertility. The current diagnostic tool should be retrained with additional patient data and excluding CR2M data should be considered to enhance clinical applicability and predictive accuracy.

Preclinical Drug Testing in Personalized in Vitro Endometrial Cancer Models

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Introduction: Endometrial cancer is the sixth most common cancer in women worldwide (Bray et al., 2024). Interestingly, Lithuania ranks in Europe's top two countries in terms of endometrial cancer incidence. A standard treatment for endometrial cancer is surgery, followed by adjuvant therapy of paclitaxel, cisplatin, or carboplatin. However, high intra- and intertumoral heterogeneity raises a challenge for developing effective personalized treatments. Thus, new targeted therapy agents, such as inhibitors of cancer-related signaling pathways, regulators of cell cycle and death constantly undergo clinical trials (Janssens and Remmerie, 2018). Our study highlights the importance of flow cytometry tools for preclinical drug testing in personalized in vitro endometrial cancer models.
Methods: Three novel endometrial cancer cell lines were established in our laboratory from the tumor tissue of Lithuanian endometrial cancer patients. We characterized the cells by determining their growth rate, colony-forming efficiency, and the expression of cancer markers. Cell viability and apoptosis induction after drug treatment were analyzed by flow cytometry coupled with propidium iodide and active caspase 3/7 staining.
Results: We analyzed cell sensitivity to different types of anticancer agents: 1) standard first-line chemotherapy drugs, currently used to treat endometrial cancer; 2) PI3K/AKT/mTOR and receptor tyrosine kinase inhibitors; 3) regulators of cell cycle and proliferation; 4) modulators of Notch signaling pathway; 5) ferroptosis inducers. The most cytotoxic compounds were nuclear transport inhibitor selinexor, PI3K/AKT/mTOR inhibitors apitolisib and MK-2206, as well as cell cycle regulator BI 2365. What is more, we showed that PI3K/AKT/mTOR inhibitors promote cell death via apoptosis pathway. However, interestingly, specific ferroptosis inducer RSL3, despite being cytotoxic, failed to induce ferroptosis in most of the tested cell lines.
Conclusions: Flow cytometry reveals diverse responses of patient-derived endometrial cancer cells treated with targeted therapy compounds.

Session 6:
Multiomics in Flow Cytometry

Genomic cytometry: promise and challenges of multiomic workflows

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The emergence of transcriptome exploration techniques at the single-cell level has led to new technological developments for more precise analyses of the immune system, including so-called "Multi-Omics" approaches such as Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq). This approach, also described as genomic cytometry, allows for a combined analysis of RNA and proteins. It is thus possible to analyze, at the single-cell level, gene expression as well as the expression of target proteins traditionally used in cytometry, while encoding the samples. Datasets produced from this technique are of great value because they allow fine developmental trajectory analysis of immune cells and represent the missing link to connect flow cytometry datasets to transcriptomic atlas ressources.
While the implementation of this technique is becoming more widespread, some common pitfall are encountered around sample preparation and cell counting. In addition, the inherent properties of certain fragile cell types remain a significant challenge for Multi-Omics sample processing. Indeed, in the case of samples sensitive to pressure or pro-apoptotic, physical sorting by Multiparametric Flow Cytometry can be detrimental and lead to increased post-sorting mortality. Likewise, labeling steps can result in the loss of the cells of interest, especially in the case of fragile samples or rare populations.
To address these unavoidable steps, we sought to establish a new cell preparation pipeline to eliminate these impacts. To this end, we tested two new sample processing devices: AttuneNxT or CytPix (ThermoScientific), Laminar Wash™ MINI (Curiox Biosystems) and the LeviCell® (LevitasBio). The aim of this presentation is to share with the baltic flow cytometry community the implementation of a less traumatic workflow in terms of viability for sensitive cells, while offering better yield than physical sorting to ensure maximal recovery of high-quality cells out of these costly workflows.

POSTER ABSTRACTS

Utilizing Flow Cytometry in Pharmaceutical Quality Control: Assessing the Stability of Probiotic Preparations

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Introduction: Flow cytometry is an advanced analytical technique that has gained significant attention in pharmaceutical quality control due to its ability to provide rapid, accurate, and comprehensive analysis of cellular properties. This abstract explores the application of flow cytometry as a valuable tool for assessing the stability of probiotic preparations, which is crucial for ensuring their efficacy and safety in pharmaceutical products. Probiotic preparations require strict quality control to guarantee that the viable bacterial count remains consistent throughout the shelf life of the product.
Aim: The study highlights the advantages of using flow cytometry in monitoring the stability of probiotics, focusing on its ability to differentiate between live, dead, and damaged bacterial cells. By using fluorescent markers, flow cytometry can assess the viability and integrity of probiotics in real time, offering a faster and more precise alternative to traditional methods such as plate counting.
Methods: The stability tests of the probiotic preparations were performed over a 24-month period with analyses at six time points: 0, 3, 9, 12, 18, and 24 months. Samples were stored in climatic chambers following ICH Q1A (R2) guidelines for stability testing of drug substances and drug products.
Results: The study demonstrates how flow cytometry can be utilized to track changes in probiotic populations under varying storage conditions and throughout product shelf life. This method also provides insights into bacterial metabolic activity and stress responses, which are crucial for understanding the long-term stability of probiotic preparations. This technique provides valuable information about the heterogeneity of bacterial populations, which is critical for evaluating the long-term quality and efficacy of probiotic products.
Conclusions: Flow cytometry has proven to be a reliable and valuable tool in the probiotic industry, supporting the development of more robust production technologies by enabling detailed and accurate monitoring of preparation stability. It offers a significant advantage over traditional methods and is an essential tool for ensuring the safety, quality, and efficacy of probiotics throughout their shelf life.

Incidence and Primary Immunophenotypic Characterization of Acute Lymphoblastic Leukemia (ALL) by Flow Cytometry at the Children's Clinical University Hospital from 2022 to 2024

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Introduction: Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in pediatric patients, accounting for more than 25% of all pediatric cancer cases. Although the etiology of ALL can be attributed to inherited genetic syndromes such as Down syndrome and Fanconi anemia, as well as congenital immunodeficiency disorders, in some patients, the underlying cause remains largely unidentified in the majority of cases.
Aim: To investigate the incidence and primary immunophenotypic characterization of pediatric ALL cases using flow cytometry at the Children's Clinical University Hospital from August 24, 2022, to January 31, 2024.
Methods: This retrospective cohort study included 26 pediatric oncohematological patients diagnosed with C91.0 (ALL according to the ICD-10). The study evaluated patient demographics including age, gender, and region of residence. Patients underwent an acute leukemia orientation tube (ALOT) protocol examination, as per the standardized NOPHO protocol. Additionally, further B- or T-lymphoblast leukemia phenotyping was conducted to ascertain the primary immunophenotype of the blasts.
Results: The incidence of ALL determined by flow cytometry at the Children's Clinical University Hospital from August 24, 2022, to January 31, 2024, revealed a total of 26 patient cases. Of these, 23 cases (~88%) were diagnosed with B-cell ALL (B-ALL), and 3 cases (~12%) with T-cell ALL (T-ALL). 16 out of 26 patients (~62%) resided in Riga/Riga district. Among the 26 patients, 16 were female (~62%) and 10 were male (~38%). At the time of ALL diagnosis, the most common age was 2-3 years old (8 out of 26 cases, corresponding to 31%). The most common positive markers identified in primary immunophenotyping for B-ALL were CD10, CD19, CD22, cyCD22, CD24, CD34, CD38, CD58, CD73, cyCD79a, CD81, and CD123, but in cases of T-ALL, the markers included CD2, cyCD3, CD5, CD7, CD99, nTdT.
Conclusions: This study demonstrated that out of 26 patient cases, B-ALL was most frequently detected, occurring predominantly in the age group of 2-3 years among females residing in Riga/Riga district. To investigate the incidence and primary immunophenotypic characterization of pediatric ALL cases more precisely, this retrospective cohort study should be extended over a longer period of time.

Analysis of Cell Viability and Apoptosis in Endometrial Cancer Cells, Exposed to Targeted Therapy Compounds

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Introduction: Endometrial cancer is the 6th most common cancer in women (Bray et al., 2024). Surgery is usually the first-line treatment for endometrial cancer, if there is any risk of cancer recurrence, chemotherapy is applied afterward. However, the most common medicines used for endometrial cancer such as paclitaxel, carboplatin, and cisplatin have many downsides, including their non-specificity. As an alternative to classical chemotherapy, targeted therapies are continuously being developed and actively investigated (Bestvina & Fleming, 2016). In this study, we aim to explore the effects of different clinically approved targeted therapy compounds - PI3K/AKT/mTOR and receptor tyrosine kinase inhibitors, regulators of cell cycle, and others - on endometrial cancer cell lines, established in our laboratory from the tumor tissue of Lithuanian endometrial cancer patients.
Methods: Three endometrial cancer cell lines, CRL-230315C, CRL-230315Dif, CRL-230407, derived in our laboratory, and one commercially available endometrial cancer cell line KLE were used in the study. Cell viability (the proportion of cells with a permeabilized membrane) after drug treatment was determined by propidium iodide staining using flow cytometry. To detect apoptosis, the cells were simultaneously stained with Caspase-3/7 Green detection reagent (Thermo Fisher Scientific).
Results: Most of the tested compounds decreased endometrial cancer cell viability in a concentration-dependent manner. The most effective compounds were nuclear transport inhibitor selinexor, PI3K/AKT/mTOR inhibitors apitolisib and MK-2206, as well as cell cycle regulator BI 2365. The most sensitive cell line was CRL-230407. In these cells, apoptosis was induced after exposure to nine of the tested compounds, however, PI3K/AKT/mTOR inhibitors such as MK-2206, apitolisib and samotolisib had the strongest pro-apoptotic activity.
Conclusions: PI3K/AKT/mTOR inhibitors promoted cell death via apoptosis pathway, while nuclear transport inhibitor selinexor and cell cycle regulator BI 2365 induced strong cell viability decrease, but apoptosis was not predominant.

Specific immune signatures of T cells as biomarkers of the neuro Post-COVID Syndrome and predictors of long-term health dysfunctions

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Introduction: A significant group of COVID-19 convalescents suffers from Post-COVID Syndrome (PCS), which manifests with several symptoms, including chronic fatigue, cognitive dysfunction ("brain fog"), and respiratory problems. Previously, we have focused on dynamics of T cells immune landscape in COVID-19 convalescent patients after different severityof infection. Here, we stratified convalescents who had mild or severe SARS-CoV-2 infections, based on neurological symptoms lasting more than 3 months.
Methods: Using multiparameter spectral flow cytometry with unsupervised clustering (FlowSOM) and data mining techniques (SPICE) we detect complex phenotypes and immune responses.
Results: Our study reveals specific, long-lasting remodeling of CD4+, CD8+, and regulatory T cells in mild versus severe COVID-19 convalescents who are affected by PCS. We observed alterations in the frequency of these cell populations, strong polarization towards exhausted (PD-1+) and senescent (CD57+) phenotypes, as well as the production of cytokine combinations (polyfunctionality) by T cells, which led to inflammation and tissue cytotoxicity in severe COVID-19 convalescent. In contrast, patients who experienced mild infection displayed different types of abnormalities related to T cells and immunosuppression. Furthermore, we analyzed vascular inflammation, and neurological markers in the serum of these patients, what was possible using the super sensitive Quanterix Simoa technology.
Conclusions: We identify immune marker signatures specific to neuro-post-COVID conditions after mild infection. By identifying these specific immune signatures, we deepen our understanding of PCS pathophysiology and move closer to developing targeted diagnostic tools and treatment strategies. These immune markers could potentially serve as early indicators of PCS risk, allowing for timelier and tailored therapeutic interventions.

Flow cytometry and scanning electrochemical microscopy for the investigation of human skeletal muscle-derived mesenchymal stem/stromal cells

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Introduction: Skeletal muscles, like other adult tissues, have the ability to regenerate and can repair micro-tears caused by intense physical activity or injuries [1,2]. The self-regeneration process of skeletal muscles involves resident quiescent skeletal muscle-derived mesenchymal stem/stromal cells (SM-MSCs), known as satellite cells [1,3]. Once isolated, these satellite cells are referred to as myoblasts or activated SM-MSCs.
Methods: In this study, the total population of primary SM-MSCs was isolated from human post-operative material, cultured at 37 °C in 5% CO_2, labeled with CD56-APC (a satellite cell marker), and separated into CD56(+) and CD56(-) subpopulations using a BD FACSAria sorter. The myogenic differentiation of the total SM-MSC population, as well as the CD56(+) and CD56(-) subpopulations, was assessed immunocytochemically. Redox activity was investigated using a non-invasive scanning electrochemical microscope (SECM) in feedback mode with the natural redox mediator menadione (MD). Additionally, SM-MSCs were seeded onto conductive indium tin oxide (ITO) glasses, and their responses to alternating current were examined using SECM and immunocytochemical methods.
Results: Flow cytometry enabled the clear identification of two subpopulations within the total SM-MSC population and their sorting into CD56(+) and CD56(-) groups. Data from this study revealed that CD56(+) cells exhibited significantly higher metabolic activity, a faster response to alternating current, and superior myogenic differentiation compared to the CD56(-) subpopulation. The increased redox activity and enhanced myogenic differentiation observed in CD56(+) cells in vitro may indicate their greater role in skeletal muscle regeneration in vivo compared to CD56(-) cells.
Conclusions: CD56(+) cells exhibited significantly higher myogenic differentiation potential in vitro than CD56(-) cells. Targeted stimulation of the CD56(+) SM-MSC subpopulation could improve the regenerative potential of human skeletal muscle and extend its functional lifespan in vivo.

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Tha association of tumor response for neoadjuvant chemotherapy with activation of monocyte CCR2-CCL2 axis in triple negative breast cancer

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Introduction: The monocyte CCR2-CCL2 axis appears to play a crucial role in forming tumor-associated macrophages (TAMs), which subsequently promotes tumor metastasis and resistance to therapy. Therefore, our study assessed the monocyte CCR2-CCL2 axis in triple-negative breast cancer (TNBC) and its ability to predict tumor response to neoadjuvant chemotherapy (NAC).
Methods: A study included forty-six female patients diagnosed with TNBC and qualified for NAC. Responses to neoadjuvant chemotherapy were based on the pathological complete response (pCR). The TNBC patients were divided into two groups: pCR and non-pCR responders. In both groups the surface expression of CCR2 on monocytes was evaluated by flow cytometry. Circulating CCL2 was measured by Luminex X-Map technology.
Results: The percentage of CD14+ monocytes expressing CCR2 increased in both groups compared to controls, but the change reached statistical significance only in non-pCR responders [94.10 (17.10-100) % versus 83.25 (48.10-98.40) %, P=0.028]. The MFI levels of CCR2 levels showed a similar increasing trend, reaching the highest statistically significant value in non-pCR responders compared to the control group [766 (189-1709) versus 423 (239-632) %, P=0.0003] . A statistically significant increase in CCL2 concentration was observed only in the non-pCR patients compared to the control group [113.9 (56.48-360.7) pg/mL versus 83.20 (36.97-153.3) pg/mL, P=0.010]. Furthermore, the increase in CCL2 in the non-pCR responders remained greater than in the pCR responders [113.9 (56.48-360.7) pg/mL versus 76.74 (35.40-409.4), P=0.034]. ROC curves showed that the optimum diagnostic cut-off value of CCL2 ≤ 89.61 pg/mL better discriminates against TNBC patients achieving pCR than the Ki-67 index. Univariate analysis demonstrated that circulating CCL2 ≤ 89.61 pg/mL was significantly associated with pCR. However, in the multivariate model, this correlation lost statistical significance.
Conclusions: Our study demonstrated the activation of the monocyte CCR2-CCL2 revealed as the increased monocytic CCR2 expression and circulating CCL2 concentration axis in TNBC. This activation occurs mainly in patients who do not respond to NAC. Circulating CCL2 levels ≤ 89.61 pg/mL were found to predict, to some extent, the achievement of pCR in TNBC patients receiving NAC.

Clonal Heterogeneity Analysis of Colorectal Cancer Cells Using Flow Cytometry

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Introduction: A few decades ago, colorectal cancer was rarely diagnosed. Today, it is the second deadliest cancer in the world, killing 900,000 people every year (Globocan 2022). Many chemotherapy drugs are used to treat colorectal cancer, but in many cases the effectiveness of the drug treatment is impaired by the tumour's innate or acquired resistance to chemotherapy treatment. Innate resistance can be caused by tumour heterogeneity, where the tumour is composed of subpopulations of cells with different sensitivity, which makes eradication of the cancer extremely difficult. However, it is known that resistant tumor cells may be susceptible to a newly discovered cell death type, ferroptosis (Dixon and Stockwell, 2019).
Methods: In this study, human colorectal cancer cells DLD-1 were used. To assess the clonal heterogeneity of this cell line, it was cloned and 26 clones were established. Sensitivity of the selected clones to anticancer drugs and ferroptosis modulators was determined by propidium iodide staining using flow cytometry.
Results: DLD-1 clones showed a range of sensitivity to anticancer drugs oxaliplatin and cisplatin, but, as well as the parental cell line, were resistant to 5-fluorouracil. 5 clones with different sensitivity to platinum drugs, F, K, M, X and Z, were chosen. Further analysis showed an increased resistance of clone K and sensitivity of clone Z to RSL3 treatment, compared to the parental line. However, ferroptosis inhibitor ferrostatin-1 failed to reverse RSL3 cytotoxicity, indicating a non-ferroptotic cell death.
Conclusions: Flow cytometry is an excellent tool to study the clonal heterogeneity of colorectal cancer cells.

Microscopy Facility – Your gateway to the Micro World

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Uncovering the role of T cell help in B cell response to SARS-CoV-2 in treatment naive chronic lymphocytic leukemia patients

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Introduction: Infectious complications are common in patients with chronic lymphocytic leukemia (CLL). Vaccines can prevent infections, but in CLL vaccination often shows suboptimal protection. Generally, effective antibody responses require germinal centers and T cell help, which is provided by a T cell subtype called T follicular helper (Tfh) cells. T follicular regulatory (Tfr) cells, which share phenotypic characteristics with Tfh cells, in turn exert suppressive functions on Tfh and germinal center B cells. Here we evaluate circulating Tfh and Tfr cell populations in treatment naive CLL patients and their association with antibody response to SARS-CoV-2 antigen.

Methods: Blood samples were collected from 38 treatment naive CLL patients (Binet stage A, n=29; stage B, n=9) and 13 age- and sex-matched healthy controls. All participants were fully vaccinated against SARS-CoV-2. Total serum immunoglobulin (Ig) M, G, A levels were measured by turbidimetry (Siemens), and SARS-CoV-2 specific IgG and IgA were determined by ELISA (Euroimmun). Peripheral blood mononuclear cells were stained for analysis of Tfh (defined as CD3+CD4+CD45RA-CXCR5+FOXP3-) and Tfr cells (CD3+CD4+CD45RA-CXCR5+FOXP3+). Cell events were collected on Navios EX (Beckman Coulter) and analyzed by FlowJo v10.9.0 Software (BD Life Sciences). This study was supported by the Latvian Council of Science Fundamental and Applied Research Project through grant No lzp-2022/1-0080.

Results: Total IgG and IgM levels were significantly decreased in CLL. However, majority of the CLL patients were able to mount a response to SARS-CoV-2, both Spike-specific IgG (94.7%, n=36) and IgA (89.5%, n=34). Tfh and Tfr frequency and absolute numbers were significantly increased in CLL. While Tfh population did not show correlation with SARS-CoV-2 specific antibody levels, Tfr cell numbers were positively associated with SARS-CoV-2 IgG levels.

Conclusions: Possibly due to early-stage disease and absence of treatment most of the CLL patients were able to produce SARS-CoV-2 specific antibodies despite reduced total Ig levels. Although Tfh and Tfr cell populations were expanded in CLL, only Tfr showed association with specific SARS-CoV-2 IgG levels. This suggests a complex interaction between circulating Tfh and Tfr cells and adaptive immune responses in CLL.

The evaluation of cytokines of the IL-1 family in monocytes and neutrophils in patients with Helicobacter pylori infection

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Introduction: H. pylori causes gastritis in 30% of children and adolescents in Poland. The bacterium induces infiltration of neutrophils, macrophages and dendritic cells into the lamina propria of the gastric mucosa. However, it is unknown how H. pylori affects the activity of peripheral phagocytes and their ability to produce IL-1 family cytokines in children and adolescents.
The aim of the study was to assess: 1) the concentration of cytokines of the IL-1 family (IL-1beta, IL-18, IL-33) and IL-10 in plasma 2) the intracellular expression of cytokines of the IL-1 family, IL-10, caspase 1, and NLR family pyrin domain containing 3 (NLRP3) in neutrophils and monocytes in peripheral blood in children and adolescents infected with H. pylori and 3) if the degree of inflammation and activity of the gastric mucosa depend on the expression of cytokines of IL-1 family, IL-10, caspase 1 and NLRP3.
Methods: A total of 49 children and adolescents (3-17 years old) with symptoms of dyspepsia were studied: 24-newly diagnosed with H. pylori infection gastritis; 25-with H. pylori-negative gastritis. In order to assess the plasma levels of IL-1beta, IL-18, IL-33 and IL-10, the ELISA was used. However, the expression of IL-1beta, IL-18, IL-33, IL-10, caspase 1 and NLRP3 in neutrophils and monocytes in blood culture stimulated with lipopolysaccharide was assessed by flow cytometry.
Results: Patients with H. pylori infection with gastritis do not affect the expression of pro-inflammatory cytokines of the IL-1 family and the anti-inflammatory IL-10 in plasma and peripheral neutrophils and monocytes. However, patients with infection are characterized by reduced expression of the NLRP3 signaling receptor in the inflammasome in the peripheral neutrophils, which negatively correlates with the degree of inflammation and activity of the gastric mucosa, as well as H. pylori density.
Conclusions: H. pylori infection in children and adolescents inhibit inflammasome activation in peripheral neutrophils by downregulating the expression of the NLRP3 signaling receptor.

Enhanced cytotoxic activity of natural killer cells in children with non-alcoholic fatty liver disease

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Introduction: Non-alcoholic fatty liver disease (NAFLD) is characterized by chronic, low grade systemic inflammation. Changes in distribution of different peripheral immune cell subsets play a role in the development of NAFLD in experimental models and in adults. The aim of the study was to establish which types of leucocytes are associated with early stages on NAFLD development (as observed in children).
Methods: We recruited 45 obese children with NAFLD (diagnosis based on Fibroscan CAP value >250 suggestive of liver steatosis in general pediatric population- Ferraioli G, BioMed Central 2017) aged 15± 1.45 years and 30 aged matched controls. In addition, clinical characteristics were obtained. Peripheral blood samples were collected into TransFix/EDTA vacuum blood collection tubes. Using nine-color flow cytometry (CytoFLEX Flow Cytometer, Beckman Coulter) the frequency of cells were assessed including, total CD8+ and CD8+ with granzyme B, perforin or CD161 expression, mucosal associated invariant CD8+ T cells (MAIT: CD161+/TCR Valfa7.2+), NK (CD3-/CD56+/CD16+), NKT (CD3+/CD56+/CD16+) (with or without granzyme B and perforin expression) and iNKT (CD3+/TCR Vbeta11+/TCR Valpha24+). Data acquisition and analysis were performed by using the CytExpert 2.4 software (Beckman Coulter).
Results: NAFLD children compared to controls presented with: a) elevation of granzyme B and perforin expressing NK cells (p= 0.02), b) increase in proportion of CD8+/CD161int T cells (p=0.02), c) decrease of frequency of CD8+/CD161- T cells (p=0.02). The following correlations with clinical parameters were obtained: : 1) CAP vs % perforin expressing CD8+ T cells (r = 0, 287, p = 0, 021) and CAP vs granzyme B (r = 0, 291, p = 0.019,) and perforin (r = 0.294, p = 0.018) expressing NK cells, 2) ALT vs granzyme B (r = 0.392, p = 0.013) and perforin (r = 0.308, p = 0.014,) expressing NK cells, 3) GGTP vs perforin bearing iNKT cells (r = 0.278, p = 0.034).
Conclusions: Proportions of peripheral, natural cytotoxic cells including NK and iNKT expressing granzyme B or perforin may be predictors of NAFLD progress and metabolic disturbances of obesity in early stages of this condition in the pediatric patients.

Basophil Activation Test (BAT) with Annexin 5 (Annexin-Basophil Binding Assay, A-BBA) in diagnosing allergy to Alternaria alternata

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Introduction: The basophil activation test is used to assess the ability of patient's basophil population to activate under the influence of a suspected allergen. The cellular assays with the markers CD63 and CD203c are most commonly utilized to evaluate basophil activation. Annexin 5 (An-5) is a multifunctional protein commonly used as a marker for an early phase of cell apoptosis. However, there are few reports of studies using Annexin 5 as a marker of basophil activation [1,2]

Aim: To investigate if Annexin 5 is an effective basophil activation marker.

Methods: The study comprised experiments on 32 patients allergic to molds with seasonal allergic rhinitis and positive skin prick tests (SPT) to the *Alternaria alternata* allergen mix and positive specific IgE (sIgE) against Alternaria and 33 controls with no allergic symptoms and with negative SPT results and sIgE against *A.alternata. A*-BBA was performed using a Flow CAST kit (Bühlmann Laboratories A.G., Switzerland) and analyzed by flow cytometry. Basophil activation was measured without any stimulation (negative control), with anti-IgE (positive control; 10 µg/ml; Dako Denmark A/S, Denmark), and with culprit allergen extracts in concentrations: 100, 10 and 1 SBU/ml. Basophils were identified with BAT protocol $CCR3^{high}/SSC^{low}$. Basophil activation was estimated as a percentage of basophils expressing An-5 binding. The data was analyzed using CellQuest software (BD Biosciences). Statistical analysis was performed with the use of STATISTICA software (StatSoft, Krakow, Poland). The optimal cut-off values for allergen stimulation, sensitivity, and specificity were determined using receiver operating characteristic (ROC) curve analysis.

Results: In our experiments, the evaluated sensitivity was in the range of 90.6-100% and 100% specificity.

Allergen extract, concentration	cut-off value [%]	Sensitivity [%]; Specificity [%]
A.alternata, 100 SBU/ml	4.95	100.0; 100.0
A.alternata, 10 SBU/ml	10.28	93.8; 100.0
A.alternata, 1 SBU/ml	9.37	90.6; 100.0

Conclusions: Annexin-Basophil Binding Assay is characterized by high sensitivity and specificity and seems to be an effective and cost-saving basophil activation test. It requires further optimization, validation, and standardization before being introduced into clinical practice. Comparative studies should be conducted on the efficacy of Annexin 5 against CD63 and CD203c markers, commonly used for *in vitro* allergy diagnosis.

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Applying Flow Cytometry to Identify New Biomarkers in Alzheimer's Disease and ALS

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Introduction: Due to increased life expectancy, the incidence of neurodegenerative diseases is rising. Deutschl et al. have shown considerable increase in all-age burden of neurodegenerative disease in EU and Europe [1]. Alzheimer's disease (AD) is the most common neurodegenerative disease worldwide but still lacks specific, sensitive biomarkers for presymptomatic detection [2]. Flow cytometry (FC), thanks to its ability to analyze many parameters at the single cell level, can be helpful in identifying biomarkers, assessing immune responses, and understanding disease mechanisms. The use of this analysis technique in AD research could potentially enable diagnosis of the disease before clinical symptoms appear.
Methods: A literature review was performed using scientific articles published on the PubMed and Google Scholar.
Results: Dayarathna, T et al. used nanoscale flow cytometry (nFC) to measure circulating extracellular vesicles (EVs) in blood samples from AD patients. These 100 to 1000 nm particles cross the blood-brain barrier, enabling easily detection in body fluids. Nonetheless, small size of EVs results in low sensitivity using common FC equipment. Thus, to ensure high quality of the results, new generation FC equipment are required [3–5]. Multiple markers were assessed using nFC, including beta-amyloid, accurately distinguished AD samples from healthy controls. In patients with mild cognitive impairment (MCI), elevated levels of p-tau181 and lower levels of neurogranin predict progression to AD [2]. In another study, FC was used to analyze inflammatory markers, including the genetic risk factors LPL and TREM2, in glial cells and synaptosomes from the cerebral cortex of AD patients. This enabled marker quantification and subsequent analysis of the co-occurrence of inflammatory markers and AD [6]. Raineri et.al have investigated using FC that GLAST+ EVs counts are significantly elevated in amyotrophic lateral sclerosis (ALS) compared to healthy control. This study provides a promising avenue for ALS diagnosis and therapy, addressing the shortage of reliable biomarkers [7].
Conclusions: The increasing incidence of neurodegenerative diseases underscores the urgent need for advanced diagnostic tools as well as specific biomarkers. FC shows great promise in identifying biomarkers of AD as well as ALS.

Tracking Mitochondria In Extracellular Vesicles From Different Types Of Mesenchymal Stem Cells

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Introduction: Mitochondrial dysfunction is implicated in the pathogenesis of various diseases. Mesenchymal stromal cells (MSCs) play a crucial role in mitochondrial transfer, including the encapsulation of whole mitochondria or mitochondrial fragments within extracellular vesicles (EVs). Mitochondria-containing EVs (mito-EVs) and mitochondria-derived EVs (mito-derived EVs) have recently been identified as important mediators of intercellular communication. Understanding the mechanisms of EV-mediated mitochondrial transfer is essential for elucidating the mechanisms behind mitochondria-related diseases and developing novel therapeutic strategies. The aim of this study was to isolate EVs from bone marrow MSC (BMMSCs) and menstrual blood MSCs (MenSCs) supernatants and characterise them for the presence of mitochondrial thiols (MitoTrackerGreenFM (MTG)) and a proton gradient (MitoTrackerDeepRed (MTDR)).
Methods: Mito-EVs were isolated by centrifugation at 12 000 x g for 20 mins from BMMSCs and MenSCs supernatants. Mito-EVs were detected by flow cytometry (Cytoflex LX) and labeled with either MTG, MTDR, and the EV markers (CD9, CD63, CD81).
Results: Both BMMSCs and MenSCs had MTG+EVs, while a portion of MTG+EVs were also MTDR+, indicating the presence of healthy mitochondria in EVs. Comparison of the MTG+EVs revealed 2 distinct populations that could be separated based on size - small EVs (SEVs, 1000 nm). LEVs had more MTG and MTDR staining than SEVs, with MTG+MTDR+EVs also having 3-fold more MTG staining than MTG+EVs regardless of EV size. MenSC LEVs had lower MTG staining compared to BMMSCs but the difference was not significant. All EV samples were positive for CD9+/CD63+/CD81+, however MenSC EVs had 8-fold more positive LEVs than SEVs, while BMMSC EVs had 15-fold more positive LEVs than SEVs. In addition, MenSC CD9+ LEVs were significantly larger than BMMSC.
Conclusions: Mito- and Mito-derived EVs were successfully isolated from BMMSCs and MenSCs supernatants, with LEVs being more likely to be positive for mitochondrial thiols and a proton gradient and EV markers than SEVs. Only LEVs are large enough to contain full, intact mitochondria, however further work is needed to determine if the presence of a proton gradient indicates these Mito-EVs are metabolically active and if they can affect the metabolism of the recipient cells.

6 WAYS TO KILL YOUR FACS STAINING

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Introduction: Dendritic cell vaccines are an established advanced therapy medicinal product (ATMP) applied for cancer treatment in many clinical settings. Manufacturing under Good Manufacturing Practice (GMP) guidelines requires stringent quality control measures to ensure safety and optimal product functionality. To that end, flow cytometry is used to assess maturation of dendritic cell-based vaccines. Variations in staining protocols and handling conditions may affect the accuracy of quality control assessments, which may directly impact patients treatment outcomes.
Aim: To identify factors that affect the accurate flow cytometry measurement of viability and CD11c, CD83, CD80 and HLA-DR marker signal strength on dendritic cells by evaluating several conditions.
Methods: We performed surface marker staining using antibodies coupled with various fluorochromes of potentially different resistance to bleaching, as well as 7-AAD staining. Cells were stained and collected on LSR II flow cytometer after being subjected to several different conditions that could result in distortion of registered signal or in decrease in sample quality. Investigated scenarios included:

1. Dependence of signal strength on light exposure during staining incubation
2. Dependence of viability on thawing temperature
3. Degradation of viability and signal strength throughout periodic measurement intervals
4. Effect of delayed staining on cell viability
5. Effect of temperature microshocks performed on frozen cells on cell viability post-thaw
6. Effect of incubation temperature on marker signal strength

Results: We validated the thawing and staining process and proved that it is resilient and can accommodate minor fluctuations in the majority of investigated factors. Viability proved to be the most time-sensitive measurement, which has a direct impact on product quality assessments. Additionally, we found that in the staining process, fluorochrome degradation in time and during light exposure can influence data analysis due to signal loss.
Conclusions: To ensure optimal measurement accuracy, each combination of fluorochromes will have to be validated separately because degradation is fluorochrome-specific.

Anti-inflammatory activity of novel amidrazone derivatives having cyclohex-3-enecarboxylic acid substituent

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Introduction: Developing new bioactive small molecules remains an important task in medical chemistry, aiming to discover novel agents with improved therapeutic activities (Beck H, Drug Discov Today. 2022). Amidrazone derivatives have gained considerable attention due to their wide spectrum of biological activities, such as antibacterial, anti-inflammatory, and antitumor effects. Notably, structural modifications of these compounds have been shown to significantly influence their pharmacological potential, particularly through the incorporation of 1,2,4-triazole ring, known for their bioactivity and stability (Habashneh AY, Med Chem. 2018; Aggarwal R J Med Chem. 2020).

Methods: New derivatives 3a-3f were obtained in reaction of amidrazones with cis-1,2,3,6-tetrahydrophthalic anhydride. Structures of new compounds were confirmed by spectral analyses (1H and 13C NMR). Freshly isolated human peripheral blood cells (PBMCs) were cultured with compounds 3a-3f (obtaining final concentrations of 10, 50 and 100 μg/mL) to study: their toxicity (by Annexin V Apoptsis Detection Kit I, flow cytometry); their antiproliferative activity towards phytohemagglutinin (PHA) stimulated PBMCs (using Violet Proliferation Dye 450 in flow cytometry);their influence on proinflammatory cytokines production (TNF-α and IL-6) and anti-inflammatory (IL-10) in PBMC cultures stimulated with LPS (by ELISA). Ibuprofen (IBU) was used as a reference anti-inflammatory drug. The study was approved by the Bioethics Committee of the Nicolaus Copernicus University in Torun (Poland).

Results:

- The compounds 3a-3f showed no toxic effect towards PBMC;
- Derivatives 3a, 3e, and 3f effectively reduced TNF production;
- 1,2,4-triazole derivatives (3a-c, 3e, 3f) significantly enhanced IL-10 production;
- Compounds 3a-3f showed low effect or lack of anti-proliferative activity against PBMC.

Conclusions: Using cytometric and ELISA techniques the novel acyl amidrazone derivatives were characterized as compounds with significant anti-inflammatory properties and no toxic effects. The newly synthesised derivative 3a-3c and 3e-3f significantly elevated IL-10 release especially in low and medium doses. The strongest effect was observed for compound 3e possessing 4-pyridine ring in R1 position. Observed effect may be related to the cyclic structure and presence of the 4-pyridine ring, but requires further investigation.

In vitro human kidney organoid model to study ischemia-reperfusion injury

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Introduction: Kidney ischemia-reperfusion injury (IRI) is important clinical problem frequently leading to acute kidney injury (AKI). The reduction and subsequent restoration of blood flow during IRI causes damage to kidney structures. Kidney organoids represent a novel yet well-established technique to study kidney diseases. Here we focused on developing robust kidney ischemia-reperfusion injury model using kidney organoids. We performed analysis to determine optimal hypoxia time followed by two hours of reoxygenation.
Methods: Kidney organoids derived from human induced pluripotent stem cell (iPSC) were used for this research according to the protocol of Garreta et al. Kidney organoids were subjected to low oxygen condition (1%O2, 37°C) and cultured for 5, 24 or 48 hours subsequently moved to standard oxygen condition (21% O2, 37°C) for another 2 hours to mimic reperfusion phase of IRI. Control groups were matched with experimental groups and were cultured in standard oxygen conditions for 5 + 2, 24 + 2 and 48 + 2 hours. Later, kidney organoids were harvested for future experiments and the culture medium was separately collected after both hypoxia and reoxygenation and frozen for LDH and proteins assessment. Pyroptosis-related protein: caspase 1 was evaluated. To determine the level of oxidative stress carbonylated protein content was determined and LDH activity was measured in the culture medium to indicate cell damage. qPCR was performed to determine the expression of KIM-1, Bax, Bcl-2 and p53 gene expression. Apoptosis and necrosis were assessed via flow cytometry.
Results: Kidney organoids subjected to 48 hours hypoxia and 2 hours reoxygenation showed intensified oxidative stress that was manifested in higher carbonylated protein concentration. LDH activity and caspase-1 concentration were increased after 24 and 48 hours of hypoxia and 2 hours of reoxygenation in comparison to corresponding control groups. qPCR proved increased KIM-1, p53 and Bcl-1 gene expression after 48 hours of hypoxia and 2 hours of reoxygenation. However, flow cytometry detected a small increase of apoptotic cells after 24 hours of hypoxia and 2 hours reoxygenation.
Conclusions: We proved that 48 hours of hypoxia and 2 hours of reoxygenation are the optimal conditions to study ischemia-reperfusion injury on kidney organoid models.

Prophylactic use of bacteriophage-derived dsRNA only slightly modulates lung macrophage response in SARS-CoV-2 infected K18-hACE2 mice

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Introduction: Alveolar macrophages play a key role in the immune response to respiratory viral infections, including SARS-CoV-2. In this study, we investigated the effects of prophylactic administration of bacteriophage-derived dsRNA (Larifan) on monocyte and lung macrophage populations in transgenic K18-hACE2 mice infected with SARS-CoV-2. The primary objective was to determine whether Larifan might provoke additional immune activation in the case of SARS-CoV-2 infection.
Methods: K18-hACE2 transgenic mice, expressing the human ACE2 gene, were intranasally infected with SARS-CoV-2 (1×10^4 PFU). The treatment group received Larifan (5 µg/kg) six hours before infection, while control mice received only the virus. In each group were five animals.Lung tissues were collected on days 3 and 5 post-infection for flow cytometry analysis. Lung cell suspensions were assessed for total, alveolar, and monocyte-derived macrophage populations using the markers CD45, CD64, F4/80, CD11c, MHCII, CD88, SiglecF, and CD163. The presence of SARS-CoV-2 in lung and brain tissues was confirmed via ddPCR and immunostaining for SARS-CoV-2 nucleoprotein.
Results: Larifan administration did not significantly alter the proportion of monocyte-derived or alveolar macrophages among total lung macrophages compared to viral infection alone. However, by day 5 post-treatment, a significant reduction in monocyte-derived macrophages ($p = 0.0003$) and total macrophages within the leukocyte population ($p = 0.0108$) was observed. These findings suggest that prophylactic Larifan administration may help shorten disease duration without inducing immune hyperstimulation.
Conclusions: Our results indicate that Larifan may modulate inflammatory responses in alveolar macrophages while maintaining their homeostatic functions, potentially contributing to improved disease outcomes in SARS-CoV-2 infection.

Bacteriophage-Derived dsRNA Improves Survival Without Inducing Hyperinflammation in SARS-CoV-2-Infected K18-hACE2 Mice

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Introduction: The SARS-CoV-2 pandemic has highlighted the need for novel antiviral therapies. While direct-acting antivirals are essential, immune-modulating agents offer an alternative approach to enhancing host defences by limiting viral replication and mitigating disease severity. One such agent is bacteriophage-derived double-stranded RNA (dsRNA), Larifan, which has demonstrated immunomodulatory properties. However, its effects on cytokine and chemokine responses, particularly regarding immune overstimulation, remain unclear. This study investigates the efficacy and immunomodulatory effects of prophylactic Larifan treatment by assessing survival and cytokine profiles in SARS-CoV-2-infected K18-hACE2 mice.

Methods: Transgenic K18-hACE2 mice, expressing the human ACE2 gene, were intranasally infected with SARS-CoV-2 (1×10^4 PFU). The treatment group (n=5) received Larifan (5 µg/kg) six hours before infection, while control mice (n=5) received only the virus. Mice were monitored for weight changes and disease progression until reaching humane endpoint criteria. Serum samples were collected on days 3 and 5 post-infection for cytokine and chemokine analysis using the LEGENDplex™ immunoassay. The levels of IFN-γ, CXCL1, TNF-α, CCL2, IL-12, CCL5, IL-1β, CXCL10, GM-CSF, IL-10, IFN-β, IFN-α, and IL-6 were measured. SARS-CoV-2 presence in lung and brain tissues was confirmed via ddPCR and immunostaining for viral nucleoprotein.

Results: Untreated mice reached the humane endpoint within 4–7 days, while Larifan-treated mice exhibited delayed symptom onset, with some surviving asymptomatically until day 16 ($p < 0.001$, Log-rank Mantel-Cox test). Eight of thirteen analysed cytokines and chemokines were detected: IFN-α, TNF-α, IL-6, IL-1β, CXCL1, CCL2, CCL5, and CXCL10. GM-CSF, IL-10, IL-12, IFN-β, and IFN-γ were below detection thresholds. No significant differences were observed between groups, suggesting that Larifan did not induce excessive immune activation. Instead, it modulated cytokine responses, potentially contributing to improved survival.

Conclusions: These findings suggest that Larifan may serve as an effective immunomodulatory agent in viral infections by regulating cytokine responses without exacerbating inflammation.

Phenotypic Assessment of Peripheral Blood NK Cells in Patients with Dilated and Ischemic Cardiomyopathy

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Introduction: Cardiovascular diseases (CVDs) are a leading cause of mortality worldwide. Heart failure (HF) affects approximately 26 million people, with 60,000 deaths and 150,000 hospitalizations annually in Poland. Early diagnosis remains challenging due to nonspecific symptoms. Recent studies suggest that immune response and inflammation play key roles in HF development. Natural killer (NK) cells are crucial in infection defense, which may lead to myocarditis and HF, making them an important subject of study in inflammation-related HF. This study aims to analyze the phenotype of peripheral blood NK cells in patients with dilated (DCM) and ischemic cardiomyopathy (ICM). Understanding the immune response regulation in HF, particularly NK cell involvement, could provide insight into their cytotoxic and immunoregulatory functions.
Methods: Peripheral blood samples (3 mL) were collected and stored at 4°C for up to 5 days. NK cell percentages and cytotoxicity were assessed using flow cytometry (CytoFLEX, Beckman Coulter) with specific antibodies. Data were analyzed using CytExpert 2.4 and Statistica 13.3. The Shapiro-Wilk test checked data normality, and the Mann-Whitney U test analyzed non-normally distributed data. Results were statistically significant at $p < 0.05$.
Results: NK Cell Population Percentage: No statistically significant differences in NK cell percentages were observed between patient groups and the control ($p > 0.05$). NK Cell Cytotoxicity: The ICM group had significantly lower perforin-expressing NK cells compared to the control ($p < 0.05$). Additionally, granzyme B and perforin gMFI were significantly reduced in ICM (p 0.05). CD161 Marker Expression: The ICM group showed a significantly reduced percentage of $CD161^+$ NK cells compared to the control (p 0.05).
Conclusions: The NK cell population did not significantly differ between groups. However, NK cell activation was markedly reduced in ICM, demonstrated by lower cytotoxic factor expression (granzyme B, perforin) and decreased $CD161^+$ NK cells. Myocardial ischemia in ICM may influence NK cell activity.

The application of machine learning and artificial intelligence in the analysis of flow cytometry data

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Advances in flow cytometry have allowed the generation of extremely complex and multidimensional data, which pose significant analytical challenges. In recent years, artificial intelligence (AI) and machine learning (ML) have revolutionized approaches to analyzing this data. Used algorithms have been instrumental in detecting relationships imperceptible to human analysis.

PubMed and Google Scholar platforms were used for the review. Search queries included phrases such as: 'machine learning in flow cytometry', "artificial intelligence in mass cytometry", and "cytometry data analysis using AI". Inclusion criteria included peer-reviewed articles from 2019-2025.

Deep learning models, including neural networks, have demonstrated considerable potential in flow cytometry in the identification of rare cell subpopulations and the prediction of immune responses in cancer and autoimmune diseases[10.1053/j.semdp.2023.02.004; 10.3791/64549]. The utilization of a diverse array of algorithms in conjunction with machine learning (ML) methodologies has been demonstrated to markedly enhance the visualization and interpretation of multidimensional cytometric data[10.1016/j.cll.2023.04.009]. AI models have the potential to be executed in real-time, thereby facilitating dynamic cell sorting and enhancing diagnostic performance [10.1038/s41598-019-47193-6]. In 2018, a clinical trial was initiated on the yPath Lung program, in which a rapid method for detecting early-stage lung cancer from sputum was developed through ML and automated cytometry. The initial results were released in 2023, demonstrating that the program accurately classified samples as either cancerous or non-cancerous[10.1186/s12931-023-02327-3]. A further study published in 2025 also underscores the significance of ML in cancer diagnosis. The authors developed a methodology that employs size-encoded gel microspheres in conjunction with flow cytometry and AI analysis to facilitate the automated diagnosis of lung cancer, along with the identification of a specific lung cancer subtype with an accuracy of 80%[10.1021/acs.nanolett.4c05233]. Ensuring the interpretability of models and their validation in a clinical setting remains one of the main challenges. The extant literature further indicates a growing need for data standardization and the development of publicly available collections that will accelerate the development of algorithms dedicated to cytometry[10.3389/fimmu.2021.787574].

The tools and strategies presented in this review demonstrate the potential of AI and ML in transforming the analysis of cytometric data and the automated identification of diagnostic biomarkers.

The Role of the Notch Signaling Pathway in KLE Endometrial Cancer Cells

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The Notch signalling pathway regulates key cellular processes such as proliferation, differentiation, and survival. It is now known that this pathway can exhibit oncogenic or tumor-suppressing functions in many types of cancer, endometrial cancer being one of the examples. However, its involvement in endometrial cancer development remains unclear. To better understand the role of Notch signalling in endometrial oncogenesis, we studied the effects of Notch pathway inhibition and activation on the viability of the KLE endometrial cancer cell line. We further examined KLE cells by studying the effect of Notch signalling inhibition on cell cycle progression, apoptosis, sensitivity to chemotherapeutic drugs and motility. To achieve that we used two Notch pathway inhibitors: Nirogacestat, a γ-secretase inhibitor, and IMR-1, an inhibitor of the Notch transcriptional activation complex. Additionally, we employed Ataluren, a probable Notch signalling pathway activator that inhibits early translation termination due to nonsense mutations. We determined that both Nirogacestat and IMR-1 caused a significant decrease in cell viability while Ataluren showed no notable impact. Furthermore, Notch signalling inhibitors did not cause major changes in KLE cell cycle progression, motility or their susceptibility to chemotherapy. However, we discovered that both nirogacestat and IMR-1 promoted KLE cell apoptosis. Our findings indicate that Notch signalling inhibition may be a potential approach to cure endometrial cancer in a targeted manner. Nevertheless, further studies are required to fully grasp the involvement of the Notch signalling pathway in endometrial carcinogenesis and to determine its effectiveness in clinical practice.

Optimizing Flow Cytometry for Accurate Hypoxia Assessment

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Introduction: Low oxygen state, or hypoxia, triggers bioenergetic stress that can lead to adaptive physiological responses. These responses play a crucial role in processes such as embryonic development and the progression of various diseases like cancer. A deeper understanding of cellular adaptations to hypoxia is essential for uncovering novel therapeutic strategies. Flow cytometry is widely used to assess the effects of hypoxia on cells, however, discrepancies between assay conditions and measurement environments—particularly variations in oxygen levels and temperature—may impact the accuracy of results. Addressing these challenges is key to improving the reliability of flow cytometry in hypoxia research.
The aims of this study were:

- identify an easy and reliable method to be able to perform the analysis of cells under growing conditions,
- analyse if the values obtained under these conditions remain stable when samples are transferred to different oxygen level conditions.

Methods: The cancer cell line (A549) cells were grown under hypoxic (0.5% O2) and normoxic (21% O2) conditions, optimising the analysis of different physiological parameters by flow cytometry under these conditions: scatter changes, cell viability with DRAQ7, mitochondrial membrane potential with JC-1, reactive oxygen species with DCFDA and hypoxic levels with HypoxiTRACK. The analysis of these physiological markers has been done during 5 consecutive days at 0.5% O2 and 21% O2. At day 5, cells were relocated to the other oxygen conditions of growth (from hypoxic to normoxic conditions and vice versa) and analysed at different time points during the first hour after the change (0, 15, 30, 45 and 60 min).
Results: Until now, the analysis of the cell physiology/responses under hypoxic conditions have been done under normoxic conditions. With this assay, we have demonstrated that when oxygen levels are dramatically altered, the physiology of the cells changes quickly and in a very dynamic way, indicating that the analysis of cell responses to drug treatment should be optimally performed under the same environmental conditions of the study.
Conclusions: In summary, our approach allows a better alternative to analyse the physiological difference of cells growing at different environmental conditions by flow cytometry.

Bacteriophage-Derived dsRNA Improves Survival Without Inducing Hyperinflammation in SARS-CoV-2-Infected K18-hACE2 Mice

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Introduction: The SARS-CoV-2 pandemic has highlighted the need for novel antiviral therapies. While direct-acting antivirals are essential, immune-modulating agents offer an alternative approach to enhancing host defences by limiting viral replication and mitigating disease severity. One such agent is bacteriophage-derived double-stranded RNA (dsRNA), Larifan, which has demonstrated immunomodulatory properties. However, its effects on cytokine and chemokine responses, particularly regarding immune overstimulation, remain unclear. This study investigates the efficacy and immunomodulatory effects of prophylactic Larifan treatment by assessing survival and cytokine profiles in SARS-CoV-2-infected K18-hACE2 mice.
Methods: Transgenic K18-hACE2 mice, expressing the human ACE2 gene, were intranasally infected with SARS-CoV-2 (1×10^4 PFU). The treatment group (n=5) received Larifan (5 µg/kg) six hours before infection, while control mice (n=5) received only the virus. Mice were monitored for weight changes and disease progression until reaching humane endpoint criteria. Serum samples were collected on days 3 and 5 post-infection for cytokine and chemokine analysis using the LEGENDplex immunoassay. The levels of IFN-γ, CXCL1, TNF-α, CCL2, IL-12, CCL5, IL-1β, CXCL10, GM-CSF, IL-10, IFN-β, IFN-α, and IL-6 were measured. SARS-CoV-2 presence in lung and brain tissues was confirmed via ddPCR and immunostaining for viral nucleoprotein.
Results: Untreated mice reached the humane endpoint within 4–7 days, while Larifan-treated mice exhibited delayed symptom onset, with some surviving asymptomatically until day 16 ($p < 0.001$, Log-rank Mantel-Cox test). Eight of thirteen analysed cytokines and chemokines were detected: IFN-α, TNF-α, IL-6, IL-1β, CXCL1, CCL2, CCL5, and CXCL10. GM-CSF, IL-10, IL-12, IFN-β, and IFN-γ were below detection thresholds. No significant differences were observed between groups, suggesting that Larifan did not induce excessive immune activation. Instead, it modulated cytokine responses, potentially contributing to improved survival.
Conclusion: These findings suggest that Larifan may serve as an effective immunomodulatory agent in viral infections by regulating cytokine responses without exacerbating inflammation.

Flow cytometry analysis of Yersinia enterocolitica O:3 outer membrane vesicles endocytosis by epithelial cells

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Introduction: Outer membrane vesicles (OMVs) are secreted by Gram-negative bacteria and are supposed to be involved in the pathogensis of systemic inflammatory response syndrome (SIRS). They were shown to spread in the host body and could be found in body fluids and tissue sections.
We investigated the mechanism of LPS structure-dependent-endocytosis of OMVs by epithelial cells. Our model bacterium was Yersinia enterocolitica O:3 (YeO3), able to grow in a wide range of temperature (4-45°C) synthesizing surface-exposed lipopolysaccharide (LPS) with an unique arrangement, where the inner core (IC) is substituted with an O-specific polysaccharide (OPS) or an outer core oligosaccharide (OC). Like other Y. enterocolitica virulence factors, LPS expression is influenced by temperature, with diminished expression of OPS at higher temperatures.
We investigated the OMVs secreted by YeO3 bacteria expressing LPS with long polysaccharide chains (S-chemotype) and its variants with truncated LPS (Ra-, Rd1-, and Re-chemotypes).
Methods: OMVs were isolated from sterile culture supernatants (late logarithmic growth phase) by ultracentrifugation. Their concentration and size were analysed by NTA and SEM/TEM. After labelling with lipophilic carbocyanine dye (DiO), the uptake of OMVs (with/without opsonisation with normal human serum) by epithelial lung cells (A549) was analysed by flow cytometry and fluorescence microscopy. Several inhibitors of endocytosis were used to investigate the mechanisms of OMVs internalization.
Results: The DiO-labelled OMVs were internalised by A549 cells within 3 hours of incubation. The presence of normal human serum affected that process: OMVs preincubation with NHS hindered their uptake by epithelial cells. The OMVs internalisation was inhibited by dynasore (dynamin-inhibitor) and cytochalasin D (actin polymerization inhibitor) but not by papain (cysteine protease) and filipin (lipid raft-inhibitor))
Conclusion: Our results suggest that OMVs secreted by Y. enterocolitica O:3 may be internalized by A549 cells through two main pathways of endocytosis: clathrin-dependent and actin-polimerization-dependent (fusion membrane and/or micropinocytosis).
The LPS-chemotype, growth temperature of bacteria as well as opsonisation with serum factors may affect the process of OMVs' internalization.