# PROCEEDINGS OF THE XXXIX NATIONAL CONFERENCE OF THE ITALIAN SOCIETY OF CYTOMETRY GIC

Società Italiana di Citometria

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# PROCEEDINGS

THE ITALIAN SOCIETY OF CYTOMETRY
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# Hematology

## FLOW-CYTOMETRIC IDENTIFICATION OF HODGKIN REED-STERNBERG CELLS AND ASSESSMENT OF PD-L1 EXPRESSION IN LYMPH NODE CELL SUSPENSIONS

Bellesi S.1, Maiolo E.1, Alma E.1, Fatone F.1, Viscovo M.1, Puggioni PL.1, Marchionni F.1, D'Innocenzo S.1, Meacci E.2, Corina L.3, Fiorita A.3, D'Alò F.1, Larocca LM.4, Hohaus S.1
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Flow-cytometric (FC) identification of Reed-Sternberg cells (HRS) in Hodgkin Lymphoma (HL) is challenging. Fromm JR described a 6-colour assay to identify cells with HRS features. By definition, a putative HRS cluster shows expression of CD30, CD40, and CD95, with increased FSC and SSC. CD3 expression is due to T-cell–HRS-cell rosettes. CD20 and CD45 are usually negative or low. The aim of our study was to assess the diagnostic power of an 8-colour HRS tube on lymph node biopsies.
Our panel derived from the tube proposed by Fromm plus CD45 and PDL1, as follow: CD20V450/CD45V500/CD64FITC/CD30PE/CD40PECy5.5/PDL1PECy7/CD95APC/CD3APCH7.
Cell suspensions were obtained with the Medimachine. Data were acquired and analyzed by DXFLEX Cytometer (Beckman Coulter).
Histological diagnosis was HL in 52/350 biopsies. We performed the HRS tube in 41 cases. According to FC limit of quantification we considered a cluster of at least 50 events with phenotypic features of HRS as diagnostic. We observed a typical HRS cluster in 29/41 (71%) cases and a cluster between 20 and 50 events in 6 cases. No HRS events were found in the remaining 6 cases. Therefore, a putative HRS cluster was observed in 35/41 cases (positive predictive value 85.3%). The median number of total acquired events was 511500 (274000-905000) in samples with a HRS cluster and only 39000 (5000-190000) in samples without a HRS cluster (p 0.0002).
The median percentage of HRS cluster was 0.04% (0.009-1.2) of total events with a median absolute number of 236 HRS events (20-3371).
CD40 and CD95 expressions were always bright separating HRS cells from the rest of leukocytes populations. The 7 decades dynamic range of DXFlex instrument optimizes the detection of brilliant CD40 and CD95 positive HRS events. Expression of CD30, CD3 and CD20 was heterogeneous. PDL1 was analyzed in 6 cases. Its expression on HRS cells was compared to expression on CD3+ T lymphocytes and myeloid cells. PDL1 was absent on T cells. It was strongly and homogeneously expressed on HRS cells in all cases and expressed with lower intensity on neutrophils and CD64+ monocytes (median MFI 403367, 7651 and 26109 respectively, Kruskal-Wallis test, p 0.002). No difference was found between PDL1 MFI on neutrophils and monocytes.
Finally, in a group of 21 NHL we observed a putative HRS cluster only in one case with a histological diagnosis of T cell lymphoma (median number of acquired events 300000; negative predictive value 95.2%)
We conclude that HRS cells can be rapidly detected by FC in lymph node suspensions in the great majority of cases when sampling yield a sufficient cellular population for analysis. PDL1 assessment may improve the accuracy of HRS detection compared to 6-colour panel. However, HRS cell detection requires high analytical sensitivity and specificity that can be reached by increasing number of acquired events. Moreover, this assay allows the quantitative assessment of therapeutic target antigens, such as CD30 and PDL1.

# THE CONTRIBUTION OF FLOW CYTOMETRY TO THE DIAGNOSIS OF AGGRESSIVE B CELL LYMPHOMA: ANALYSYS OF 97 LYMPH NODE BIOPSIES. A SINGLE CENTER EXPERIENCE

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We describe our flow-cytometric (FC) diagnostic approach to lymph node biopsies in patients (pts) with histological diagnosis of aggressive B cell lymphomas, in particular diffuse large B cell lymphoma (DLBCL) to evaluate the correlation between FC and immunohistochemistry (IHC).
Cell suspensions were prepared by mechanical disaggregation of 97 solid tissues (65 surgical resections, 19 radiologically guided biopsies and 13 endoscopic core biopsies) using the Medimachine. The cell suspensions were incubated with 8 surface markers including the main diagnostic antigens of B-cell lymphomas (KappaV450/CD45V500/CD20FITC/CD79bPE/ CD5PerCpCy5.5/CD19PECy7/CD10APC/LambdaAPCH7). Data were acquired with BDFACSCantoII (BD) and DXFlex (Beckman Coulter) cytometers.
Our FC algorithm to detect an aberrant CD19+ B cell population suggestive for DLBCL consisted in the identification of a surface immunoglobulin light chain clonality or the absence of light chains expression in combination with increased FSC-A and SSC-A physical parameters. We observed a phenotypically aberrant mature B cell cluster in 77/97 cases. A clonal B cell population was observed in 71 cases. In 6 cases we noted the absence of surface light chain on pathological B cells and four of these had an histological diagnosis of primary mediastinal large B cell lymphoma.
WBC count of the cell suspension was an important factor for the diagnostic sensitivity. The median leukocyte count was 68000/$\mu$l in diagnostic cases and 1350/$\mu$l in non-diagnostic cases respectively (p=0.04).
FSC-A and SSC-A median values were evaluated on pathological CD19+ B cells and CD5+ T cells. We defined FSC-ratio and SSC-ratio as the ratio between the median value of FSC-A and SSC-A in these two populations respectively. By calculating the ROC curve, the best cut-off value to differentiate DLBCL from 34 cases of different B-cell NHL (28 FL, 4 MCL, 2 MZL) were FSC-ratio >1.28 and SSC-ratio >1.44, with an AUC=0.90. This cut-off value provided a sensitivity of 76% and a specificity of 94%. The positive predictive value was 82%.
Moreover, we compared expression of markers assessed in FC with IHC results; there was a high concordance for CD20, CD10 and CD5 positivity (Fisher test p<0.0001).
CD5 expression, that defines a subgroup of DLBCL with an inferior prognosis, was identified in 18% of cases. Moreover, we observed that CD20 MFI value was significantly lower in CD5+ DLBCL (Mann-Whitney p 0.02).
No correlation was found between CD79b expression in FC (positive in 55/77 cases) and CD79a expression in IHC (positive in 75/77 cases).
We conclude that FC is a useful and rapid tool to identify with a high sensitivity and specificity aggressive B cell lymphomas and can support the IHC work-up in the diagnosis of DLBCL. Therapeutic targets, such as CD20 and CD79b can be detected in a quantitative approach with the perspective to identify markers not only for prediction of the pts's prognosis but eventually to guide therapeutic choices.


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# MORE THAN A SURFACE MARKER: THE RELEVANCE OF CD200 SERUM LEVELS IN PREDICTING PROGNOSIS OF CHRONIC LYMPHOCYTIC LEUKEMIA

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The evaluation of CD200 expression has shown to be a useful tool to better classify chronic lymphoproliferative diseases. Chronic lymphocytic leukemia (CLL) overexpresses surface CD200 with respect to other lymphoid leukemias, in particular mantle cell lymphoma. Moreover, there is some evidence that serum levels of soluble CD200 (sCD200) could be related to disease progression in pts with CLL. However, very little is known about sCD200 prognostic significance.
In light of this, serum samples were collected at diagnosis from 272 pts with CLL (median age 66 yrs, range 33-90; 58% males) and from 78 age and sex-matched healthy subjects (median age 63 yrs, range 42-100; 58% males), used as normal controls. Human CD200 (OX-2 membrane glycoprotein) ELISA kit (Wuhan Fine Biotech Co., Ltd., Wuhan, Hubei, China) was used to quantify sCD200 in serum samples.
A significantly higher concentration of sCD200 in CLL pts than in controls (median, 1281 pg/ml vs 799 pg/ml; p=0.0002) was found. In pts with CLL, sCD200 was significantly higher in those ≥66 vs <66 yrs old (median, 1560 pg/ml vs 1193 pg/ml; p=0.0001), in those with Binet stage C vs A/B (2055 pg/ml vs 1274 pg/ml; p=0.0045), in those with unmutated vs mutated IgVH (1601 pg/ml vs 1131 pg/ml; p<0.0001), and in those with unfavorable (del11q or del17p) vs favorable (normal or del13q or tris12) FISH (1897 pg/ml vs 1239 pg/ml; p=0.0077). On the contrary, gender, bulky disease, whole blood cell or lymphocyte count, β2-microglobulin serum levels and presence of autoimmune complications did not significantly correlate with sCD200. Time-to-first-treatment (TTFT) was shorter in pts with higher sCD200 levels (sCD200 >1281 pg/ml vs <1281 pg/ml, median TTFT, 61 vs 109 months; p<0.001). Furthermore, baseline sCD200 values appear to have an impact on response to therapy (median in CR vs PR/NR pts, 1308 pg/ml vs 1590 pg/ml; p 0.0468), and this difference seems to increase if only pts who received chemotherapy or chemo-immunotherapy are considered (1244 pg/ml vs 1602 pg/ml; p=0.0193). However, we did not find an association between baseline sCD200 values and response to targeted agents. Finally, sCD200 also had an impact on overall survival (OS) (sCD200 >1281 pg/ml vs <1281 pg/ml; median OS, 222 vs 299 months; p=0.005).
A correlation between sCD200 serum levels at diagnosis and the prognosis of pts with CLL was found: higher sCD200 correlated with more aggressive clinical and biological features and was able to predict a worse prognosis. It has been previously shown that CD200 can be released from CD200+ neoplastic cells by ectodomain shedding. Both the membrane and soluble forms of CD200 are also able to engage CD200 receptor, which in turn can result in increased tumor growth, by means of a negative impact on tumour immunosurveillance. Our data further support the relevant role of CD200 not only as a diagnostic tool but also as a prognostic indicator and a potential therapuetic target in CLL.

# WHAT DOES ATYPICAL CHRONIC LYMPHOCYTIC LEUKEMIA REALLY MEANS? MORPHOLOGY VS IMMUNOPHENOTYPE

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Atypical chronic lymphocytic leukemia (CLL) is defined according to the morphologycal features of peripheral blood lymphocytes well established by French-American-British (FAB) in 1989. However, no commonly accepted criteria have been proposed so far to differentiate atypical from typical CLL only on the basis of the immunophenotypic profile. Neverthless, some abnormalities of the typical immunophenotypic profile of CLL (i.e., high expression of CD20 and/or CD22 and/or surface membrane immunoglobulins [smIg], CD79b and/or FMC7 expression) are commonly used to identify immunophenotypic atypical form of CLL.
May-Grunwald Giemsa peripheral blood smears collected and stored at diagnosis from 72 patients (mean age 68 years; range 48-89 years, 46 [61%] males) with CLL diagnosed at our Institution and with a follow-up longer than 12 months were reviewed by two of us (G.D. and G.P.). Medical records of these patients were also evaluated aiming at establishing if there is a correlation between the morphological and immunophenotypic definition of atypical CLL and if the so-called discordant cases (patients without such a correlation, i.e., typical for morphology but with atypical immunophenotype, or otherwise) displayed relevant biological and clinical features. We have arbitrarely chosen to classify as immunophenotypic atypical CLL all cases in which the deviation from typical immunophenotypic profile was found for at least two antigens.
Twenty-eight (39%) patients were found discordant for morphology and immunophenotype and 48 (61%) concordant. No differences were found according to Binet clinical stage, leukocyte and lymphocyte count, hemoglobin levels and platelet count, IgVH (mutated or unmutated) status, FISH abnormalities according to Dohner classification, LDH and beta2-microglobulin serum concentrations. Finally, time-to-first-treatment and overall survival were also found not different.
In our hands, the discrepancy of neoplastic B lymphocytes for morphological and immunophenotypic features did not appear as a category risk of CLL. However, the small series of patients evaluated does not allow us to draw firm conclusions. Further evaluation are currently ongoing to try bo better define the significance of "atypical" CLL and the role of immunophenotype or morphology, or both, to identify patients with a better or worse prognosis.


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# HODGKIN (HL), FOLLICULAR (FL) AND DIFFUSE LARGE B CELL (DLBCL) LYMPHOMAS MICROENVIROMENT : CD26, CD38 AND CD39 ANALYSIS BY FLOW CYTOMETRY (FC)

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Introduction: Within the tumor microenvironment the adenosine, an immunosuppressive metabolite, is produced at high levels by CD38 and CD39 ectoenzymes and is accumulated due to the absence of the CD26 molecule. In a paper we have already showed how CD3+CD4+CD26-CD38+ lymphocytes characterize the immunosuppressed microenvironment of HL. We have analyzed CD26, CD38 and CD39 expression on CD4 T lymphocytes in order to verify whether they can characterize and distinguish the HL, FL and DLBCL microenvironment.
Materials and methods: Cell suspensions obtained by dissociating 21 non-neoplastic lymph nodes (N-N), 21 DLBCL, 23 FL and 15 HL were acquired by NAVIOS (BC) cytometer using the combination CD38Fitc/CD26Pe/CD3PC5.5/CD4PC7/CD39APC in order to analyze the positivity and intensity of antigen expression in CD4 T.
Results: CD38 was expressed on 28% of CD4 cells in N-N, 31% in DLBCL, 27% in FL and 52% in HL which significantly differs ($p<0,05$) from the other groups. All the subtypes significantly differed from N-N (11%) in the CD26-CD38+ subset and the high value (42 % of the CD4 cells) distinguishes HL from both FL (20%) and DLBCL (23%). This test resulted to have a high accuracy as obtained by the ROC curve analysis: the AUC was 0.9429 (95% CI: 0.876-1) with a cut off value of 21.6%. The percentage of CD39-positive CD4 cells is similarly significantly higher in all types of neoplastic samples (with mean values from 34 to 46%) than in N-N (19%) without differences between them. While maintaining the same significant differences with the N-N (32%) group, CD39 proportion in the CD26-CD38+ subset distinguishes the DLBCL (74%) from the other histotypes (FL 50% and HL 57%). DLBCL and HL express CD38 at a higher intensity on CD4 cell surface [value measured as the Mean Fluorescence Intensity (MFI)] with significant difference ($p<0.05$) between them and between FL and N-N. CD39 MFI is higher in DLBCL both in the all CD4 T and in the CD4+CD26-CD38+ subsets with significant differences ($p<0.05$) among all the other subtypes. CD39 MFI has a high capability to discriminate DLBCL from N-N, both in the CD4+ cells with AUC 0.8381 (95% CI: 0.7073-0.9689) and in CD4+CD26-CD38+ with AUC 0.781 (95% CI: 0.6339-0.928).
Conclusions: In the HL microenvironment a higher percentage of CD4+CD26-CD38+ is present, while CD38 MFI on all CD4 T defines the HL and the DLBCL neoplastic microenvironments and differentiates them from FL. In addition, a significant percentage of CD39 positivity characterizes CD4 T lymphocytes of the three neoplastic microenvironments but does not distinguish between them, while CD39 MFI distinguishes only DLBCL from the other lymphomas. The analysis of the percentage and intensity of CD38, CD26 and CD39 on CD4 T could be a quick diagnostic tool that might contribute both to the characterization of the neoplastic microenvironment and the discrimination between lymphoma histotypes.

# APPLICATION OF FLOW CYTOMETRY FOR THE DETECTION OF THE ABUSE OF BLOOD TRANSFUSIONS IN SPORT DOPING: STATE OF THE ART AND NEW PERSPECTIVES

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Background and Aims
In doping control, anti-doping laboratories introduced flow cytometry techniques and instrumentation for the detection of blood transfusion (BT) abuse among cheating athletes. The method for detecting Homologous Blood Transfusion (HBT) abuse is based on flow cytofluorimetric analysis of phenotypic mismatches between minority blood group antigens on surface of donor and recipient erythrocytes. Since minor blood groups mismatch approach obviously cannot be applied when autologous blood transfusion (ABT) is used, several alternative strategies are currently being tested with the aim of identifying possible markers that are able to reveal an ABT. One of the most promising strategy concerns the analysis of the morphological and biochemical changes that erythrocytes undergo during storage in the blood bags conditions before being reinfused for transfusion purposes.
The aim of this work is to highlight how flow cytometry is used in the wide context of blood doping abuse detection. The strategies applied over the years in improving the sensitivity and the reliability of the results of the HBT method and the recent advances in the identification of ABT abuse are highlighted.
Methods
Erythrocytes (RBC) surface markers are detected by immunohematology techniques. Primary antibodies for the target of interest are used after titration in order to identify the best workflow concentration. Staining of RBC with fluorochrome-bound secondary antibodies are used in all those cases where the primary antibody linked to a detector fluorochrome is not commercially available. Minor blood group antigens currently being tested are big-C, small-c, big-E, small-e, Jka, Jkb, Fya, Fyb, big-S, small-s and big-K. More surface antigens are screened to assess the modification undergoing on RBC during storage in blood bags conditions: Glycophorin-A, CD47, Band3, CD55, CD59. Characterization of erythrocyte-derived microparticles (EMPs) is obtained by both morphological gating and immunological staining. Counting of EMPs is achieved by both absolute counting and relative percentage with respect of mature erytrhocytes.
Results and Discussion
A panel of 8-12 blood group antigens allows achieving an adequate global sensitivity to detect HBT abuse even many days after its execution. Accurate gating strategy together with high-brightness fluorochromes allow the clear separation of the donor and recipient erythrocyte cells populations. As for ABT, the counting of EMPs is emerging as the most effective strategy to identify the autologous transfusion as it is more sensitive than the monitoring of the decrease in expression of surface antigens in RBC subjected to storage.
Conclusion
Flow cytometry has become a basic technique in doping control for all aspects related to the detection of doping practices with the use of blood cells and at present the only technique suggesting the possibility of developing a direct method of identifying ABT abuse in sport doping.


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# A NEW NON-INVASIVE METHOD FOR ISOLATION OF CIRCULATING EXTRACELLULAR VESICLES AND EVALUATION OF ITS SUITABILITY FOR HEMATOLOGICAL MALIGNANCY BIOMARKER DISCOVERY

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Introduction and aim: Extracellular vesicles (EVs) are naturally secreted cellular lipid bilayer particles, which carry a selected molecular content. Due to their systemic availability in biological fluids and to their role in tumor pathogenesis, circulating EVs (cEVs) can be a valuable source of new biomarkers useful for tumor diagnosis, prognostication and monitoring of tumors, probably alternative to traditional biopsy.
However, a precise approach for isolation and characterization of cEVs as tumor biomarkers, exportable in a clinical setting, has not been conclusively established.
This study was conceived to demonstrate the feasibility/employment of serum cEVs as a source of tumor biomarkers by using simple, fast and sensitive procedures that can be complemented by other in-depth analyses.
Methods: We developed a novel and laboratory-made procedure performing a bench centrifuge step which allows the isolation of serum cEVs suitable for subsequent characterization of their size, amount and phenotype by nanoparticle tracking analysis (NTA), different microscopy and flow cytometry, and for nucleic acid assessment by digital PCR.
Results: Applied to blood from healthy subjects (HSs) and tumor patients, our approach, permitted from a small serum volume: i) the isolation of a great amount of EVs enriched in small vesicles, free from protein contaminants; ii) a suitable and specific cell origin identification of EVs, and iii) nucleic acid content assessment. In clonal plasma cell malignancy, like multiple myeloma (MM), our approach allowed to identify specific MM EVs, and to characterize their size, concentration and microRNA content allowing to significantly discriminate between MM and HSs. Finally, EV associated biomarkers correlated with MM clinical parameters.
Discussion and conclusions: EVs are highly stable and easily quantified in serum. Therefore, biological samples can be analyzed with minimal sample processing and the combined use of NTA, flow cytometry and digital PCR for EV routine screening could become a reality for monitoring tumor patients. To our knowledge, this is the first approach that, using only a bench centrifugation step, allows the efficient isolation of protein contaminant-free EVs suitable for subsequent characterization (size, count, surface antigens and nucleic acids).
Overall, our cEV based procedure can play an important role in malignancy biomarker discovery and then in real-time tumor monitoring using minimal invasive samples. From a practical point of view, it is smart (small sample volume), rapid (few hours), easy (no specific expertise required) and requirements are widely available in clinical laboratories.

# CYTOFLUORIMETRIC ANALYSIS OF TWO BONE MARROW COLLECTION SYSTEMS AND THE IMPORTANCE OF CLINICAL RESULTS

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Aim: We analyzed whether two different approaches for bone marrow aspiration (Harvest H vs. Aspire Medical incA) had an effect on the number of stem cells and the purity of bone marrow (BM) harvested. Methods: endpoint of study was occurrence of leg amputation after injection of stem cells obtained with two different sampling techniques for bone marrow aspiration (HvsA); 240 cc of BM are harvested and centrifugated with a standard needle for a final quantity of 40cc. Due to different construction of Marrow Cellution needle 40cc of BM were obtained and centrifugation was not necessary. We compared baseline characteristics of two pts group detect factors, beside technique used for bone marrow aspiration. Continuous variables were reported as median with interquartile range (IQR), and categorical variables were reported as proportion and percentages. Continuous variables were compared using the Mann-Whitney U test for independent samples and Wilcoxon signed rank test for paired samples, while categorical variables were compared using Chi square test. Statistical significance was set at $p<0.05$. Results: we included n=58 patients in the H group and n=17 patients in the A group. Major risk factors for artery disease and comorbidities were balanced in two groups. We analyzed following cell populations by flow cytometer (Beckman Coulter Navios) CD34, CD133, CD117 and CD309. HCT value was also assessed. There purity of BM with H was 73% before centrifugation and 88% after. Purity of BM with A was 93.4 % and is statistically superior to H both pre and post centrifugation. Cell lines CD133 of patients in the A group were significantly different in respect to ones of group H after centrifugation with both approaches (Mann- Whitney-U test CD133% H 0.10 (0.06-0.20); A 0.01-0.02 < 0.0001).Leg amputation was significantly more frequent patients in H group (20/58, 34.5%) as compared to the patients in the A group (1/17, 5.9%; p=0.0209).Multivariable logistic regression analysis confirmed that the A technique was independently associated with a 88% reduced probability of leg amputation as compared to the H technique, also when corrected for the age of the patients (OR 0.12, 95% confidence interval:0.006 to 0.7001, p=0.057).Discussion and conclusion: Bone marrow aspirate which contain a complex mix of nucleated cells, platelets, and growth factors, is known to promote angiogenesis in ischemic tissue. Bone marrow harvesting and preparation is important for the different localization of the cells. Moreover manipulation of bone marrow, as with centrifugation impact the presence and number of stem cells. Increased number of red blood cells alter the function of stem cells. This innovative method (A) allows to harvest only 40cc of bone marrow at different level reducing the contamination of BM from peripheral blood and avoids. Cell population avoid the manipulation of centrifugation and are better preserved. This novel approach appears to improve clinical results.



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# CYTOKINE ANALYSIS IN PLATELET RICH PLASMA OF PATIENTS WITH OSTEOARTHRITIS VS HEALTHY SUBJECTS.

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Aim: Platelet-rich plasma (PRP) and its platelet-poor plasma (PPP) by product are rich sources of cytokines. The use of PRP in osteoarthritis (OA) is receiving increased attention. However few data are available on the cytokine presence in PRP of OA pts. The aim of our study was to investigate the biological presence of some cytokines in the PRP of patients with osteoarthritis (30) and in the PRP of healthy subjects (6). Methods: The blood (27cc plus 3cc of trisodium citrate) is centrifuged twice with a Kit (TriCellPRP REV-MED Inc - first round 5min 3200 RPM, second round 6min 3400 RPM); 1 ml of PRP was sent for analysis. Cytokine assay was performed using the ELISA method and the absorbance reading was performed at 450nm. Cytokine panel has been the following: IL 6, VEGF, IGF-1, G-CSF, TNF- , TGFβ-1, FGF-1, SDF-1 .Results: overall, we included n=29 patients with osteoarthritis and 7 healthy subjects. Median IGF1 levels in the PRP of patients with OA were significantly lower (30.5 pg/ml, IQR: 9.9 -57.9 pg/ml) compared to healthy subjects (median 443.0 pg/ml, IQR 214-481.5 pg/ml; p=0.0001). Similarly, TNFα levels in the PRP in patients with OA were significantly lower (median 173.5 pg/ml, IQR 130.3 -276.5 pg/ml) compared to healthy subjects (median 436.0 pg/ml, IQR 354.5 -1037.8 pg/ml; p=0.0012). In patients with osteoarthritis compared to healthy patients there is a marked down-regulation for TNF alpha and IGF-1.Discussion and conclusion Cytokines, released from activated platelet are essential for paracrine action of platelets. Few data are present in literature that analyze cytokines present in the PRP of patients with OA versus healthy subjects. Different balance between cytokines could impact clinical outcome of patients. IGF-1 induces stem cell differentiation toward chondrogenic lineage and cartilage extracellular matrix deposition and stabilizes chondrocyte phenotype in pathological conditions where homeostasis is perturbed. Presence of IGF-1, even if down-regulated, is important in environment of the OA cartilage.IL-6 and TNF-alpha are a proinflammatory cytokines and their reduced presence in the PRP of OA pts is of benefit.
VEGF expression levels correlate with the pathogenesis of osteoarthritis. The increased level of VEGF in the PRP represents the blood value. This preliminary data show that the cytokines, in the PRP of patients before platelets activation, correlate with their blood level and could be used to assess the severity of OA and influence the PRP protocol for number of injections during the follow up.

# MUSASHI-2 SUSTAINS THE GROWTH OF MLL-REARRANGED ACUTE LYMPHOBLASTIC LEUKEMIA AND IT IS INVOLVED IN GLUCOCORTICOID RESISTANCE

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Despite the recent improvements achieved during the last decades in the field of pediatric leukemia, some patients (defined as “high-risk”) still suffer from a dismal prognosis. B-cell acute lymphoblastic leukemia with MLL gene rearrangement occurring within the first year of life (infant ALL MLL-r) is a rare but very aggressive form of leukemia, typically associated with poor outcome (with an event- free survival of 20-40% compared to 80% in pediatric patients > 1 year). The failure of the current therapies is mainly due to the drug resistance of ALL MLL-r cells and the high incidence of disease relapse observed in infant patients (after having achieved an early but transient remission). Therefore, nowadays the identification of (potentially targetable) genes involved in the pathogenesis of leukemia and responsible for chemoresistance is essential for the development of novel therapeutic strategies aimed at improving the clinical outcome and survival of these very young patients.
The RNA-binding protein Musashi-2 (MSI2) has a crucial role in regulating cell proliferation, differentiation and maintenance of the self-renewing stem cell pool, both in normal as well as in malignant hematopoiesis. Although the importance of MSI2 in myeloid leukemia has been extensively ascertained, the functional role of MSI2 in ALL still remains largely unknown, and this represents the main purpose of my thesis. In particular, my thesis is focused on ALL MLL-r. To study the function of MSI2 gene, a human ALL MLL/AF4+ cell line (SEM) was engineered by CRISPR/CAS9 genome editing in order to obtain MSI2 knock out (KO) clones.
Herein we observed that the abrogation of MSI2 in ALL MLL/AF4+ cells: I) confers a proliferative disadvantage in a long-term competition assay in vitro; II) impairs the leukemia-initiating capacity in a xenotransplantation mouse model in vivo; III) sensitizes the cells to glucocorticoids (both Prednisolone and Dexamethasone) in vitro.
Overall, our results demonstrate that MSI2 plays a crucial role in ALL MLL/AF4+, by sustaining the proliferation of leukemic cells in vitro, their leukemogenic potential in vivo and being responsible for glucocorticoid resistance of leukemic cells.
In conclusion, this study is particularly relevant for clinical translation, as not only it sheds light onto the functional role of MSI2 in the pathogenesis of ALL MLL-r, but also it lays the bases for the use of MSI2 inhibitors (such as Ro 08-2750) as a novel therapeutic strategy in the future for the treatment of high risk ALL MLL-r in infants.


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# FUNCTIONAL CHARACTERIZATION OF GENETIC VARIANTS, IDENTIFIED THROUGH NEXT GENERATION SEQUENCING, WITH A ROLE IN THE PREDISPOSITION TO PEDIATRIC HEMATONCOLOGICAL DISEASES

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AIM

This study is focused on the identification of somatic and germline variants, in cohesin genes and in other selected genes associated with paediatric tumours, to analyse in detail their potential role in the predisposition to leukemias and other hematoncological diseases. A better understanding of the pathogenesis and the identification of new potential molecular targets could allow the improvement of the efficacy and reduction of the toxicity of the treatments.

METHODS

We set up a targeted Next Generation Sequencing (NGS) Nextera Flex DNA panel of 40 genes known to be associated to ALL predisposition, as well as all known cohesin genes. Bioinformatic analysis has also been carried out by the Sophia DDM software. We included certainly pathogenic, potentially pathogenic, variants of unknown significance (VUS) or NOVEL ones.

A peculiar variant (Arg1187Gln) was found in STAG1 and functional studies were performed on a lymphoblastoid cell line, derived from patient's peripheral blood's B lymphocytes carrying this mutation. A phenotype analysis was performed by labeling with specific antibodies and preliminary cell cycle assays were then performed on these cells by fixing them in ethanol and labeling with Propidium Iodide.

RESULTS

A retrospective analysis was performed on 40 hypodiploid paediatric patients which returned 19 variants in TP53, confirming the results of previous studies and allowing us to validate the sensitivity and effectiveness of the NGS technology developed, which was then used to extend the screening.

Overall, 118 ALL patients have been screened and a total of 465 variants were identified. Among them, 28 mutations in cohesin genes were found. 131 paediatric patients with ALL relapse or other hematopoietic diseases were also considered and this new screening resulted in a wider cohort of 39 mutations in cohesin genes.

All the lymphoblastoid cells resulted to be B-cells CD45+, CD19+ and CD10-, with a low percentage of myeloid CD33+ and CD13+. So were demonstrated to be a well representing in vitro model for the diseases studied.

Cell cycle assay resulted in a significant reduction of cycling cells, 48 hours after the seeding the median of the 4N cells was 11,6% in the controls while was only 8,1% in the patient's derived LCL line, with a relevant statistical significance (p value = 0,00018).

DISCUSSION

Mutations in the cohesins genes, so far studied only as causative of genetic syndromes or somatically mutated in pathologies of myeloid origin, may also be implicated in predisposition to haematological diseases, including Acute Lymphoblastic Leukemia. Further studies are needed to clarify their role in ALL.

CONCLUSION

NGS screening showed several variants among cohesin genes, with either a potentially pathogenic or unknown role. Functional studies on a selected patient with a mutation in STAG1 confirmed the alteration of fundamental cellular processes and this supports its involvement in the predisposition and progression of leukaemia.

# CIRCULATING REGULATORY T-CELL NUMBER DOES NOT PREDICT PROGNOSIS OF MONOCLONAL GAMMOPATHIES OF UNCERTAIN SIGNIFICANCE

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Abstract
Background: FOXP3-expressing regulatory T-cells (Tregs), which suppress aberrant immune response against self-antigens, also suppress anti-tumor immune response. It has been shown that there is an increased proportion of Tregs in several different human malignancies, although the actual mechanism remains unclear.
Aims: The research aims to explore the relationship between the number of Tregs and a predict prognosis in particular hematological diseases as monoclonal gammopathies of uncertain significance (MGUS).
Methods: Tregs were evaluated by means of flow cytometry (CD4+ CD25high/+CD127low/-) in whole peripheral blood of 56 patients with MGUS to predict progression to overt multiple myeloma (MM).
Results: In two groups of patients, MGUS versus MGUS evolved to MM, we found a significative difference for the number of white blood cells, but not in terms of clinical and laboratory features evaluated at diagnosis.
Conclusions: The study demonstrated the absence of a prognostic relevance of Tregs in MGUS. Nevertheless, their role in these disorders is still to defined.
Keywords: regulatory T cells, multiple myeloma, monoclonal gammopathy of undetermined significance, prognosis.

# Immunology

## THE ASSESSMENT OF CD8+ DR+ T LYMPHOCYTES CORRELATES WITH THE SEVERITY OF COVID-19 PATIENTS. A POTENTIAL THERAPEUTIC TARGET TO HYPERIMMUNE PLASMA THERAPY

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Purpose
In this study we wanted to evaluate the peripheral blood lymphocyte subpopulations of patients affected by interstitial pneumonia linked to Sars-CoV-2 infection. The aim was to search of identifying the predictive parameters of severity and mortality in order to identify patients who could use hyperimmune plasma therapy on the initial phase of hospitalization.
Methods
All patients affected by related Covid-19 interstitial pneumonia, admitted to our hospital, carried out a sample for the evaluation of lymphocyte subpopulations. The assessments were performed on day 1 and repeated at 4 and 7 days after admission. Was evaluated T lymphocytes (CD3, CD4, CD8), B lymphocytes (CD19) and NK lymphocytes (CD16 + CD56). Particular attention was paid to activated cytotoxic T cells (CD3+ CD8+ DR+). Data were evaluated as percentage and absolute number.
Results
The results are referred to 130 consecutive patients admitted to our hospital. The evaluation of the ROC curve regarding the absolute number of CD8+ DR+ T lymphocytes allowed to divide the patients into 2 groups: Group 1 (n=66) with CD8+ DR+ T cells ≤30/µl and a Group 2 (n=64) with value >30/µl. The ratio (Δ) between the values of CD8+ DR+ T cells of day +4 and the values of day +1 was also evaluated and the ROC analysis showed an area under the curve of 0.776 (sensitivity 84.6% and specificity 77.8%). The overall mortality of the 130 patients was 26.9% (35/130). In the Group 1 the mortality was 40.9% (27/66) compared to 12.5% (8/64) of Group 2. The patients in Group 1 with Δ≤1.2 showed a mortality of 63.2% (12/19).
Discussion
The respiratory disease induced by SARS-CoV-2 infection is probably determined by T cell immunity mediated pathogenic damage occurring in lungs of COVID-19 patients. The data here reported suggest that a value of CD8+ DR+ T cells ≤30/µl, shown on admission of these patients, correlates with a worse prognosis. More precisely, the poor prognosis is related to the failure to increase this value in the days following hospitalization (Δ ≤1.2). Most likely, the finding correlates with the displacement of these activated cytotoxic T cells from peripheral blood to the lung. The data would therefore identify patients with the worst prognosis and could therefore represent the target for the use of hyperimmune plasma in the first days after hospitalization.
Conclusions
There are not yet declare the causes which involve a progressive worsening of respiratory conditions in a proportion of Covid-19 patients with interstitial pneumonia; at the same time is not yet defined a viable therapy for these patients. The hyperimmune plasma employment results are contradictory, but it is accepted by all that the plasma should be administered within a few days of admission. The assessment of CD8+ DR+ and Δ provide an opportunity to identify, from the first days of hospitalization, patients with worse prognosis and is in such patients that can be reserved therapy with hyperimmune plasma

# B CELL RELATED PREDICTIVE BIOMARKERS OF TREATMENT RESPONSE IN MYASTHENIA GRAVIS

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Background and aims
Myasthenia Gravis (MG) is a B cell-mediated autoimmune disease characterized by muscle weakness and fatigability, mostly associated to antibodies against the acetylcholine receptor (AChR). MG patients are chronically treated by immunosuppressants and 10-15% are treatment refractory1. The purpose of our study is to identify changes in B-cell subsets that could predict treatment response in MG subgroups with different treatment-related status, with the aim of improving MG management, leading to personalized therapy.
Methods
Peripheral blood mononuclear cells (PBMCs) were isolated from 79 AChR-MG patients for the characterisation of transitional (CD19+CD20+CD24+CD38+), naïve (CD19+CD20+IgD+CD27-), double negative (CD19+CD20+IgD-CD27-), unswitched memory (CD19+CD20+IgD+IgM+CD27+), switched memory (CD19+CD20+IgD-CD27+IgG+) B-cells and plasmablasts (CD19+CD27+CD38+) by multicolour flow cytometry.
Results
Thirty/79(38%) patients were women and mean age at onset was 48 years. At sampling, 15/79(19%) patients were immunotherapy-naïve, 38/79(48%) were immunotherapy-responders, 13/79 (16%) were refractory to standard immunotherapy and 25/79(36%) were in clinical stable remission (CSR). The frequency of total B-cells in the lymphocyte gate did not differ among the clinical subgroups. Naïve B-cells were lower (Fig.1A) in immunotherapy-responders compared to immunotherapy-naïve (p=0.005), refractory and patients in CSR (p=0.009). Transitional B-cells were increased in refractory MG compared to immunotherapy-naïve (p=0.01) and responders (p=0.029). Surprisingly, plasmablasts were lower in refractory patients than immunotherapy-naïve patients (Fig.1C) (p=0.018).
Discussion
The persistence of transitional B-cells, rather than antigen experienced B-cells, might predict unresponsiveness to immunotherapy in a subgroup of patients2. In these cases, B cell-directed therapies could restore the balance between regulatory and inflammatory B-cells in the pre-germinal compartment.
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## SPIKE-SPECIFIC IMMUNE RESPONSE INDUCED BY BNT162B2 MRNA VACCINE IN FORMER COVID-19 PATIENTS AND HIGH RESPONSIVE SUBJECTS

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Background: The worldwide escalation of Coronavirus Disease 2019 (COVID-19) has urgently required the development of safe and effective vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causative agent of disease. The BNT162b2 (Pfizer–BioNTech) RNA-based vaccine confers 95% protection against COVID-19 by encoding a mutated isoform of SARS-CoV-2 full-length spike (S) protein.
Objective: Here, we report the antigen-specific immune profile against SARS-CoV-2 S protein after vaccination with a single dose of BNT162b2 in order to define the immunological landscape required for an efficient response to the SARS-CoV-2 vaccine.
Methods: We determined the levels of antibodies and antigen-specific B, T and NK-T cells against a recombinant GFP tagged SARS-CoV-2 S protein in subjects up to 20 days after injection of a single dose of BNT162b2 vaccine using a combined approach involving serological assays and flow cytometry analyses. Former COVID-19 patients have been also included in this study to evaluate the effect of vaccine after exposition to SARS-CoV-2.
Results: The level of antigen-specific helper T-cells against SARS-CoV-2 S protein was reduced in subjects, low responsive or unresponsive to vaccination with respect to the highly responsive individuals, while the numbers of antigen-specific regulatory and cytotoxic T-cells were comparable. Of interest, in former COVID-19 patients, a single dose of BNT162b2 vaccine induced a significant increase of antibody production simultaneous with an antigen-specific B and NK-T cell response.
Conclusion: Taken together, these results suggest that favorable immune profiles support the progression and an effective reaction to BNT162b2 vaccination.

## DIRECT SORTING OF RECOMBINANT MVA BY FLOW VIROMETRY

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ABSTRACT
In the field of non-replicating virus-vectored vaccines, recombinant Modified Vaccinia Ankara (rMVA) production is a crucial translational technology for both infectious diseases and cancer. Methods based on cell sorting, although better than all other published methods, are still complex and time consuming, since involve the selection of cells infected by recombinant constructs, virus lysates production, multiple rounds of infections and sorting and of virus terminal dilution in order to produce recombinant untagged rMVAs. In this work, we have moved the selection step from the infected cells to the virions themselves, tagging the surface of recombinant virions by a fluorescent protein which allows direct sorting by a high-resolution flow cytometer and an appropriately modified protocol. Thus, the term "flow virometry" defines virus analysis and subsequent sorting. Advances in sorting technology allow the distinction between fluorescent and non-fluorescent micro-vesicles down to 100 nm in diameter and thus the direct sorting of large viruses, such as vaccinia viruses, distinguishing fluorescent from non-fluorescent virions. To exploit this opportunity, the gene of a fluorescent fusion protein (Flu hemagglutinin-EGFP) is associated to the transgene in the DNA construct that leads to the formation of recombinant virus. The fluorescent protein is integrated in the external membrane of the extracellular MVA virions (EEVs) so that the fluorescent sorted virions would also carry the transgene. A second (programmed) recombination event eliminates the DNA of the fluorescent fusion protein and untagged rMVA can be sorted by flow virometry and cloned by terminal dilution. In summary, two rounds of virus sorting separated by a single round of infection lead to the production of untagged rMVA, whose genotype and phenotype can be easily analyzed by PCR and Western blot analysis. Compared to our previous method based on the sorting of infected cells, the flow virometry method is more reliable and reproducible and allows to save time and labor. In practice, the schedule is cut down from 24 working days (and 57 hours of bench work) to 13 working days (and 38 hours of bench work). Morover, cutting down the number of cell cultures involved in the process, a larger number of recombinant viruses can be processed in the same facility, thus allowing the production of more vaccine candidate rMVAs at the same time.

# CD169 EXPRESSION ON MONOCYTES WAS INVOLVED IN SARS-COV-2 INFECTION AND WAS ASSOCIATED TO CLINICAL FEATURES OF COVID-19

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Aim: The COVID-19 is an acute infectious disease caused by the SARS-CoV-2 virus. To date, a standard therapeutic approach for COVID-19 patients (COV) has not been established and the identification of early biomarkers to predict disease progression is needed. Recently, was suggest that SARS-CoV-2 infects CD169 macrophages in the spleen and lympho-nodes playing a central role in mediating SARS-CoV-2 translocation. Moreover, CD169 was strongly overexpressed in the blood of confirmed COV. We analysed CD169 in blood cells of COV admitted to the hospital during the COVID-19 outbreak and correlated its expression with clinical characteristics. Methods: The ratio of the Median Fluorescence Intensity (MFI) of CD169 between monocytes and lymphocytes (CD169 RMFI) was used to screening blood samples of COV and Healthy Donors (HDs) by flow cytometry, and its correlation with clinical signs, inflammatory markers, cytokines mRNA expression, and disease progression was evaluated. To clarify whether CD169 was directly activated by SARS-CoV-2 stimulation, Peripheral Blood Mononuclear Cells (PBMCs) from HDs were stimulated in vitro with SARS-CoV-2 Spike protein for 24 hours and CD169 RMFI and mRNA expression were analysed. Results: CD169 RMFI was highly expressed in the macrophages of COV but not in those of HDs especially in untreated patients at sampling. In CD4+ T cells of untreated patients, CD169 RMFI inversely correlates with the expression of central memory (CD45RA- CCR7+) and effector memory (CD45RA- CCR7-) cells and directly correlated with exhaustion markers (CD57+ PD1+). In CD8+ T cells, its expression was associated with the decrease of naive (CD45RA+ CCR7+) and increase in EM (CD45RA- CCR7-) cells. Finally, CD169 RMFI positively correlated with the senescence marker CD57+. Moreover, the CD169 RMFI correlated with inflammatory markers, blood cytokine levels, and pneumonia severity in the untreated group of COV at sampling. Notably, in this group, CD169 reflects the respiratory outcome of patients during hospitalization. In vitro stimulation of PBMCs from HDs with SARS-CoV-2 Spike protein induced an elevated expression of the activation marker HLA-DR in monocytes and a significant increase in CD169 RMFI in a dose-dependent manner with a significantly increase of IL-6 and IL-10 gene expression. Discussion: Considering the central role of CD169 macrophages in SARS-CoV-2 infection and subsequent cytokine storm, our data highlighted the association between CD169 expression and clinical status, inflammatory markers, and respiratory outcome. Conclusion: Considering the immunological role of CD169 and its involvement during the infection and the progression of COVID-19, it could be considered as an early biomarker of disease progression.

## CHARACTERIZATION OF T LYMPHOCYTES IN SEVERE COVID-19 PATIENTS

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Introduction. The SARS-CoV-2 infection can lead to different clinical pictures (from asymptomatic/pauci-symptomatic infection to moderate/severe forms of disease), suggesting that the clinical picture of the infection might strictly depend on the outcome of the SARS-CoV-2-immune system interaction in the patient . Therefore, it is conceivable that patients with the worst forms of disease could have an immunological imbalance due to hampered virus eradication and overwhelming inflammatory response. In order to investigate on the immunological dynamics in SARS-CoV-2 infected patients with severe disease, we performed a study on peripheral T lymphocytes from patients with severe COVID-19 in comparison to T cells from healthy controls.
Methods. In this observational study, blood samples from 13 patients with severe COVID-19 and 10 healthy controls untreated HNSCC patients were evaluated by multicolor flow cytometry to delineate: CD4+ and CD8+ T cell maturation and activation levels, frequency of CD4+ and CD8+ T regulatory cells (Treg), dynamic of Th subsets by espression of chemokine receptors.
Results. The data show that a robust immune response develops in patients with severe COVID-19 characterized by relative expansion of T cell subsets typical of persistent viral infection and prone to sustain inflammation (Th2- and Th17-oriented TFH) as well as T cell subtypes devoted to regulation (CD8+ Treg), and by relative reduction of Th1 cells, which are those associated with eradication of a viral infection. This pathogenic mechanism could lead us to envisioning possible new form of biological target therapy.

## DEVELOPMENT OF EXHAUSTION AND ACQUISITION OF REGULATORY FUNCTION BY INFILTRATING CD8+CD28- T LYMPHOCYTES DICTATE CLINICAL OUTCOME IN HEAD AND NECK CANCER

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Introduction. CD8+ T lymphocytes are among the immune cells reputed to kill tumor cells. Head and neck squamous cell carcinoma (HNSCC) has a poor clinical outcome despite the presence of a rich CD8+ T cell tumor infiltrate. This may be due to alterations of tumor infiltrating CD8+ T cells. Here, we performed a characterization of infiltrating CD8+ T cells in a cohort of 30 HNSCC patients.
Methods. Blood and tumor biopsies from untreated HNSCC patients were evaluated by multicolor flow cytometry to delineate: CD4+ and CD8+ T cell maturation, frequency of CD4+ and CD8+ T regulatory cells (Treg), expression of immune checkpoints (CD39, PD-1, CTLA-4, TIM-3). Combination of traditional analysis and computational tools based on particular algorithms (i.e., t-SNE) have been used to dissect the complexity of the data.
Results. The results showed that differential intratumoral frequency of CD8+CD28+ T cells, CD8+CD28- T cells, and CD8+CD28-CD127-CD39+ Treg distinguished between HNSCC patients who did or did not respond to treatment. Moreover, we identified an intratumoral CD8+CD28- T cell subpopulation, which expressed markers of both exhausted (i.e., with impaired effector functions) and regulatory (i.e., exerting suppressive activities) cells. This suggests that in HNSCC effector T cells progressively undergo exhaustion and acquisition of regulatory properties, hampering their anti-tumor functions.

# BASOPHIL CELL ACTIVATION TEST BY FLOW CYTOMETRY IN THE MANAGEMENT OF IMMEDIATE DRUG HYPERSENSITIVITY TO CARBOPLATIN IN OVARIAN CANCER PATIENTS

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Hypersensitivity reactions (HSRs) to carboplatin (CARB) chemotherapy are becoming increasingly frequent in the clinical practice of ovarian cancer (OC). Longer survival time of patients and availability of ancillary treatments make it possible administration of multiple treatment cycles of CARB, thus facilitating presentation of HSRs that tipically occur after 5/6 therapy cycles. Discontinuation of treatment and standardized protocols of desensitization represent the standard of care for OC patients after HSRs to CARB, since alternative drugs for OC treatment are not available. The basophil activation test (BAT) is considered useful in the diagnosis of HSRs to several chemotherapeutics. Thus, we set out to verify whether BAT might help in the management of OC patients who had presented HSR to CARB and had to receive desensitization to this agent. Eleven patients with diagnosed HSR to CARB were enrolled in the study. Patients were free from steroid treatment in the last 2 wks. We optimized a BAT protocol to detect sensitivity/desensitization to CARB. Peripheral blood was obtained from patients after at least 2 wks from HRS. Blood samples (100µl) were incubated for 20 min in Ca++ activation solution (Beckman Coulter) with three different concentrations of CARB, anti FcεR (positive control) or PBS (negative control). A drug (taxol) was also included for comparison, with which patients had no history of previous HSR. All the experimental conditions were run in the presence/absence of IL-3. Cells were then stained with previously titrated BV-421 anti-CD63 and APC anti-CCR3 (BioLegend), FITC anti-CRTH2, PE anti-CD203c, APC-AF750 anti-CD45 and KO anti-CD3 (Beckman Coulter) for 20 mins at room temperature. One ml Fix and Lyse solution (Beckman Coulter) was added to lyse erythrocytes and fix samples. Flow cytometry run was performed on Cytoflex LX (Beckman Coulter). Positive reactions were evaluated by computing the stimulation index (SI) for CD63 and CD203c expression. Both CD63 and CD203c SI increased in a CARB dose-dependent fashion, but not in the presence of taxol. The SI was further increased in the presence of IL-3. Interestingly, we observed a relationship between extent of CD63 SI and HSR severity grading. Conversely, no correlation was found between CD203c SI and severity grading. CD63 and CD203c SI were larger in patiets tested after at least 21 days from HSR.
In conclusion, the BAT seems to be a feasible and minimally invasive assay to monitor risk of HSR to CARB in OC patients who had HSR in response to previous treatment with this agent. These preliminary results are based on a limited number of patients and may not be extrapolated to the general population. Introduction of BAT in the range of assays performed in OC patients referred for desensitization protocol for HSR to CARB is currently being investigated.

## CELL-MEDIATED AND HUMORAL ADAPTIVE IMMUNE RESPONSES TO SARS-COV-2 ARE LOWER IN ASYMPTOMATIC THAN SYMPTOMATIC COVID-19 PATIENTS, BOTH DURING INFECTION AND AFTER RECOVERING

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PURPOSE The characterization of cell-mediated and humoral adaptive immune responses to SARS-CoV-2 in the acute and early convalescent, as well as in recovered individuals, is fundamental to understand COVID-19 progression and the development of immunological memory to the virus (1,2).
METHODS Multiparametric flow cytometric characterization of antigen specific T cells response and Ig specific serum levels were evaluated in 22 SARS-CoV-2 infected patients (8 asymptomatic and 14 hospitalized) during the infection phase, in 30 recovered patients after 5 months from SARS-CoV-2 infection and in 15 uninfected healthy controls.
RESULTS We detected T cells reactive to SARS-CoV-2 proteins M, S and N, as well as serum virus-specific IgM, IgA, IgG, in nearly all SARS-CoV-2 infected individuals, but not in healthy donors. More importantly, symptomatic patients displayed a significantly higher magnitude of both cell-mediated and humoral adaptive immune response to the virus, as compared to asymptomatic (3). Then we found a heterogeneous magnitude of immunological memory at five months post infection since 20% of the subjects displayed a weak cellular and humoral memory to SARS-CoV-2. In particular individuals with an history of symptomatic COVID-19 was associated to higher levels of SARS-CoV-2 reactive CD4+ T cells and specific antibody levels compared to asymptomatic individuals (4).
DISCUSSION The different levels of both cell-mediated and humoral immune responses to SARS-CoV-2 in symptomatic versus asymptomatic patients, suggest that a possible dysregulation of adaptive immunity in COVID-19 that could be related to different immunopathology. On the other hand, the divergence in antigen specific immune response observed in recovered patients might reveal subjects with higher risk of reinfection.
CONCLUSIONS These results suggest that monitoring SARS-CoV-2 specific immune response in recovered patients could be important to develop effective timing in vaccination strategies.
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## RESPONSIVE NANOPARTICLES FOR EFFICIENT GENE DELIVERY IN IMMUNO-ONCOLOGY

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ABSTRACT
AIM: Here, we present a targeting strategy to overcome actual clinical therapeutic problems associated with viral safety, developing a capsid-like nanoparticle, which combines into one system the sensitivity of polycations used to complex the acid nucleic molecules, with surface degradation of the outer layers of the nanoparticles.
METHODS: Our nanosystems are composed by an outer shell of FDA approved polymers with a soft and stiff domain and by an inner core of acid nucleic molecules complexed with different responsive polycation polymers in order to guarantee high loading, stability, and controlled plasmid release over time.
RESULTS & DISCUSSIONS: Our nanoparticles are hemocompatible, not cytotoxic and have the ability to protect the gene cargo from DNase and serum protease action. Further, our nanoparticles show a controlled and sustained transfection of different human cells, as human T lymphocyte cells, human neuroblastoma cells and human cervix carcinoma cells, respect to transfection with commercial Lipofectamine 3000. In addition, our nanoparticles showed the ability to penetrate into 3D spheroids allowing transfection of inner cells.
CONCLUSIONS: Our capsid-like NPs thanks their properties, as biocompatibility, biodegradability, hemocompatibility, sustained plasmid release can be used as efficient tool of transfection overcome viral vector problems.

# EXPRESSION OF THE PATHOGENIC HERV-W ENVELOPE IN T LYMPHOCYTES IN ASSOCIATION WITH THE RESPIRATORY OUTCOME OF COVID-19 PATIENTS

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Aim: The identification of early biomarkers for predicting coronavirus disease 2019 (COVID-19) progression and of new therapeutic intervention for patient management are needed, considering that no standard therapeutic approach has been established yet. As recent findings that the Human Endogenous Retrovirus-W Envelope (HERV-W ENV) is activated in response to infectious agents and leads to various immune-pathological effects, the present study aimed to evaluated HERVs involvement during severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Methods: HERV-W ENV expression in blood samples of COVID-19 patients and Healthy Donors (HDs) was analyzed by flow cytometry and quantitative RT-PCR, and was correlated with clinical signs, inflammatory markers, cytokine expression, and disease progression. To address the contribution of SARS-CoV-2 on the activation of HERV-W ENV, in vitro experiments were performed stimulating Peripheral Blood Mononuclear Cells (PBMCs) from HDs with SARS-CoV-2 Spike protein and monitoring the induction of HERV-W ENV and IL6 expression after 3, 24 h and 5 days. Results: HERV-W ENV has been found expressed both as mRNA and protein, in blood samples from COVID-19 but not in HDs. A high percentage of HERV-W ENV positive leukocytes has been found in COVID19 patients. Lymphocytes displayed the highest values among all leukocytes, and in particular, CD3+ T cells showed the highest percentage of HERV-W ENV positive cells. HERV-W ENV expression correlated with the T cell differentiation, exhaustion, and senescence markers: in particular, in CD3+CD8+ T HERV-W ENV expression was associated with a decrease in naïve (CD45RA+CCR7+) and central memory (CD45RA-CCR7+) cells and positively correlated with effector memory (CD45RA-CCR7-) and terminal effector memory (CD45RA+ CCR7-) cells. Furthermore, HERV-W ENV positively correlated with CD3+CD4+PD1+ and CD3+CD8+CD57+ T cells. Moreover, the percentage of HERV-W ENV-positive cells in CD4+ T cells significantly correlated with coagulopathy markers and biochemical parameters associated with COVID-19 severity. Interestingly, a significant increase in the percentage of HERV-W ENV-positive lymphocytes across groups with different pulmonary involvement was observed. Finally, by in vitro stimulation with SARS-CoV-2 Spike protein of PBMCs of HDs, an early mRNA production of HERV-W ENV was already observed at 3h, and ahead of the induction of IL6 which was significantly detected from 24h onwards. After 5 days, a significant increase in the percentage of CD3+ HERV-W ENV positive cells was observed by flow cytometry. Discussion: The data demonstrated a close association between the expression of HERV-W ENV and several immunological and clinical markers related to the severity of COVID-19 disease. Conclusion: The obtained data support the role of HERV-W ENV as contributing factor in the development and progression of COVID-19 and candidate it as a new potential therapeutic target.


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## PHENOTYPIC AND FUNCTIONAL HETEROGENEITY OF LOW-DENSITY AND HIGH-DENSITY HUMAN LUNG MACROPHAGES

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Abstract: Background: Pulmonary macrophages are a highly heterogeneous cell population distributed in different lung compartments. Methods: We separated two subpopulations of macrophages from human lung parenchyma according to flotation over density gradients. Results: Two-thirds 65.4% of the lung macrophages have a density between 1.065 and 1.078 (high-density macrophages: HDMs), and the remaining one-third (34.6) had a density between 1.039 and 1.052 (low-density macrophages: LDMs). LDMs had a larger area (691 vs. 462 _m2) and cell perimeter (94 vs. 77 _m) compared to HDMs. A significantly higher percentage of HDMs expressed CD40, CD45, and CD86 compared to LDMs. In contrast, a higher percentage of LDMs expressed the activation markers CD63 and CD64. The release of TNF-α, IL-6, IL-10 and IL-12 induced by lipopolysaccharide (LPS) was significantly higher in HDMs than in LDMs. Discussion: The human lung contains two subpopulations of macrophages that differ in buoyancy, morphometric parameters, surface marker expression and response to LPS. The density distribution of lung macrophages is clearly bimodal on continuous gradients and it is maintained during short-term culture in vitro.
Conclusion: These subpopulations of macrophages probably play distinct roles in lung inflammation and immune responses. Our study shows that LMDs and HDMs produce different amounts of cytokines upon activation with the same stimulus. In fact, LPS-stimulated HDMs produce significantly higher levels of classical proinflammatory cytokines (i.e., IL-6 and TNF- α) compared to LDMs. This difference is even more marked in the case of the immunoregulatory cytokines IL-10 and IL-12. These observations support the concept that differences in the intensity rather that the quality of the response to LPS is a feature of the two subpopulations of human lung macrophages.

## A CASE OF REFRACTORY CYTOPENIA AND INFLAMMATORY SYNDROME CAUSED BY HETEROZYGOUS MUTATION OF AIRE GENE: IS GENETICS ALWAYS RIGHT?

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Key words: AIRE mutations, PHD1, refractory cytopenia, inflammatory syndrome, immune dysregulation.
Autoimmune regulator (AIRE) is the cause of APECED, a rare monogenic recessive disorder of immune dysregulation. AIRE plays a key role in central immunological tolerance, inducing T lymphocytes negative selection in the thymus. Mutations located in SAND and PHD1 domains cause milder forms of autoimmunity.
We report a case of a 9 years-old boy affected by autoimmune cytopenias and inflammatory symptoms. At four years he presented with fever, conjunctivitis, arthritis, and IBD. Laboratory findings showed: neutropenia, increased inflammatory indices and ANA positivity. Peripheral blood immunophenotyping revealed normal T cell subsets and decreased B memory cells with increased mature naïve B cells, possibly associated with polyreactive clones. Bone marrow aspirate revealed normal cellularity. He was started on prednisone and IVIG, with good control of inflammation for two years. Then he developed severe thrombocytopenia, refractory to first line therapy. Rituximab was administered with partial response, then mycophenolate mofetil and later sirolimus, with remission.
Assuming immune dysregulation, specific gene panel studies revealed a heterozygous variation in PHD1 domain of AIRE gene (c.901G>A; p.V301M).
Mutations in PHD1 cause different autoimmune phenotypes, including late onset APECED and isolated organ-specific autoimmunity; so, genotype needs to be related with clinical evolution. Further studies are required to understand the possible role of genetic findings in our patient. Moreover, based on ongoing NGS studies we might reconsider the role of AIRE variation from disease causing to disease modifying.

# IDENTIFICATION OF FUNCTIONAL SUBSETS OF T LYMPHOCYTES BASED ON THE EXPRESSION OF IMMUNE-CHECKPOINTS, IN JUVENILE IDIOPATHIC ARTHRITIS

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PURPOSE
Juvenile idiopathic arthritis (JIA) is a persistent arthritis of unknown cause in children. T cells are certainly involved in its pathogenesis, but functional analyses of T cells subset of JIA patients are needed to better characterize the role of these cells in disease development.
METHODS
T cells gene expression in JIA samples was investigated trough single-cell RNA sequencing (scRNA-seq) analysis. Peripheral blood (PB) and Synovial Fluid (SF) T cells from 12 children with JIA were analyzed by flowcytometry for the expression of immune-checkpoint (IC) molecules TIGIT and PD1. T cells cytokine production profile was analyzed as well.
RESULTS
Data obtained from scRNA-seq analysis showed that T cells of PB and SF samples clustered separately and among the genes differentially expressed between clusters emerged TIGIT and PD1. These two ICs identified four different subsets. The expression of ICs was correlated with T cells functionality checking each subsets for cytokines genes expression. The TIGIT-PD1+ subset shows the highest expression of cytokines, followed by TIGIT+PD1+, TIGIT+PD1- and TIGIT-PD1-. In order to study the temporal relation between the different subsets, it was made a trajectory analysis, focusing on SF samples. Data showed that PD1 is more expressed at the beginning and next slightly reduce. TIGIT is less expressed at the beginning but next increase more than PD1 and then undergoes to reduction. ScRNA-seq data were confirmed by flowcytometry analysis, which showed that both TIGIT and PD1 expression was higher in SF compere to PB. Moreover, among the different subsets the maximum production of all cytokines was observed in TIGIT-PD1+ subset.
DISCUSSION
Cytokines and scRNA-seq analysis confirm the hypothesis that in SF recently activated T cells after repeated stimulation acquire an higher effector function and express PD1. The persisting stimulation, due to pro-inflammatory molecules, induces on T cells the expression of other inhibitory molecules such as TIGIT and decreases T cells effector capacity.
CONCLUSIONS
The results confirmed that PD1 is one of the mainly expressed molecules by active cells in SF, suggesting that it can be considered as a valid therapeutic target in chronic inflammation. In this context, anti-PD-1 antibodies that mimic activity of natural checkpoint ligands, may have the potential to restore and maintain immune balance in inflammatory diseases.

# TCRγδ+ AND IL4+ T CELLS AS BIOMARKERS OF MUCOSAL LESION IN CELIAC DISEASE

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Celiac disease (CD) is a chronic intestinal inflammation caused by intolerance to gluten in genetically predisposed individuals. All CD patients are characterized by the presence of HLA DQ2/DQ risk alleles and anti-tissue transglutaminase antibodies (anti-tTG2 IgA), but they can be divided on the basis of the intestinal damage in overt and potential-CD. Overt-CD patients show an atrophic intestinal mucosa at histological analysis of duodenal biopsy samples, according to the histological Marsh-classification (Marsh type 3 lesion - M3), while potential CD patients are identified by a morphologically normal intestinal mucosa with low or mild lymphocyte infiltration (Marsh type 0 - M0 or type 1 lesion - M1, respectively). Recently, we have observed that the villous atrophy of patients with overt-CD is characterized by an intestinal expansion of TCRγδ+ T cells and a low frequency of IL4 producing T cells compared to the morphologically normal intestinal mucosa of potential-CD patients. The changes in the frequency of these two lymphocyte populations suggest their involvement in the transition from potential to overt-CD.
In order to demonstrate that TCRγδ+ and IL4+ T cells could be novel cell biomarkers for the different CD forms, the phenotype and cytokine production of these two intestinal lymphocyte populations were investigated by flow cytometry in gut biopsies of children with overt- or potential-CD, and in healthy controls. In particular, a multiparametric flow cytometric analysis was performed on two different experimental systems: in vitro approach on gluten-raised short-term T cell lines (st-TCLs) and ex vivo approach on intestinal cells freshly isolated from the mucosal samples. Moreover, the possible correlations between the intestinal frequency of TCRγδ+ or IL4+ T cells and the disease indices, such as the serum titers of anti-tTG2 IgA and the degree of mucosal lesion, were evaluated in overt and potential-CD patients.
An intestinal expansion of TCRγδ+ T cells combined with a disappearance of IL4+ T cells characterize the transition from potential to overt-CD. More specifically, the frequencies of IL4-producing T cells inversely correlated with TCRγδ+ T cell expansion ($p<0.005$) and with titers of anti-tTG2 IgA antibodies ($p<0.05$). The changes of these cell subsets density strongly correlated with mucosal lesions, according to the histological Marsh-classification, as the transition from M0 to M3 was associated with a significant reduction of IL4+ T cells and increase of TCRγδ+ T cells.

# Oncology

## CHARACTERIZATION OF SMALL EXTRACELLULAR VESICLES SPECIFICALLY SECRETED BY HUMAN TUMOR CELLS IN ORTHOTOPIC PRECLINICAL MODELS IN VIVO

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During the past few years, the new concept of "liquid biopsy" has emerged opening up previously unexpected perspectives. A liquid biopsy is a revolutionary minimally-invasive technique performed on samples of blood or other human biological fluids to detect and quantify circulating tumor cells (CTCs) or other tumor-derived elements, such as cell-free tumor DNA (ctDNA) and, more recently, extracellular vesicles (EVs), which are released in the bloodstream or other fluids by cancer cells. The presence of EVs in biological fluids and their distinctive composition make them candidate liquid biomarkers of great significance for cancer. Tumor cells produce large amounts of EVs and are able to select the EV cargo, which is constituted by proteins, RNA and lipids, thus safeguarding them from degradation and conveying to nearby or distant cells. miRNAs contained into tumor-secreted small EVs (sEVs) are crucial component of this cargo, since they reflect the pathophysiological state of the primary affected tissue and mediate the interaction between cancer cells and their microenvironment, thus contributing to tumor progression.
Recently, we developed a human metastatic melanoma xenograft mouse model to identify circulating sEV-enriched miRNAs secreted in the blood specifically by tumor cells injected in the mouse brain (Guglielmi et al., Cancers 2020). We characterized the miRNA expression profile of sEVs released by the brain xenograft in the mouse blood. We identified 25 most abundant human miRNAs contained in the sEVs that were mostly related to stage IV metastatic disease. Since all the cells in an organism secrete EVs, it is of great importance to identify the sEVs specifically released by tumor cells in the blood.
The glioblastoma multiforme (GBM) brain tumor is an incurable disease with one of the worst prognoses among all human cancers. In order to more accurately identify the sEVs specifically secreted in the bloodstream by the tumor cells from the brain, we have developed a human GBM experimental model using the U87MG GBM cell line orthotopically injected in the brain of NOD scid gamma (NSG) mice. To improve our model, we traced the secreted EVs by transfecting the U87MG cells with a construct coding for a red fluorescent (dTomato, dT) CD81 fusion protein. CD81 is a tetraspanin specifically expressed on the sEV surface. After transfection, to enrich the dT-CD81 cell population, we sorted the fluorescent cells and expanded them in vitro. We then characterized the sEVs released by the U87 MG-dT-CD81 cell clone by microscopy, WB and FACS after immunocapturing the vesicles with magnetic beads. Then, we injected the U87MG-dT-CD81 cells in NSG mouse brains and purified circulating fluorescent sEVs. FACS analysis revealed the presence of about 1% of human fluorescent sEVs in the mouse plasma.


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# VOACAMINE, BISINDOLE ALKALOID, ACTS AS CHEMOSENSITIZING AGENT ON MULTIDRUG RESISTANT TUMOR CELLS OVEREXPRESSING P-GP

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The use of natural compounds in combination with conventional chemotherapy, now widely used in integrated cancer protocols, requires an in-depth study of the cellular and molecular effects in the combined treatment.
The aim of our work was to verify the chemosensitizing effect of the bisindole alkaloid voacamine (VOA), extracted from the Peschiera fuchsiaefolia plant on drug-resistant human cancer cells of different histotype (osteosarcoma, melanoma, colon carcinoma and ovarian cancer). Our goal was to analyze the target's resistance mechanisms.
We performed MTT analysis, light and electron microscopy, confocal laser scanning microscope (LSCM) and flow cytometry.
Selected cell lines overexpressed P-gp on the cell surface, which was responsible for the increased efflux of chemotherapeutic agents (such as doxorubicin), reduced drug intracellular concentration and reduced the toxic effect.
The MTT test on drug-resistant cancer cells showed that cell treatment with 1 $\mu$g/ml of VOA alone was not toxic. When VOA was administered in combination with doxorubicin (DOX, 1 $\mu$g/ml) on osteosarcoma (U-2 OS/DX and SAOS-2-DX) and melanoma (Me30966) cells, we observed increased DOX accumulation by flow cytometry and an intracellular distribution DOX by confocal microscopy. Consequently, cell viability significantly decreased compared with DOX treatment alone. Scanning electron microscope observations confirmed the MTT data and highlighted evident morphological alterations (Meschini et al., 2003; Condello et al., 2014).
UIC2 shift assay performed by flow cytometry demonstrated that VOA was a substrate of P-gp and, as a competitive antagonist of cytotoxic drugs (such as DOX), it interfered with P-gp mediated efflux (Meschini et al., 2005).
Furthermore, LSCM observations showed that VOA depolymerized microtubules blocking DOX-mediated efflux vesicles (Condello et al., 2020).
Next, we investigated if VOA was a chemosensitizing compound when administered in combination with other P-gp substrate drugs, such as paclitaxel (PTX), or possibly with drugs that are not P-gp substrates. We analyzed the combination VOA plus PTX on tumor resistant ovarian cells (A2780 DX); as a positive control we used a colon cancer resistant cell line (LoVo DX) treated with VOA plus 5-Fluoracil (5-FU). The MTT test demonstrated that VOA has a chemosensitizing effect on resistant cells when treated in combination with P-gp substrate drugs. Optical and electron microscope observations showed irreversible cell damage induced by VOA plus PTX on A2780 DX cells. The Annexin V / IP assay and cell cycle analysis by flow cytometry and the expression of PARP cleaved by western blot showed induction apoptosis cell death.
We concluded that VOA specifically exerts its chemosensitizing function only against drugs known to be substrates of P-glycoprotein.
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# NEW MOLECULAR TARGETS FOR OVERCOMING DRUG RESISTANCE IN TRIPLE NEGATIVE BREAST CANCER

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Triple negative breast cancer (TNBC) is an aggressive cancer with patient survival rarely exceeding five years. TNBC is also the most difficult breast cancer subgroup to treat due to its unresponsiveness to current clinical targeted therapies (e.g., hormonal therapy protocols or chemotherapeutics), high rate of recurrence, and poor prognosis. The lack of a targeted therapy and the TNBC heterogeneity highlighted the urgent need to identify novel therapeutic targets and develop effective medicines capable to overcome drug resistance also hindering the typical TNBC metastatic pattern (i.e., frequent occurrence of distant metastases, mainly localized in lung, central nervous system, and bones) often associated with poor prognosis. One of the most promising sources for potential drug discovery in cancer therapy is compounds of fungal origin, i.e., medicinal mushrooms, which display anti-cancer, onco-immunological, and immuno-modulating activities, also improving quality of life during chemotherapy. "Micotherapy U-care" is a novel medicinal mushroom blend supplement consisting of a mixture of mycelium and sporophores extracts of five species (Agaricus blazei, Ophiocordyceps sinensis, Ganoderma lucidum, Grifola frondosa, and Lentinula edodes), which has been proposed as a promising anti-cancer adjuvant. In particular, our previous studies testing the effect of Micotherapy U-care oral supplementation in a 4T1 triple-negative mouse BC model, demonstrated both an indirect action, i.e. decrease of inflammation and oxidative stress, paralleled by a direct effect on cancer cell leading to reduction of lung metastases and apoptotic pathway activation.
In the present investigation, we deepened the knowledge about the alternative molecular mechanisms by which Micotherapy U-care could be able to overcome drug resistance in TNBC. In particular, using complementary techniques, alternative cell death pathways will be explored including mitophagy, and, contextually, mitochondrial fission and fusion dynamics will be investigated, as crucial events involved in TNBC growth and metastatization.

# MULTIPARAMETRIC FLOW CYTOMETRY HIGHLIGHTS B7-H3 AS A POSSIBLE NOVEL DIAGNOSTIC/THERAPEUTIC TARGET IN GD2NEG/LOW NEUROBLASTOMA VARIANTS

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High-Risk neuroblastomas (HR-NB) relapses in more than 30% of cases, despite using aggressive therapies including targeting of GD2. The presence of GD2neg/low NB variants and/or the surface expression of different immune checkpoints ligands including B7-H3 might contribute to therapy resistance. The main aim of this study was to set a fast, unbiased method to unequivocally identify, quantify and characterize Bone Marrow (BM) infiltrating tumor cells. In this study we used a Multiparametric Flow Cytometry (MFC) panel to analyze 41 BM aspirates from 25 NB patients, comparing results with Cytomorphological Analysis (CA) and/or immune-histochemical analysis (IHC). Spike-in experiments assessed the sensitivity of MFC. To find novel prognostic markers possibly integrating the MFC panel, Kaplan-Meier analysis on 498 primary NBs has been performed. No false-positive were detected, and MFC showed high sensitivity (0,0005%). Optimized MFC identified CD45negCD56pos NB cells in 11 out of 12 (91.6%) of BM indicated as infiltrated by CA, 7 of which co-expressed high levels of GD2 and B7-H3. MFC detected CD45negCD56posGD2neg/low NB variants which, importantly, expressed high surface levels of B7-H3. Kaplan-Meier analysis highlighted an interesting dichotomous prognostic value of different ligands involved in NB recognition by the immune system.
To conclude this study describes a specific, sensitive and fast MFC analysis allowing a precise quantification of i) BM tumor burden; ii) surface expression of GD2; iii) surface expression of different immune checkpoint ligands. MFC might usefully support other routinely used diagnostic and prognostic tools, improving diagnosis, prognosis, and orienting novel personalized treatments in patients with GD2neg/low NB, who might benefit from innovative therapies combining B7-H3 targeting.

# A DIETARY GANODERMA LUCIDUM-BASED SUPPLEMENTATION AS A CHEMOTHERAPEUTIC ADJUVANT THERAPY IN GLIOBLASTOMA

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The current standard oncotherapy for brain cancer is limited by several adverse side effects. Complementary and Integrative Medicine (CIM) was adopted as an innovative approach in oncological care, often associated with better response in cancer treatment. Promising sources for potential drug discovery in oncotherapy are compounds coming from phyto and mycotherapy. Thus, aiming at exploring the potential contribution of these therapies with those in clinical use and to improve the living conditions of glioblastoma patients, in the present study we exploited the effect of a novel phyto-mycotherapic supplement, namely "Ganostile", in U251 human glioblastoma cell line, at a dose range which mimics the oral supplementation in humans (about 1.5 g/day). The supplement contains Ganoderma lucidum, Eleutherococcus senticosus, Echinacea purpurea, Astragalus membranaceus, all known to stimulate the immune system. A battery of complementary techniques, i.e. MTS assay, cytofluorimetry and immunofluorescence, were adopted to evaluate cell cycle and viability, oxidative stress pathway, and cytoplasmic organelles function as uptake/cell metabolism marker. After 48hr-continuous exposure to "Ganostile", a drastic decrease in cell viability was measured, further confirmed by cytofluorimetric data showing a bulk cell number blocked in the G2/M phase. The observed effects induced on proteins involved in metabolic function and cell homeostasis regulation could support the supplement direct effect on the immune system modulation through beta-glucan contents and on intracellular mechanisms. It was also evaluated its effect combined with compounds currently used in oncotherapy, such as Temozolomide and Platinum based drugs. Our data suggest a preliminary effect of the supplement on cell cycle. Therefore, our data support the use of "Ganostile" in integrative oncology protocols as a promising adjuvant able to amplify conventional drug effects and reducing resistance mechanisms often observed in brain tumours, e.g. glioblastoma multiforme.

# FLOW-CYTOMETRY APPLICATIONS TO CHARACTERIZE THE RADIATION RESPONSE OF COLORECTAL ADENOCARCINOMA CELLS

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According to the Global Cancer Observatory (GCO), colorectal cancer (CRC) is the third most common in terms of incidence and the second leading cause of cancer death. It is usually treated with surgery, with chemotherapy and radiotherapy as adjuvant/neoadjuvant therapies, and radiation can also be used for palliation of symptoms. Despite advances in treatment modalities however, patients' survival remains poor. This requires further research efforts to better understand the in vivo response to treatment, and well-characterized in vitro cell systems represent important pre-clinical tools to study the main mechanisms underlying cell response to therapeutic agents, in particular ionizing radiation (IR). Based on these premises, we present an experimental characterization of the in vitro response to X-rays of Caco-2 cells, a cell line derived from human colorectal adenocarcinoma. We measured a variety of radiobiological endpoints with different techniques, including flow-cytometry applications.

Material and method

Caco-2 cells were exposed to X-rays from 2 Gy up to 10 Gy from a LINAC (linear accelerator) used in radiotherapy. Clonogenic survival was assessed at 2 weeks after irradiation. We further focused on measuring several endpoints in the early timepoints after exposure up to 48 hours: cell-cycle perturbations and cell death were investigated via flow cytometry, respectively using a triple staining with PI (Propidium Iodide), EdU (5-ethynyl-2´-deoxyuridine) and anti-pH3, and with Annexin-V/PI staining. We also scored micronuclei and atypical mitosis with immunofluorescence microscopy. To complement and better interpret flow-cytometry data on cell cycle from a molecular point of view, we quantified via Western Blot techniques several proteins involved in the regulation of the G2/M checkpoint, among which FoxM1, Chk2, Cdc25C, CyclinB1, and in the DNA response by γH2AX signal. Finally, activation of matrix metalloproteases (MMPs) was measured via gelatin zymography.

Results and discussion

We found that: Caco-2 clonogenic potential is conserved up to 5 Gy; radiation mainly induces an arrest in the G2-phase, peaked at 24 hours, confirmed by the associated molecular markers from the integrated analysis of flow cytometry and Western blotting; the number of apoptotic cells increases as a function of the dose and the dependency of the death pathways on dose and time correlates with the inhibition of MMPs; genomic instability markers, as the number of atypical mitosis and micronuclei, are also dose-dependent.

Conclusion

Integrating flow-cytometry applications and other experimental techniques, we achieved a more detailed characterization of Caco-2 early response to radiation, with further correlation to their long-term replicative potential. This integrated analysis offers the chance to gain knowledge on mechanisms underlying radiation effects on such model, that can be exploited in perspective to identify therapeutic targets for colorectal cancer treatment.

# IONIZING RADIATIONS INDUCE MITOCHONDRIAL IMPAIRMENT IN NEUROBLASTOMA CELL LINES

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Aim. Ionizing radiations may induce in tumor cells unwanted responses leading not only to radio-resistance but also to an increase in the aggressiveness, that are often associated to metabolic changes. The aims of this work were to analyze in human neuroblastoma cell lines treated with X rays, the mitochondrial status and the effects induced by the secreted vesicles on the non-irradiated cells.

Materials and methods. Human neuroblastoma cell lines (SH-SY5Y) were irradiated with increasing doses of X-rays (1 to 10 Gy). The secreted vesicles were purified by the ultracentrifugation / filtration method and analyzed by flow cytometer. Then, cells irradiated with 10 Gy of X-rays after 24 hours were incubated with two fluorescent probes - i.e. MitoTracker™ Green and MitoTracker™ Red, capable of detecting respectively the mass of mitochondria and their transmembrane potential. The fluorescence intensity in the irradiated and non-irradiated cell populations was analyzed by flow cytometer and with confocal microscopy. The mass of mitochondria was further assessed through the determination of mtDNA by quantitative PCR analysis. These results were compared with the mitochondrial functionality by Seahorse Analyzer. This experimental approach was also applied to the non-irradiated neuroblastoma cells after their treatment with the vesicles secreted by the irradiated cells.

Results and discussion. Data obtained by flow cytometry indicate that X-rays treated neuroblastoma cells release an increased number of extracellular vesicles. Flow cytometry and confocal microscopy showed that X-rays induce a mitochondria impairment. Finally, the production of ATP and the consumption of oxygen decrease both in cells irradiated with X-rays and in those treated with extracellular vesicles.

Conclusions. The obtained results showed that X-rays induces a reduction in the involvement of mitochondria in cellular metabolism which leads to the establishment of a more glycolytic phenotype.


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# Methodology and Microscopy

## THE NUCLEAR ENVELOPE: A TOLLBOOTH FUNCTION FOR SPLICING FACTORS

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AIM OH THE WORK
Pre-mRNA processing factors are main components of the complex cellular transcriptional machinery, holding a central position in the delicate process of gene expression. Hence, their access to the nuclear compartment is a fundamental aspect at the very basis of transcription regulation. Moreover, elucidating which proteins may be involved in promoting this molecular traffic could evidence new outcomes and strategies to operate in specific diseases or conditions. For these reasons, we assessed how pre-mRNA processing factors traffic is affected during thermal insult and the possible activity of heat-shock proteins as nuclear envelope molecular chaperones.
METHODS
Immunocytochemistry for transmission electron microscopy has been performed. We targeted SC35 and the Sm antigen, being markers of mRNA splicing, and the Heat-Shock Protein 71 (HSP71).
RESULTS
Our analysis showed that HSP71 interacts with the nuclear envelope with a significantly higher frequency during the heat shock stress rather than in the recovery condition and the control, that quantitatively shows the lowest of the values.
In parallel, we found a significantly increased presence of the Sm antigen on the nuclear envelope during the thermal stress. Its lowest level is again found in the control case. Remarkably, SC35 shares the Sm antigen localization and distribution.
Moreover, we performed double labelling, that granted us the possibility to verify a hypothesized colocalization of HSP71 with both the pre-mRNA processing factors at the level of the nuclear envelope.
DISCUSSION
The increased concentrations of HSP71, SC35 and Sm antigen in the nuclear envelope surroundings during the thermal shock may be considered as an attempt carried out by the cell to overcome the damages caused by high temperature, which is correlated to degradations of high amount of proteins. This result suggests that several proteins covering the same function may be preferentially recruited during this type of stress and, possibly, be sharing HSP71 chaperone activity to access nucleus.
CONCLUSION
During this work, we assessed a level of transcription regulation based on the cytoplasmic-nuclear traffic of splicing factors. It was possible to colocalize the pre-mRNA processing factors and HSP71 on the nuclear envelope, supporting the idea that the latter acts as a chaperone to drive the former proteins as its cargo inside the nucleus. Evaluating whether other proteins share the same condition as cargo molecules and if this situation is kept also during different type of cell stresses or treatments, for instance chemicals or drugs, may represent a valid implement to overcome many diseases that hold their roots in dysregulations at the level of transcription.

# OXIDATIVE STRESS IN VIABLE SPERMATOZOA: DETECTION IN NATIVE SEMEN SAMPLES

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Abstract
Oxidative stress is involved in many known causes of impaired sperm function. It is of great importance to have methods able to detect oxidative stress in native semen samples to be used in male infertility work-up. In this study, we challenged three probes for sperm ROS generation: CM-H2DCFDA, CellROX Green and MitoSOX Red. Each probe was coupled by flow cytometry to a suitable stain to exclude non-sperm elements and focus viable spermatozoa, the clinically most relevant sperm fraction. We found that the three probes have different cell localization: in the midpiece (CM-H2DCFDA) and in the head of spermatozoa (CellROX Green and MitoSOX Red). In addition, H2O2 treatment highly increased MitoSOX Red fluorescence (14.18±4.6 vs 31.13±9.74, %, $p<0.01$), but not, or only slightly, the labelling with CM-H2DCFDA and CellROX Green, respectively. Menadione treatment highly increased CellROX Green (1.98±0.28 vs 7.71±4.53, mean fluorescence intensity, $p<0.05$) and MitoSOX Red fluorescence (20.38±10.31 vs 65.34±32.34, %, $p<0.05$), but not that one of CM-H2DCFDA. Finally, only MitoSOX Red resulted able to detect spontaneous sperm ROS generation during a short-term in vitro incubation. We also found that MitoSOX labelling is strictly associated to sperm DNA fragmentation, as assessed in sorted spermatozoa by Comet and SCD Assay. In conclusion, the three probes showed different localization and specificity in human spermatozoa. MitoSOX Red appears a probe able to detect both spontaneous and H2O2- and menadione-induced ROS production. Labelling with this probe also identifies a viable sperm fraction susceptible to sDF, a clinically important semen parameter.
Key words: Native semen, Flow cytometry, Oxidative stress, DNA fragmentation.

# OXIDATIVE STRESS AND NEUROINFLAMMATION EFFECTS IN HIPPOCAMPAL DEVELOPMENT, AGEING, AND DISEASE

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The purpose of this work was to evaluate inflammation and oxidative stress, as a common thread in three different pathologies, using both animal models and human biopsies. Prolidase deficiency (PD) is a rare genetic disorder caused by mutations in PEPD gene. Prolidase enzyme can selectively cut proline dimers located at the C-terminal end of a polypeptide, crucially participating in collagen turnover. Recently, cerebellar morphological alterations in PD mice have been demonstrated, characterized by extracellular matrix disorganization, tissue damage and cell migration anomalies. Ageing is a physiological condition closely related to oxidative stress and a chronic inflammatory state called "inflammaging". A broad consensus exists that medicinal mushroom Hericium erinaceus (He1) exerts antioxidant activity also promoting cell proliferation in some brain areas of aged individuals. Eating Disorders are complex and difficult-to-cure diseases, mostly arising during adolescence. Among them, Anorexia Nervosa (AN) is characterized by insufficient energy supply for survival with severe consequences affecting the whole organism. To investigate onset and progression of inflammation and oxidative stress in the above reported pathological conditions, a battery of complementary techniques was used, followed by statistical evaluations. In particular, morphological and immunohistochemical reactions were conducted on mouse and human hippocampal specimens considering different markers: superoxide dismutase 1 and 2 (SOD1, SOD2), cyclooxygenase 2 (COX2), nitric oxide synthase 2 (NOS2), interleukin 6 (IL-6), and transforming growth factor β (TGF-β). Parallely, Bax, Bcl-2, p62, PINK1 and PARKIN were employed to investigate cell death pathways. Haematoxylin & eosin and Nissl staining were performed for histological and cytoarchitectural analyses. The results demonstrated that, in the presence of high inflammation/oxidative stress levels, the cells undertake different response mechanisms aimed at cell survival or death. Therefore, these data could provide novel insight allowing to develop new therapeutic protocols targeting inflammatory/oxidative stress pathway through activation of programmed cell death for cell survival.

# KNOCKING DOWN THE METABOTROPIC GLUTAMATE RECEPTOR 5 IN A MOUSE MODEL OF AMIOTROPHIC LATERAL SCLEROSIS REDUCES THE REACTIVE PHENOTYPE OF EX-VIVO CULTURED SPINAL CORD ASTROCYTES

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Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disease characterized by a selective death of upper and lower motor neurons (MNs). ALS is known to be a multifactorial and non-cell autonomous disease, where the non-neuronal supporting cells are directly involved in MNs degeneration. Glutamate-(Glu)-mediated excitotoxicity has been proposed as one major cause that trigger MNs degeneration. Although the etiopathogenesis is not completely understood, in-vitro and in-vivo studies demonstrated that damage within MNs is sustained by the degeneration of non-neuronal cells such as microglia and astrocytes. Group I metabotropic glutamate receptors (mGluR1, mGluR5) likely play a role in ALS, since they are over-expressed and functionally altered in different experimental model of ALS. In a previous work, we demonstrated that knocking-down mGluR1 or mGluR5 significantly prolongs survival and ameliorates the clinical progression in the SOD1G93A mouse model of ALS.
The aim of this work is to investigate the effects of mGluR5 down-regulation on the reactive phenotype of astrocytes in ALS. We used here spinal cord astrocyte cell cultures from adult B6SJL-TgN (SOD1-G93A)1Gur mice expressing high copy number of mutant human SOD1 with a Gly93Ala substitution (SOD1G93A) and mGluR5+/- mice expressing half dose of mGluR5. The two strains were appropriately crossed to obtain mice carrying the SOD1G93A transgene and lacking one allele of the mGluR5 encoding genes.
Experiments with the FURA-2 dye showed a significantly higher cytosolic calcium concentration ([Ca2+]i) in SOD1G93A than in WT mice, both under basal condition and after exposure to a 30 $\mu$M concentration of the Group I mGluRs agonist 3,5-DHPG (3,5-dihydroxyphenylglycine). mGluR5 knocking-down significantly reduced the excessive [Ca2+]i.
Confocal microscopy revealed that the astrogliosis markers GFAP (Glial fibrillary acidic protein), vimentin and S100β (S100 calcium binding protein B) were more expressed in SOD1G93A respect to WT mice and decreased in SOD1G93AmGluR5+/- mice. The same was true for the expression of the autophagy activation marker LC3-II (Microtubule-associated protein light chain 3). Of note, mGluR5 knocking-down also translates in a significant lower presence of misfolded-SOD1 protein when comparing SOD1G93AmGluR5+/- and SOD1G93A mice.
To conclude, a lower constitutive level of mGluR5 had a positive impact in SOD1G93A mouse astrocytes, supporting the idea that mGluR5 may be a potential pharmacological target for cell specific therapeutic approaches in ALS, aimed at preserving MNs by acting at the neighboring astroglial cell.


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# WORKSHOP EXOFLOWMETRY2 - 18 Giugno 2021

## CHARACTERIZATION OF EXTRACELLULAR VESICLES IN SEMINAL LIQUID BY NANOCYTOMETRY

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Key words: flow cytometry, extracellular messengers, virus
Extracellular Vesicles are vesicles produce by cells that carry proteins and nucleic acids that can work as extracellular messengers. Depending on cargo and origin can be classified. But one main characteristic is their size. Always bellow 500 nm. That size is the size limit detection for the majority of flow cytometers. By using fluorescence as trigger you can overlook the problem, but then, you can lose information. Recently, the development of new equipment (Nanocytometers) has allowed the analysis of extracellular vesicles and the development of a new technique: Nanoflowcytometry. In this talk we will show the limitations and the technical requirements to perform this type of analysis, with special emphasis in the flow cytometer setup and the application of Nanoflowcytometry to the immunophenotypical analysis of extracellular vesicles from pork and human Seminal Liquid. Also, we will demonstrate that Nanocytometry can be applied to other biological nanoparticles like virus.

## FLOW CYTOMETRY IN FOOD MICROBIOLOGY

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key word: VBNC status, phyiological parameters, probiotics

The flow cytometry applications in the food microbiology field are not limited to the cell counting even if this simple parameter is far to be easily obtained due to the presence of complex cell morphologies and aggregating structures among microorganisms associated to food matrixes. Beside the difficulties in microbial cell counting, other problems arise when the viability of microbial population has also to be measured. In this context, the viable but not cultivable fraction of the microbial population has important effect both in terms of quality and safety when pathogenic bacteria were analyzed. Flow cytometry has been also applied for the evaluation of several physiological parameters such as: membrane potential, cell membrane integrity, intracellular pH, activity of efflux systems involved in cell detoxification, ability to form aggregates among cells belonging to different taxa. The presentation will be focused on flow cytometry applications in food microbiology and probiotics.

## FLOW-SORTED MITOTIC CHROMOSOMES AS A TOOL TO UNCOVER THEIR 3D ARCHITECTURE

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Key words:
Despite the chromosomes were observed and described in 1882, their top level organization and 3D topography are being unravelled in detail in the recent years. The ability to flow-sort millions of mitotic chromosomes enabled performing chromosome conformation capture study that revealed their helical organization and allowed precise calculation of chromatin turn length along the chromosome body. Even higher numbers of sorted chromosomes needed for proteomic analyses allowed de-novo identification of barley chromosome-specific proteins by mass spectrometry and confirmed the presence of nucleolar proteins on mitotic chromosomes. The flow-sorted chromosomes can also be highly purified for observation by environmental scanning electron microscopy – a method that allows direct observation of their surface structures without any metal coating in conditions that keep the chromosome as close to its native state as possible, bringing view that challenges previous observations.

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## SEMI-AUTOMATED, PULSE-SHAPE SCANNING FLOW CYTOMETRY FOR THE MONITORING OF PHYTOPLANKTON AT FINE SPATIO-TEMPORAL SCALES

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Key words: high-frequency flow cytometry; CytoSense; phytoplankton; functional traits; functional groups; marine ecology

The ecology of phytoplankton remains elusive at the finest scales (hourly to daily variations), due to technical limitations. Our understanding of its dynamics is limited to broad spatio-temporal definitions. Because phytoplankton is significantly involved in CO2 uptake, this lack of understanding increases greatly the uncertainty of biogeochemical models. Quasi-continuous sampling of the whole phytoplankton community along the track of a ship, or from a fixed platform, has become possible with the advent of the semi-automated pulse-shape recording flow cytometers CytoSense (Cytobuoy B.V., the Netherlands). This instrument is specialized for the study of phytoplankton cells and its large dynamical size range (1 $\mu$m to 1 mm) opens to the possibility of describing phytoplankton dynamics at an unprecedented resolution in terms of optically-defined functional groups. Two high-frequency phytoplankton data sets generated with a CytoSense will be presented, in different contexts. The first data set has been generated during a 2-days long sampling, with a frequency of 10 minutes, from the Acqua Alta oceanographic platform located 8 miles from Venice in the Northern Adriatic Sea. Few changes were observed at the community level, but a significant variability of optical parameters was observed depending on the light field and the vertical movements of the water masses, also dependent upon the tide. The second dataset has been generated during an oceanographic cruise from the Azores to Sicily, sampling every 4 minutes while the ship was cruising through different water masses. Phytoplankton patchiness was observed down to the sub-mesoscale. Significant differences in phytoplankton functional group assemblages were assessed, dependent on the physico-chemical characteristics of the water masses crossed during the track. High-frequency sampling helps to understand phytoplankton ecology at a finer scale than before, highlighting changes occurring at different time and space scales in terms of functional traits (fluorescence, size of cells) and population dynamics. The large data sets generated will need powerful statistical analyses to fully appreciate the large optical diversity present in phytoplankton and to relate it reliably to environmental parameters.

## ANTIBODY-BASED FLOW CYTOMETRY METHOD FOR THE DETECTION OF A. MUCINIPHILA IN COMPLEX CELL SUSPENSIONS

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Akkermansia muciniphila is a mucin-degrading bacterium abundantly present in the human gut. Since its discovery, A. muciniphila has been recognize as a beneficial intestinal symbiont and is considered a promising next-generation probiotic. However, an efficient strategy for isolation of new strains is needed. In this study an antibody-based flow cytometry (FCM) method, for the specific detection of A. muciniphila in complex cell suspensions, was developed.
Rabbit polyclonal antibodies were produced against whole cells of A. muciniphila type strain MucT and used, after their purification, as primary antibodies. Optimal concentration of the primary antibodies was defined by titration experiments. Alexa-488 goat anti-rabbit IgG was used as secondary antibody. Briefly, A. muciniphila cells, in pure culture or in mixed cell suspensions, were incubated for 30 min at room temperature in Phosphate Buffer Saline (PBS, pH 7.4), 5% (v/v) goat serum for blocking potential nonspecific antibody-binding sites. Anti-A. muciniphila whole cells purified IgGs (1 µg/ml) were added to samples, then incubated for 1h at room temperature. Cells were washed once and resuspended in PBS, 3% (v/v) bovine serum albumin (BSA) and the fluorochrome-labeled secondary antibody (1 µg/ml) was added. Samples were incubated for 1 h at room temperature in the dark. Finally, samples were diluted in PBS and analyzed by FCM (C6 plus BD Accuri) (excitation: 488 nm; emission: 530 nm).
Our assays confirmed the antibodies specificity at species level. The antibodies obtained from the strain MucT were able to detect five newly isolated A. muciniphila strains. Conversely, no signals were detected with other intestinal species. The FCM method was applied to quantify A. muciniphila in pure culture and in mixed populations, containing the target bacteria and E. coli or Lactobacillus acidophilus at different ratio. The accuracy of the absolute quantification made by antibodies labelling was compared to SYBR Green I staining and resulted variable depending on cell density. A. muciniphila was specifically detected in all mixed populations, even at the ratio 1:1000 and a cell density of 105 FU/ml. The developed method was also able to detect A. muciniphila in enrichment cultures obtained by inoculating fecal samples in minimal medium supplemented with mucin.
Our method could be applied for the detection and quantification of A. muciniphila in multi-species samples. Moreover, considering that antibody-labelled cells showed to retain their viability, our protocol will be further combined with fluorescence-activated cell sorting (FACS) for isolation of new A. muciniphila strains from fecal samples of healthy donor. Improving the isolation process (rate and selectivity) could greatly enhance availability of new strains and, consequently, our knowledge of this bacterial species and its diversity.

## FROM CYTO2021 TO EXOFLOWMETRY2: EXPLORING FRONTIERS IN ENVIRONMENTAL CYTOMETRY

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Key words: Non-mammalian Cytometry, Environmental Cytometry (food, water, agriculture, wild areas).
As pioneered in Rome in 2019, interest groups have started to provide cytometric forums that focus on underserved areas of cytometry, "Exoflowmetry", roughly speaking all applications that deal with non-mammalian organisms, including analyses of model and non-model plants, algae, aquatic organisms, and microbes (bacteria and viruses). An assortment of otherwise unconventional samples, such as subcellular organelles from cellular homogenates, also present unique challenges to cytometric assay development and analysis, as do non-cellular particulate suspensions such as microplastics, and these challenges will be defined and discussed.
At CYTO2021 (June 7- June 9/10), the forums addressing these underserved areas are to include plenary speakers, breakout discussion groups, and poster presentations. I will summarize some notable contributions in this area.
I will also discuss considerations, approaches, and techniques to optimize the analysis and maximize the data utility from non-mammalian and unconventional samples, highlighting the importance of, wherever possible, defining Best Practices. This presentation also aims to inspire new connections within our community to support future work, and will include discussion of innovative technical directions for the field, including applications in spectral cytometry and sorting.

## MOLECULAR FLOW CYTOGENETICS IN PLANTS

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The molecular flow cytogenetic combines the molecular cytogenetic characterization of chromosomes, traditionally performed on slide by using methods as fluorescence in situ hybridization (FISH), with the flow cytogenetic, performed by flow cytometric analysis (and sorting) of chromosomes floating in suspension. The two approaches have been merged by the development of FISHIS (fluorescence in situ hybridization in suspension), a simple and fast method which enables the labelling of chromosomes in suspension with simple sequence repeats (SSR) as probes. FISHIS opened the way to a real bivariate flow karyotyping in plants and extended the chromosome approach to potentially any plant species for which a good chromosome suspension and genome widespread SSR probes are available.
The FISHIS method will be described together with some applications of the "sorted chromosomes" mainly in wheat plant breeding studies.

## HOW AUTOMATED FLOW CYTOMETRY CAN IMPROVE OUR UNDERSTANDING OF THE PLANKTON STRUCTURE, DISTRIBUTION AND FUNCTIONING?

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Key words: single cell; marine ecosystem; Lagrangian deconvolution
Flow cytometry has greatly contributed to improve our knowledge of aquatic trophic food webs through the exploration at the single-cell level of the various planktonic communities such as phytoplankton, bacteria, marine viruses. Apart from counting these planktonic particles the analysis of variations in autofluorescence, light scatter and the use of fluorescent stains has demonstrated its power in elucidating the physiology and adaptations of organisms to the natural environment both in natural samples and in culture. Automated in situ flow cytometry makes it possible to sample the Ocean with a high spatial and temporal resolution, offering a new opportunity to address complex questions and bring new answers to better understand the functioning of the marine ecosystem. For instance, model simulations and satellite observations have shown that ocean dynamics at fine scales (1–100 km in space, day–weeks in time) strongly influence the distribution of phytoplankton. This temporal scale is similar to that of many biological processes, such as phytoplankton growth, suggesting a physical and biological coupling. To better characterize this coupling, both physical and biological measurements in situ are mandatory. However, the observations of fine scales constitute a challenge due to the difficulties of sampling at high spatio-temporal frequency. In this presentation, we'll present how a satellite-based adaptative and Lagrangian strategy coupled with a high-resolution physical-biological sampling, using an automated flow cytometer installed onboard, has been performed to follow and describe fine-scale structures in the Mediterranean Sea.


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## TIPS AND TRICKS FOR A SUCCESSFUL ISOLATION OF NUCLEI IN PLANTS

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Key words: best practices; flow cytometry; plant sciences; nuclear suspensions; nuclear isolation; genome size; ploidy level

A critical aspect for obtaining accurate, reliable, and high-resolution estimations of nuclear DNA content is the successful isolation of nuclei from plant tissues. In theory, any plant tissue providing intact nuclei in sufficient quantity is suitable for nuclear DNA content estimation using flow cytometry (FCM). The most common method is very simple, rapid, and effective and consists of chopping up the plant material with a sharp razor blade to release nuclei into an isolation buffer, filtering the homogenate to remove large particles, and staining the nuclei in buffered suspension with a particular fluorochrome of choice. However, despite of the simplicity of the methods, it is clear from recent publications that experimental design and procedures have not followed the best practices, and this may affect the quality of the results and the conclusions drawn. In this presentation, I will discuss the peculiarities of nuclei isolation buffers, the distorting effects that secondary metabolites may have in nuclear suspensions and how to test them, and the singularities of optimal plant tissue selection for FCM analysis.

## EXOFLOWMETRY, is it real?

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Key words: flow cytometry, flow sorting, microbiology, ecology, biotechnology, plants

High throughput technologies have to be accurate rather than faster, but both features have to be coupled to get a successful machinery. Computer scince and engineering can help substantially to generate an effective analytical system, but are the physics and chemistry at the bases of the technology which would allow to generate meaningful and useful data to investigate Nature. Next Generation Sequencing and Flow Cytometry (FCM) are a clear example of what light and chemistry can create as information from objects in suspension by means of highly specific fluorescence and optical properties generated from each single particle. FCM features a low analytical background, uses a highly specific fluorescence emissions and a distinctive light diffraction patterns join up with a fast particle analysis, and eventually single particles flow sorting, all of this might explain the why FCM widespreads successfully from human blood cells to virus, bacteria, algae, fungi and all sort of cellular organelles and vesicles. Often, the analysis of different kinds of particles have required the development of new methods or protocols, so contributing to a more and more useful and intelligent use of FCM. From 1980 to early 2021, more than 330,000 papers have been published describing FCM uses and more than 15% of them are papers related to non-bio-medical applications, and both curves show an exponential growth. Going in “instrumental”, what was hegemony of less than five companies now see more than fifteen different firms and instruments available, and some of them have peculiar features developed just for flow cytometry applied to marine biology or microbiology or ecology. So, what was exotic because of its origin in a different “word or country”,  say a non-biomedical field, now fully belongs to the vast area of exotic flow cytometry or ExoFlowMetry, where FCM biodiversity is well preserved.

# DEVELOPMENT OF A NEW APPROACH FOR ENVIRONMENTAL STATE ASSESSMENT: FLOW CYTOMETRY AND STABLE ISOTOPE ANALYSIS OF THE EPILITHON COMMUNITY AT LAKE BRACCIANO (ROME, ITALY)

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key words: biofilm, micro algae, autofluorescence, water ecosystems

The anthropogenic nutrient enrichment of rivers and lakes is one of the major problems affecting freshwater ecosystems over recent decades. Elevated Phosphorus and nNtrogen concentrations can cause excessive phytoplankton and periphyton growth, resulting in a loss of invertebrate and macrophyte communities, low oxygen concentrations, fish kills, and loss of ecosystem services. Nutrient enrichment can also lead to shifts in microbial community structure, with plankton blooms becoming dominated by toxic cyanobacteria, which can have serious impacts for human health and drinking water supply. Therefore, the development of early warning ecological indicators that can help arrest the phenomenon in its early stages is highly desirable. Among the anthropic nutrient inputs, Nitrogen loadings are spreading with human-related loads. Stable isotope analysis of Nitrogen in algal primary producers has proved useful in determining the origins of Nitrogen inputs in several marine and freshwater ecosystems.
The characterization of freshwater photoautotrophic micro-communities is not an easy task, due to the time and specialist taxonomic knowledge required for morphological determination of algal species. High-throughput methods such as flow cytometry (FCM) have the potential to provide a much-needed rapid quantification and characterization of lake microalgae.
In this study, we present a flow cytometric methodology coupled with stable isotope analysis to characterize epilithic associations (aquatic biofilm that colonize submerged rocks surface). Samples of biofilm and green filamentous algae from rocks were collected in different seasons of 2020 in shallow water at six stations along the entire perimeter of Lake Bracciano. Biofilm was analysed for the autofluorescence emission from different pigments characterizing microalgae genera, while green filamentous algae were analysed for Nitrogen elemental content and stable isotope signature. Such combined approach was able to trace related changes among cyanophyceae and clorophyceae population densities in the community compared with the Nitrogen signature and concentration, highlighting a correlation between Nitrogen load that insist on a site and the related epilithic photoautotrophic community.
Using this approach, we confirm FCM as a sensitive method for microalgae monitoring and its use, in synergy with the stable isotopes analysis technique, could led to the emergence of an interesting indicator of environmental status in freshwaters.


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## FLOW CYTOMETRY APPLIED TO THE STUDY OF PLANT-MICROBE INTERACTIONS

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Key words: Arbuscular mycorrhizal, chromatine condensation, nucleus hypertrophy

Arbuscular mycorrhizal (AM) fungi are able to form symbiosis with the roots of more than 80% of land plants. Arbuscules are their typical and exclusive structures, originating from the dichotomous ramification of the hyphae in contact with the plasma membrane of the host root cortex cell. They receive plant carbohydrates and in exchange provide the plant with water and mineral nutrient, especially phosphorus, collected thanks to their extraradical mycelium. Moreover, the AM symbiosis improves plant tolerance versus both biotic and abiotic stresses. Modifications to the root system following the symbiosis include increased branching as well as structural and quantitative changes of the host cell nuclei. In fact, a central position inside the cell combined to a significant nucleus hypertrophy was observed in different AM systems. Depending on the plant host, this can be explained by chromatin decondensation or by polyploidization.
Flow cytometry is a good technique in order to investigate the nucleus modifications induced by AM colonization alone or in combination with different stress types, as phytoplasmas or pathogenic fungi (biotic) or heavy metal pollution (abiotic). Plant nuclei were extracted from the root systems - deprived of the apices - of three plant species (Allium porrum, Pisum sativum and Solanum lycopersicum) characterized by different DNA contents and polyploidization levels. Depending on the experimental system, nuclei were stained with different dyes [4',6-diamidino-2-phenylindole (DAPI), Propidium Iodide (PI)] and analyzed by flow cytometry. The fluorescence intensity was correlated to DNA quantity or to chromatin accessibility either at saturating or under-saturating dye concentrations, respectively. Changes in the percentages of the different ploidy populations, following treatments, were also investigated. Finally, variation coefficients (CV%) were considered as homogeneity indexes of nuclear populations. Interesting information about modifications induced by AM fungi at nucleus level were obtained, as well as about their positive effects in contrasting changes induced by pathogens and metals. Altogether the obtained results confirm that flow cytometry is a fast and useful tool for the study of plant-microbe interactions.
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## AN EFFICENT AND RELIABLE METHOD FOR A HIGH YIELD EXTRACTION OF NUCLEI FROM MICRO AMOUNT OF FRESH PLANT TISSUE

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Nowadays in plant flow cytometry, several advanced techniques are available to investigate and sort almost all components of the cell, but one request largely outperforms all others which is ploidy evaluation and DNA content estimation. Plant nuclei isolation in suspension for FCM analysis is usually performed by the Galbraith method (Galbraith et al. 1983) consisting of chopping small pieces of fresh tissues into a properly selected isolation buffer (Loureiro et al. 2021). But, plant breeding and genomic or genetic manipulations often result in a very large number of regenerating plants each one likely owing a DNA content change, and so chopping becomes a limiting factor in terms of time and efforts to manipulate tens or hundreds of samples to get all of them as nuclei suspension. We have developed a fast and effective method for plant nuclei isolation based on the use of 0.5-1mg of leaf or root tissue distributed into a 96 wells plate filled with 100$\mu$l of an appropriate isolation containing dye buffer (mostly LB01, Doležel et al, 1989), homogenized at low speed (3,000-5,000 rpm) by an Eppendorf stick mixer for 10" to 15" and diluted with a multichannel pipet with an equal amount of buffer. Samples are immediately analysed with a Cytoflex S (Beckman Coulter, USA) equipped with the automatic plate reader unit without further manipulations, such as any sample filtration. Averaged time sample preparation is of 30" about, being homogenization the most time-demanding manipulation. About 5,000 to 10,000 nuclei/mg, depending on tissue, can be isolated for FCM, which are sufficient for cell cycle analysis, ploidy evaluation and DNA content estimation. To prove the robustness of the method, a number of species have been tested with satisfactory results in term of reproducibility and quality of the measure. Our analyser equipped with the plate reader showed to be very effective in plant nuclei characterization, and not a single clogging happened during hundreds of FCM analysis. We forecast the same speedy highly reliable methodology could also be applied to other species and tissues.

## WHAT CULTURE-BASED ANALYSES DON'T TELL AND FLOW CYTOMETRY DOES ABOUT VIABLE BUT NON-CULTURABLE MICROORGANISMS

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Key words: VBNC, flow sorting, E. coli, oregano essential oil

Microbiology pays a main attention to characterize and limit bacterial contaminations in food, and the viable but non-culturable (VBNC) state of microorganisms cause a great threat to food safety and public health as the use of culture-based only microbiological methods might direct to a risky under-appreciation of a food contamination. New fast and accurate analytical methods for VBNC bacteria are needed to assess the effectiveness of treatments to ensure healthy food preservation. We have investigated flow cytometry (FCM) as a fast and consistent method to evaluate the induction of E. coli ATCC 25922 strain into a VBNC state after mild heat treatment of fruit juices in combination with oregano essential oil (OEO). At first, E. coli was grown in Brain Hearth Infusion (BHI) medium at 37 °C for 24 h and treated at 55 °C, 60 °C and 65 °C for 30 min with and without OEO. Subsequently, microbial challenge test was performed by artificially contaminating commercial and organic fruit juices with E. coli strain at 5 Log CFU/mL. The combination of mild heat treatment (65 °C) and OEO (100 ppm) for 5 min was applied and samples were stored at 4 °C and 37 °C up to 48h. Bacterial survival ratio was estimated using culture-based methods and flow cytometry. Cell sorting of the different subpopulations in terms of viability obtained after treatments was also performed and their growth on culture media was assessed using a single-cell approach. Results obtained in vitro revealed that the samples incubated at 37 °C for 24h after the treatments at 55 °C for 30 min with OEO (50 and 100 ppm), showed a long-term inactivation of bacterial growth, although a percentage of damaged cells is able to recover growth activity at optimum temperature for the strain. Instead, combined treatments at 65°C for 5 min led only to a temporary reducing effect of the alive population; indeed, it was found to be ineffective to stop long-term contaminations. Results obtained in vivo revealed no bacterial growth of the challenge microorganisms after the combined treatment both in culture-based and in FCM assay, while curing with OEO only was not sufficient to reduce the microbial load below the limits by law for bacterial contamination. Our FCM results show adding OEO to fruit juice rise the efficiency of mild heat treatment, and low thermal treatment alone can be considered as a VBNC inducing factor.

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# Others

## FLOW CYTOMETRY AT SCHOOL: A SCHOOL – WORK ALTERNATION EXPERIENCE

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Abstract
School–Work Alternation is an innovative education method brought to Italy by Law no. 53/2003 and disciplined by the Legislative Decree no. 77/2005. It is addressed to students of upper secondary schools and allows them to "alternate" periods of training in the classroom to working experiences inside companies or other hosting establishments.
Law no. 107/2015 emphasized the importance of bringing together knowledge with know-how, intensifying the relations of the schools with their surroundings and the worlds of production, services, academia, research and public administration. This can encourage innovative teaching and the dissemination of educational processes that focus on acquiring useful skills for the world of work, while promoting educational and professional guidance, self-entrepreneurship, active citizenship, and greater involvement of the young in learning processes, also thanks to new technologies.
Our experience in collaboration with the Italian Society of Cytometry and Dr. Giovanni D'Arena can be collocated in this framework.
The project was carried on between November 2019 and February 2020. Some activities were approached in a different perspective, due to Covid-19 lockdown. A group of about 15 motivated students in STEM disciplines and teachers of Mathematics, Physics, Chemistry and Biology took part in the activities organized in 5 lectures and workshops, held in school classrooms and labs.
The aim of the project was twofold: on the one hand there was the idea to enhance the "skill exchange" between school, academia and research; on the other hand to orient students towards a mature choice for their future studies.
In the first part of the project, school teachers introduced the prerequisites for understanding how a cytometer works. They gave lectures on "Physics of LASERs" and "How radiation interacts with matter". The Math teacher also explained how to read histograms, dot, density and contour plots, output of the cytometer. In the second part of the activities, the cytometer and its applications were shown to the students: the first lecture was held by Dr. D'Arena, who introduced the main features of the instrument and its applications in the field of hematology; the second activity focused on a practical demonstration of the cytometer given by Dr. Stefano Amalfitano, who invited the students to use it analyzing specimens of tap water and bottle water. Finally, Dr. Casotti showed the applications of cytometry in marine biology. In May 2021, the group of participants organized the presentation to the conference.
In conclusion, thanks to a project in line with the study plan of the school, the students had the opportunity to consolidate the knowledge acquired at school and to test their aptitudes in the field of science, to enrich their training and to orient their study path. Given the positive outcome of the project, the school will encourage such kind of experiences in the future.

# BUILDING ARNO, TIME-RESOLVED FLOW CYTOMETER FOR COMPENSATION-FREE ASSAYS

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In flow cytometry, compensation is required to account for and remove the contribution of signal spillover between spectral channels. The problem worsens as the level of multiplexing increases and more wavelength bands are crowded into a limited spectral window. While the technique of spectral flow cytometry can resolve some overlapping fluorophores, it requires spectral unmixing, which is simply another term for compensation; and it does not reduce the population spread associated with the overlaps. To solve these problems, we have developed time-resolved flow cytometry technology that distinguishes completely overlapping fluors based on differences in their fluorescence decay lifetimes.
In order to address to need of high complexity assays, increasing the multiplexing capabilities and avoiding spectral spillover, as much as possible, a novel platform was designed.
The new 15-parameter “Arno” platform has two spatially separated, pulsed excitation sources (Toptica iBeam 405 and 488); one FSC and two SSC channels; and 12 fluorescence detection channels (6 per laser), from violet to infrared. The 12 fiber-coupled fluorescence signals are sensed by only 6 physical detectors (Hamamatsu PMTs). Each detector collects light from two spectrally overlapping fluors, and the contribution from each fluor is resolved using Kinetic River proprietary time-domain multiplexing technology . Ultrastable sheath flow is established with our custom-built Shasta fluidic control system. Data acquisition is performed using 8-channel 1.25-GHz sampling on a National Instrument PXI platform and custom-written LabVIEW code. Signal processing is performed on a dedicated computing platform running custom algorithms.
The Arno system has been fully characterized with respect to sensitivity, dynamic range, CVs, and time resolution in each of the 12 fluorescence ‘channels’ (six spectral bands, three from each laser, each with both a short- and a long-lifetime component). By having only three detectors per laser, each wavelength band can be spectrally well-separated, leading to less than 5% spillover in all channels, with most below 1%. We are in the process of full system validation with sequentially more complex cell-based assays using 4-, 8-, and 12-color panels.
We have expanded proof-of-concept Arno system by designing and building a 15-parameter, compensation-free cell analyzer. Our Arno platform leverages Kinetic River’s proprietary time-resolved flow cytometry technology to achieve compensation-free 12-color (plus one forward and two side scattering channels) using only two lasers and six spectral detectors.


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## DEVELOPMENT OF A TRIPLE-UV/VIOLET EXCITATION ANALYZER FOR LABEL-FREE FLOW CYTOMETRY

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Standard off-the-shelf flow cytometers have limited flexibility, leaving little choice to the user interested in more exotic excitation sources, or requiring users to buy a “bundle” of lasers, simply to get the single laser line desired. There is unmet demand for flow cytometry systems that are customized and modular; and in particular, for analyzers tailored to operate in the violet, UV, and deep UV, from customers interested in label-free analysis of cells, bacteria, and algae.
Using Kinetic River Potomac™ flexible architecture, we have designed and built a Potomac analyzer customized for label-free analysis of cell samples for the Italian National Research Council (IREA-CNR, Naples, Italy).
It uses three interchangeable excitation sources: two built-in (405 and 375 nm, both from Pavilion Integration Corporation), and one external add-on (266 nm, from CryLas). It has six detectors (all PMTs for increased nanoparticle sensitivity): FSC, SSC, and four fluorescence channels (Hamamatsu Corp.), with three extra channels built-in to allow for future expansion. Detection channels (UV to IR) use high performance removable Semrock dichroics and filters (IDEX Health & Science).
Ultrastable sheath flow is established with Kinetic River Shasta™ fluidic control system. It incorporates our always-on flowcell monitoring Cavour™ system for simple optimization and troubleshooting. Data is collected with proprietary electronics and a PXI National Instruments data acquisition system. Proprietary Panama software™ operates the system, performs a full suite of sample processing and data visualization, and is intuitive and user-friendly. The system has built-in capability to expand the number of lasers and/or detection channels.
The system was tested for performance using a variety of dyes, microspheres, and cell-based assays, including Coefficients of variation (CVs) for the scatter channels and each fluorescence channel, 8-peak beads dynamic range, Megamix-Plus SSC nanoparticles beads and cell cycle using DAPI.
We have designed, built, and delivered a completely customized commercial flow cytometry solution that enables researchers at IREA-CNR to perform label- free excitation of algae and bacteria for water quality analysis. The system includes only those features required to meet the end-user’s unique needs, with built-in room to grow.

## NEW EYES FOR FLOW CELL MONITORING

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For many researchers, particularly those working on cutting-edge research in flow cytometry, the ability to monitor the flowcell can be crucial to the success of your experiments. However, with most flow cytometry instruments, this is difficult and user restricted .
We have developed a dedicated, always-on flowcell monitoring solution, dubbed the Cavour, to address this need. The system is designed using standard off-the-shelf components. The system uses 3D-printed mounts which can be customized, allowing the device to be adapted to most commercial flow cytometry systems.
Based on the specific requirements of flow cytometry users, we have implemented a proprietary microscope design which allows remote monitoring of an instrument without removing the safety cover.
Coupling intuitive digital microscope software (DinoCapture) with a tablet provides a continuous live feed of the flowcell and enables always-on monitoring. Our unique optical design provides a large field of view (600 µm x 400 µm) with 50x equivalent magnification, optimized for flowcell viewing. The software allows for image and video capture, and on-screen measurements.
Cavour device provide the way for remote troubleshooting and optimizing own system’s optical alignment, including tasks such as laser realignment, maximizing signal prior to a key experiment, or identifying deposits in the flowcell that can cause increased background due to light scattering, are all able to be addressed easily avoiding any exposure to eyes to laser beams.
Flowcell Monitoring Module was developed with that in mind. The module is designed to allow it to be adapted to most flow cytometers on the market. Implementing the device should allow researchers to avoid unnecessary service calls and reduce system downtime.

## ANTIBODY-BASED FLOW CYTOMETRY METHOD FOR THE DETECTION OF A. *MUCINIPHILA* IN COMPLEX CELL SUSPENSIONS

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Akkermansia muciniphila is a mucin-degrading bacterium abundantly present in the human gut. Since its discovery, A. muciniphila has been recognize as a beneficial intestinal symbiont and is considered a promising next-generation probiotic. However, an efficient strategy for isolation of new strains is needed. In this study an antibody-based flow cytometry (FCM) method, for the specific detection of A. muciniphila in complex cell suspensions, was developed.
Rabbit polyclonal antibodies were produced against whole cells of A. muciniphila type strain MucT and used, after their purification, as primary antibodies. Optimal concentration of the primary antibodies was defined by titration experiments. Alexa-488 goat anti-rabbit IgG was used as secondary antibody. Briefly, A. muciniphila cells, in pure culture or in mixed cell suspensions, were incubated for 30 min at room temperature in Phosphate Buffer Saline (PBS, pH 7.4), 5% (v/v) goat serum for blocking potential nonspecific antibody-binding sites. Anti-A. muciniphila whole cells purified IgGs (1 µg/ml) were added to samples, then incubated for 1h at room temperature. Cells were washed once and resuspended in PBS, 3% (v/v) bovine serum albumin (BSA) and the fluorochrome-labeled secondary antibody (1 µg/ml) was added. Samples were incubated for 1 h at room temperature in the dark. Finally, samples were diluted in PBS and analyzed by FCM (C6 plus BD Accuri) (excitation: 488 nm; emission: 530 nm).
Our assays confirmed the antibodies specificity at species level. The antibodies obtained from the strain MucT were able to detect five newly isolated A. muciniphila strains. Conversely, no signals were detected with other intestinal species. The FCM method was applied to quantify A. muciniphila in pure culture and in mixed populations, containing the target bacteria and E. coli or Lactobacillus acidophilus at different ratio. The accuracy of the absolute quantification made by antibodies labelling was compared to SYBR Green I staining and resulted variable depending on cell density. A. muciniphila was specifically detected in all mixed populations, even at the ratio 1:1000 and a cell density of 105 FU/ml. The developed method was also able to detect A. muciniphila in enrichment cultures obtained by inoculating fecal samples in minimal medium supplemented with mucin.
Our method could be applied for the detection and quantification of A. muciniphila in multi-species samples. Moreover, considering that antibody-labelled cells showed to retain their viability, our protocol will be further combined with fluorescence-activated cell sorting (FACS) for isolation of new A. muciniphila strains from fecal samples of healthy donor. Improving the isolation process (rate and selectivity) could greatly enhance availability of new strains and, consequently, our knowledge of this bacterial species and its diversity.

## UNCOVERING THE RELEASE OF MICRO/NANOPLASTICS FROM DISPOSABLE FACE MASKS AT TIMES OF COVID-19

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In this study, we aimed to assess the environmental impact of discarded face masks, that are a source of emerging concern as indicated by most recent literature, although still little investigated. We evaluated micro- and nanoplastic particles that can be released from face mask once subject to environmental conditions. Exposure to simulated-low shear forces demonstrated to be effective in breaking and fragmenting face mask tissue into smaller debris. Even at low shear energy densities, a single mask could release in water thousands of microplastic fibers and up to 1011 submicrometric particles. The latter were quantified using flow cytometry that was proven to be a promising technique for nanoplastic counting, thus improving our understanding on distribution and fate of NPs still representing a great analytical challenge in plastic pollution research.

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