Special Supplement 1 SS1

BC

biochimica clinica

RIASSUNTI II RIUNIONE NAZIONALE ISCCA ITALY - SOCIETÀ ITALIANA PER L'ANALISI CITOMETRICA CELLULARE

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Nota dell'Editore: i riassunti sono stati riprodotti senza alcuna revisione dal materiale direttamente fornito dagli autori.

R-1

SIGNIFICATO CLINICO DEL RAPPORTO BAX/BCL-2 DETERMINATO IN CITOMETRIA A FLUSSO NELLA LEUCEMIA LINFATICA CRONICA (LLC)

G. Del Poeta[1], M.I. Del Principe[1], M. Dal Bo[2], F. Buccisano[1], M. Postorino[1], F. Pozzo[2], L. Maurillo[1], A. Venditti[1], S. Amadori[1], V. Gattei[2]

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Nella LLC l'equilibrio tra le proteine pro-apoptotiche e quelle anti-apoptotiche della famiglia Bcl-2 determina sia la sensibilità alla chemioterapia che la sopravvivenza libera da progressione (PFS) e la sopravvivenza globale (OS). La disponibilità nell'uso clinico di nuovi farmaci BH3 peptidomimetici pro-apoptotici come ABT-199 ci ha stimolato ad analizzare l'impatto del rapporto bax/bcl-2 sulla prognosi della LLC. Gli obiettivi principali della nostra ricerca sono stati: 1) correlare il rapporto bax/bcl-2 con gli altri fattori prognostici; 2) valutare PFS e OS in base a bax/bcl-2; 3) confermare bax/bcl-2 come fattore prognostico indipendente. Pertanto abbiamo studiato 502 pazienti con età mediana di 65 anni (170 stadio Rai basso, 318 intermedio e 14 alto). Bax/bcl-2 è stato calcolato mediante citometria a flusso, dividendo l'intensità di fluorescenza media (rMFI) di bax con rMFI di bcl-2 su cellule CD19+CD5+ di LLC. La soglia di positività è stata fissata al valore mediano >1.5. I pazienti bax/bcl-2 positivi erano 266/502 pari al 53%. Bax/bcl-2 >1.5 era associato ad uno stadio Rai basso, ad un tempo di raddoppiamento linfocitario >12 mesi, a beta$_2$-microglobulina <2.2 mg/dl e a valori di CD23 solubile <70 U/ml (p<0.0001). Bax/bcl-2 >1.5 era significativamente rappresentato nel sottogruppo a basso rischio citogenetico (221/341; p<0.0001). Correlazioni significative erano riscontrate tra bax/bcl-2 <1.5 e stato IGHV non mutato (130/168; p<0.0001) o le mutazioni di *NOTCH1* (49/58; p<0.0001) o di *TP53* (29/37; p=0.00007). Inoltre abbiamo osservato brevi PFS e OS in pazienti con bax/bcl-2 <1.5 (10% vs 52% a 16 anni; p<0.0001 e 46% vs 79% a 16 anni; p<0.0001). Infine abbiamo studiato la sua espressione nei pazienti *IGHV* non mutati (168) e nei casi con *TP53* mutato (37), notoriamente a peggiore prognosi. Bax/bcl-2 >1.5 identificava pazienti con una più lunga PFS (43% vs 10% e 50% vs 10% a 7 anni; p=0.00002 e p=0.039), avvalorando così il suo altissimo impatto prognostico. In analisi multivariata della PFS (489 pazienti), bax/bcl-2 (p<0.0001) insieme a stadio Rai modificato (p<0.0001), alla citogenetica (p=0.0001), allo stato *IGHV* (p<0.0001) e a *TP53* (p=0.001) è stato confermato come fattore prognostico indipendente[1]. L'indice apoptotico bax/bcl-2 è un potente indicatore prognostico che permette anche di poter identificare pazienti a diversa prognosi anche all'interno dei sottogruppi con *IGHV* non mutato e *TP53* mutato, già di per sè a rischio clinico molto elevato. I moderni farmaci anti-bcl-2, quali ABT-199, potrebbero essere monitorati con questo semplice ma potente approccio di laboratorio in citometria a flusso.

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C-1

PHOSPHO-SPECIFIC FLOW CYTOMETRY FOR CHARACTERIZING REDOX SIGNALING SENSITIVITY ASSOCIATED WITH LEUKEMIA DISEASE PROGRESSION

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Background. B-cell receptor (BCR) signaling is a key determinant of variable clinical behavior and a target for therapeutic interventions in chronic lymphocytic leukemia (CLL). Endogenously produced H_2O_2 is thought to fine-tune the BCR signaling by reversibly inhibiting phosphatases. However, relatively little is known about how CLL cells sense and respond to such redox cues. Aims. In this study, we used phospho-specific flow cytometry to characterize redox sensitivity of BCR signaling in CLL patients. Methods. Phosphorylation levels of SYK, ERK1/2, p38, NF-κB p65, and JNK, were analyzed at the single-cell level in 42 CLL cell samples using phospho-specific flow cytometry. Phosphorylation was measured in the basal condition and following H_2O_2 stimulation. Circulating B cells from healthy individuals were analyzed as controls. Results. H_2O_2 induced a significant increase in phosphorylation of BCR signaling proteins, which is higher and more heterogeneous in CLL than normal B cells. Phosphorylation response of SYK was significantly higher in the patient subsets defined by the mutated IGHV status (M-CLL) (P=0.032), ZAP70 (P=0.020) and CD38 (P=0.005) expression. Phosphorylation response of ERK1/2 was significantly higher in patients expressing ZAP70 (P=0.040). To assess the impact of redox signaling sensitivity on disease progression, measured as time to first treatment (TTFT), we examined time-to-event modeling utilizing signaling data and currently used prognostic parameters. Univariate time-to-event analysis identified low pSYK (P=0.005) and pERK1/2 (P=0.015), UM-IGHV (P<0.001) and ZAP70-positive (P=0.001) as significant, independent predictors of shorter TTFT. In a bivariate time-to-event analysis using low pSYK as common predictor, low pSYK (P=0.020) and UM-IGHV (P=0.002), low pSYK (P=0.033) and ZAP70-positive (P=008)

showed to be independent, significant parameters of disease progression. Kaplan-Meier curves showed statistically significant slower progression (longer time to first treatment, TTFT) in patients with higher pSYK (log-rank test P=0.003) and pERK1/2 (log-rank test P=0.012). Conclusions. Redox signaling hypersensitivity measured by phospho-specific flow cytometry is an intrinsic characteristic of CLL with favorable prognosis and is predictive of a slower clinical progression. Overall, these results show that phospho-specific flow cytometry enables to capture biologically and clinically relevant information of pathologic cells.

C-2

miRNAs PROFILE OF BONE MARROW FIBROBLASTS IN MULTIPLE MYELOMA: RELATIONSHIP WITH DISEASE PROGRESSION AND DRUG-RESISTANCE

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Purpose. microRNAs (miRs) regulate gene expression at post-transcriptional level modulating several biological processes. Bone marrow (BM) fibroblasts (or cancer associated fibroblasts, CAFs) from active multiple myeloma (MM) patients present an activated phenotype (FSP1+/FAP+/αSMA+), with higher proliferative rate compared to monoclonal gammopathy of undetermined significance (MGUS) CAFs1. BM CAFs from bortezomib (bort)-resistant patients are resistant in vitro to the drug and prevent bort-induced apoptosis of co-cultured MM cells2. Our purpose was to investigate whether a specific miR profile is associated to the phenotype and functional activities of BM CAFs in MGUS to MM transition and drug resistance. Methods: miRs expression was analyzed by microarray and validated by qRT-PCR and flow cytometry on CAFs purified from BM aspirates of MGUS and MM patients. miRs target genes were identified by interrogating different tools commonly used to predict human miR gene targets and validated by western blot analysis. miRs functional effects were analyzed in CAFs transiently transfected with miRCURY LNA inhibitors and mimics. Results and discussion: MM and MGUS CAFs showed a different miRs profile, including 9 up-regulated and 17 down-regulated miRs. Among the over-expressed miRs, we focused on miRs showing a major significant p-value: miR-27b-3p and -214-3p. Target genes of miR-27b-3p and -214-3p were FBXW7 and PTEN, respectively, involved in cell apoptosis, proliferation and CAFs activation. Inhibition of miR-27b-3p induced the over-expression of FBXW7, an ubiquitin ligase, which negatively modulated the expression of MCL-1, NOTCH and Cyclin E1/2. miR-214-3p inhibition increased PTEN levels down-regulating the AKT/GSK3 pathway and Cyclin D1. Finally, co-cultures of MM cells with CAFs and bort treatment increased miRs expression. Conclusions: MGUS to MM transition and drug resistance is related to a specific miRs profile. Over-expression of miR-27b-3p and -214-3p induces cell proliferation and resistance to spontaneous and bort-induced apoptosis in MM CAFs.

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C-3

FREQUENCY AND CLINICAL RELEVANCE OF PNH CLONES IN A POPULATION OF LOW-RISK AND INTERMEDIATE-1 RISK MDS PATIENTS: A GROM-L STUDY

C. Conti[1], L. Maurillo[1], P. Niscola[2], G. Cimino[3], L. Fianchi[4], S. Bellesi[4], A. Campagna[5], R. Amodeo[5], C. Sarlo[6], S. Gumenyuk[7], S. Fenu[8], A.L. Piccioni[8], R. Latagliata[9], S. Mancini[10], C. Armentano-Conte[10], R. Sala[11], M. Irno Consalvo[1], C. Attrotto[1], D. Fraboni[1], F. Buccisano[1]

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Paroxysmal nocturnal hemoglobinuria (PNH) is a acquired non-malignant stem cell disease characterized by the expansion of a population of glycosyl phosphatidylinositol anchor protein-deficient cells, leading to chronic, uncontrolled complement activation leading to intravascular hemolysis and an inflammatory prothrombotic state. PNH is clinically heterogeneous due to the relationship with bone marrow failure syndromes like aplastic anemia and myelodysplastic syndromes (MDS). The detection of a PNH clone in the latter disease may suggest a common immunomediated physiopathology and a better response to immunosuppressive therapy. The aim of the study was to evaluate by high sensitivity multiparametric flow cytometry (MFC) the frequency of PNH clone in patients with a confirmed diagnosis of IPSS low- and Intermediate-1 MDS. From April 2014 to March 2017, 130 peripheral blood (PB) samples have been collected at diagnosis, in 11 Hematological Centers (5 University hospitals and 6 community-based hospitals) located in Rome and in the Latium region. The most commonly used markers were CD59 for red blood cells (RBC),

CD24, CD66b, Fluorescent Aerolysin (FLAER) and CD157 for granulocytes (PMN), CD14, FLAER and CD157 for monocytes. The most common gating strategy was based upon morphological parameters, Glycophorin-A and/or CD45 for detecting RBC, CD45, CD33 and CD15 for PMN, CD45, CD33 and CD64 for monocytes. Clone size was assessed by enumerating the percentage of GPI-negative PMN. The target sensitivity was 0.1% and a cluster of 30 events was required to define the clone. Median age was 69 years (range 25-94), 27 pts were RAEB1 (21%), 27 pts RA (21%), 61 pts were RCMD (46%), 4 pts were RARS (3%), 2 pts were 5q- Syndromes (2%), 9 pts were RCUD (7%). With a sensitivity of 0.1%, 8/130 clones (6%) were detected. Seven out of 8 clones were represented by PNH3 cells, with 1 case showing a PNH2 clone. Median size of the clones was 3.9 (range 0.1-36.7). No thrombotic events were recorded. No statistical correlation between the clone and LDH or PMN, Hb or PLT counts was observed. To our knowledge this is one of the largest series of MDS patients prospectively screened by high sensitivity MFC for the presence of a PNH clone. However, the frequency of PNH clones in this category of patients is low and the size of the clones is small. Possible prognostic or therapeutic implication of these observation deserves further observation and a longer follow-up.

C-4

DIABETIC BONE MARROW DIFFERENTIATION IN DENDRITIC CELL AND THEIR RESPONSE TO LPS AND GLUCOSE STIMULI

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Introduction: Diabetic foot lesion is a diabetic complication caused by ischemia, hyperglycemia, neuropathy and wounded feet. Dendritic cells (DC) are important sentinels in peripheral tissues, where they come into direct contact with invading pathogens during the infection. Most peripheral DC appear to be myeloid lineage cells and they can be differentiated in vitro using GM-CSF, TNF-α and IL-4 or CSF from separated BM CD34+ cells or PB CD14+ cells. AIM: We evaluated DC differentiation from BM and PB using cytokines and their response to exogenous stimuli in presence of glucose in healthy (C) and diabetic (D) patients. Methods: PB from C (n=2) and BM from D with foot lesions (n=3) were processed to obtain PB mononuclear cells or BM mononuclear cells (PBMC or BMMC). CD14 cells were separated from PBMC following the manufacture's instruction. To induce DC differentiation, 5*10 ^{5} CD14 positive cells and BMMC were seeded and cultured for 6 days in RPMI supplemented with 10% FBS, GM-CSF 50 ng/mL, IL-4 50ng/mL, 1% glutamine and 1% antibiotics. On day 6 cells were treated for 24h with LPS 0,1 mg/mL and/or Glucose 30 mM to see DC activation. After the treatment cells were detached, counted and analyzed by flow cytometry studying the expression of CD14, CD16, HLA- DR, Lineage marker, CD11c, CD123 and CD1c, and viability. Results: PBMC and BMMC changed their morphology in five days. Cells number increased on day 7 respect to T0 in control PB and BM cells ($p<0.05$). The treatment with LPS and/or glucose significantly reduced cell number versus untreated cells. No difference was found on viability in all the treatments in PB control and BM cells, (Viability >92%). Data showed significant decreased of CD14 and CD14 CD16 expression on BM cells and a significant increase of dendritic cells versus T0 similar to PB cells ($p<0.05$). At the same interval time, LPS and/or Glucose induced an increase of CD14 expression in BM cells but not in PB, and significant reduction of CD16, DC and mDC cells in the double treatment in BM and PB. mDC1 were significant increased only in Glucose treatment. Conclusions: BMMC differentiate in DC similarly to PB CD14+ cells. LPS and Glucose can activated DC and mDC respect to untreated in BM and PB, inducing the expression of mDC1. Under these conditions, CD14+ replicate in BM with LPS and glucose, but not in PB, with possible diabetic bone marrow exhaustion which could explain foot lesions.

C-5

MAST CELL PRECURSORS AND DIABETIC NEUROPATHY: IS IMMUNITY THE CONNECTION?

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Introduction: Diabetic neuropathy and distal foot lesions are common complications of type two diabetes, however the underlying pathophysiology remains uncertain. Mast cells (MCs) provide a possible link with immunity and neuroinflammation. These cells of innate immune system can be found in proximity with sensory nerves of peripheral tissues or the endoneurial compartment, where they differentiate and participate in innate host defense reactions. Interestingly, MCs play a role in wound healing, but their exact role in diabetes is still unknown. Aim: The aim of this study was to evaluate a possible connection between peripheral bone marrow derived blood MC precursors and diabetic neuropathy without and with skin

lesions. Methods: The study enrolled 6 healthy controls (C), 7 diabetic patients with peripheral sensimotor neuropathy (N) and 10 diabetic patients with neuropathy and foot lesions (N1). Peripheral blood was collected and analysed by flow-cytometry using CD34, CD117, lineage cocktail 1 and FcεRI antibodies (FACS CANTO II and Diva software). Results: We found a significant decrease in the percentage of MC precursors (LIN-, CD34+, CD117+, FcεRI+ cells) in the N1 group (0.00026% of mononuclear cells vs 0.001% in C, $p<0.005$). No differences were seen between C and N and between N and N1. A similar decrease in MC number/mm^3 was observed between C and N1 (0.028 and 0.005, $p<0.005$). The link with inflammatory and immune cells was assessed by simple regression: MC number directly correlated with total lymphocyte (C 2299 vs N1 1749 cells, $p<0.005$, $R^2=0.474$) T-lymphocyte (C 1565 vs N1 1218 cells, $p<0.005$, $R^2=0.396$) and CD4+ T-lymphocyte (C 1024 vs N1 805 cells, $p<0.0005$, $R^2=0.515$) numbers. There was an inverse but not significant correlation with monocytes (C 448 vs N1 552 cells, $p=0.073$, $R^2=0.197$). Conclusions: Our results show a possible involvement of MC precursors in the evolution of human diabetic foot lesions. Conceivably, MCs could have a protective role in favoring wound healing by interaction with other cells such as fibroblasts, immune, nervous and epithelial cells and by release mediators and neurotransmitters. The direct correlation with T-lymphocytes might explain the impaired wound healing in N1, suggesting a direct contact between the two populations at the lesion site. Although not significant, the inverse relation with monocytes could be indicative of dysregulation in the inflammatory compartment, up-regulated by the decrease in MC precursors.

C-6

CELL DEATH OF PERIPHERAL BLOOD MONONUCLEAR CELLS INDUCED BY IONIZIONG RADIATION IS INHIBITED BY CELL FREEZING

L. Zamai[1], M.G. Nasoni[1], G. Cugia[1], A. Lucarini[1], F. Centis[2], M. Valentini[2], E. Argazzi[3], M. Bono[3], F. Bunkheila[3], F. Luchetti[1], B. Canonico[1]

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Cryopreservation of cells, tissues and organism in liquid nitrogen (LN) offers the most secure form of conservation, nevertheless frozen biological materials are exposed to natural background of ionising radiation (IR). It is known that IR can induce cell death and tumors in living cells. The aim of this study is to evaluate the effects of IR on frozen and unfrozen peripheral blood mononuclear cells (PBMCs). PBMCs were directly irradiated at room temperature, then immediately frozen, or frozen and then irradiated in LN with different (0, 0.1, 0.3, 0.9, 3.0, 18,6 Gy) doses of IR. After thawing, cells were incubated and percentages of cell death were evaluated by flow cytometry at different time points, using both hypodiploid peak detection and supravital propidium iodide staining. Interestingly, PBMC cell death gradually increased both with dose radiation and incubation time and was relevantly higher in PBMCs irradiated at room temperature than in those frozen. In conclusion, these results suggest that cryogenic temperature protects biological material from gamma ray induced effects.

R-2

PD-1 IS A NOVEL INHIBITORY IMMUNE CHECKPOINT IN HUMAN NK CELLS

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Immune checkpoints are a series of inhibitory pathways hardwired into the immune system that play a crucial role in modulating the duration and amplitude of physiological immune responses in peripheral tissues. PD-1 is a major immunological checkpoint that limits immune responses by delivering potent T cell inhibitory signals after interaction with its ligands expressed on target cells such as APC. However it has been shown that these ligands can be frequently expressed also by tumor/virus-infected cells thus contributing to an important mechanism of tumor escape. Remarkably therapeutic PD-1 blockade was shown to accelerate tumor eradication with impressive clinical results. Little is known on the expression/function of PD-1 on human NK cells.
In the present study by multiparametric cytofluorimetric analysis we characterized a novel PD-1+ NK subset in normal donors as well as in cancer patients. In particular we show for the first time that PD-1 is expressed at high levels (PD-1bright) on an NK cell subset from approximately one fourth of normal individuals. This receptor is expressed by the $CD56^{dim}$ NK subset but not by $CD56^{bright}$ cells and is confined to a cell subset displaying the phenotypic features of fully mature NK cells being homogeneously $KIR^{+}NKG2A^{-}CD57^{+}$. Remarkably the frequency of this PD-1bright NK subset was increased in the ascites of a cohort of ovarian-carcinoma patients suggesting a possible enrichment of $PD\text{-}1^{+}$ NK cells in tumor-associated environments. Functional analysis indicated that $PD\text{-}1^{+}$ NK cells displayed reduced proliferative capability in response to cytokines as well as lower degranulation and cytokine production in response to tumor targets.

R-3

VALUTAZIONE DELLA RISPOSTA IMMUNITARIA NEL SUINO IN CORSO DI INFEZIONE DA VIRUS PESTE SUINA CLASSICA MEDIANTE "FLUORESCENCE ACTIVATED CELL SCANNING" (FACS)

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La Peste Suina Classica (PSC) rappresenta ancora oggi una patologia caratterizzata dall'avere un ruolo cruciale e condizionante rispetto alla zootecnia di moltissimi paesi del mondo. Il progetto di ricerca finanziato dal Ministero della Salute (IZSUM 02/2010 RC) grazie al quale è stato possibile realizzare questo primo studio di citofluorimetria aveva l'obiettivo di valutare i meccanismi che sono alla base della regolazione e della modulazione della risposta immunitaria del suino in corso di infezione da PSC. Tale indagine è stata svolta impiegando tecniche FACS ed immunoenzimatiche. Lo studio ci ha consentito la messa a punto e la standardizzazione di protocolli operativi da utilizzare in ricerche e indagini successive. Per 5 settimane 12 suini sono stati coinvolti nella sperimentazione suddivisi in due gruppi: 6 vaccinati (V) e 6 non vaccinati (NV) e successivamente sottoposti ad infezione con virus PSC "strain" ISS60. La percentuale di cellule T della memoria "attivate" risultava essere aumentata nel sangue dopo la vaccinazione e mostrava un incremento consistente dopo l'infezione. Tutti i suini NV mostravano invece una grave leucopenia dopo la prima settimana d'infezione. E' stato inoltre possibile evidenziare un aumento significativo nella percentuale di cellule CD4+ nel gruppo dei suini V ed una contemporanea importante diminuzione nel gruppo dei NV. Una riduzione significativa della "mean" dei CD4 è stata rilevata nel gruppo NV. Per quanto riguarda la popolazione $CD21^{+}$ si è riscontrato, dopo l'infezione, un aumento percentuale negli animali V e una diminuzione nei suini NV con indicazioni tali da riportare il ruolo di questa popolazione linfocitaria soprattutto nelle ultime fasi della patologia. Parallelamente alle indagini tramite FACS è stata svolta un'analisi qualitativa e quantitativa su un panel di citochine (IL-1beta, IL-6, IL-10, IL-12/IL-23p40, TNF-alfa e IFN-gamma). L'IL-1 beta aumentava progressivamente, dopo l'infezione, solo nel gruppo dei suini NV. Nel gruppo NV l'infezione induceva l'aumento di 7-8 volte dei valori nel siero di IL-6 rispetto agli animali V; tali valori raggiungevano un picco una settimana dopo l'infezione e poi diminuivano fino alla morte degli animali. L'IL-12/IL-23p40 presentava, nel gruppo NV, un aumento a seguito dell'infezione e tornava ai livelli basali nel periodo che precedeva la morte degli animali. Il TNF-alfa aumentava invece, in entrambi i gruppi di animali, dopo l'infezione con valori più elevati nel gruppo V. Tali dati, incrociati ed elaborati con quelli ottenuti con il FACS, hanno consentito di delineare un primo quadro sulla risposta immunitaria del suino in corso di vaccinazione e successiva infezione da virus PSC. Numerose ed evidenti sono state infatti le correlazioni riscontrate tra l'andamento delle popolazioni linfocitarie e la produzione delle citochine.

R-4

CITOMETRIA E SPERMATOZOI: UN NUOVO APPROCCIO ALLA VALUTAZIONE DELLA FUNZIONALITÀ SPERMATICA IN CAMPO ANIMALE

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Il citofluorimetro è applicato nella diagnostica andrologica grazie alla disponibilità di nuovi probe fluorescenti. Infatti, l'uso contemporaneo di diversi fluorocromi permette una valutazione simultanea di diversi attributi su un numero elevato di spermatozoi, fornendo un alto livello di ripetibilità e oggettività del risultato su un campione caratterizzato da un'elevata eterogenicità . Esiste, comunque, una differenza nell'uso della citofluorimetria in andrologia umana ed animale: infatti sebbene le metodiche citometriche possano essere applicate al seme umano, non sono molto usate a causa del successo avuto dalla fertilizzazione in vitro e iniezione intracitoplasmatica degli spermatozoi nella riproduzione assistita. Mentre, nell'andrologia animale, le tecniche di riproduzione assistita, ad eccezione dell'inseminazione artificiale (AI), non possono diffusamente essere usate per l'elevato costo e per la necessità di eliminare riproduttori con ridotta fertilità, per la possibile trasmissione alla progenie maschile di caratteri legati all'ipofertilità. Inoltre il seme di riproduttori con subfertilità è più sensibile alle condizioni di crioconservazione incidendo ulteriormente sulla qualità spermatica post congelamento. La valutazione della vitalità, funzionalità mitocondriale, stabilità cromatinica, reazione acrosomiale, capacitazione e valutazione dei ROS permettono di ampliare il pannello dei parametri funzionali dei soggetti riproduttori superando i limiti di un esame microscopico soggettivo e parziale, basato prevalentemente sulla morfologia e su alcuni parametri di motilità (1), permettendo di ottenere campioni crioconservati con una buona capacità fecondante. Ad esempio il test della stabilità cromatinica (SCSA) è risultato molto sensibile per evidenziare variazioni stagionali della condensazione cromatinica e per valutare l'effetto della refrigerazione e crioconservazione (2). Sono state inoltre definite alcune "soglie" dei parametri SCSA capaci di identificare bovini e suini subfertili. In un recente studio uso combinato di tecnologie computerizzate per la valutazione della qualità del seme (citometria e sistema Computerizzato della cinetica CASA) ha permesso di costruire dei modelli predittivi sulla fertilità di spermatozoi crioconservati bovini, selezionando i parametri più

correlati ai dati di fertilità (3). Per ultimo, la citofluorimetria è stata anche utilizzata negli studi di tossicologia riproduttiva e ha permesso di esplorare, su spermatozoi animali, l'effetto tossico determinato dall'esposizione *in vitro* di contaminanti alimentari (4,5), riducendo l'uso di animali, condizione fortemente voluta dalla Comunità Europea.

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C-7

FLOW CYTOMETRIC ASSESSMENT OF LEPR EXPRESSION IN BUFFALO PERIPHERAL BLOOD LEUKOCYTES

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Leptin performs a variety of biological actions on feeding, metabolism and neuroendocrine axis and mediates its effects by binding with the leptin receptor (LEPR), a transmembrane protein member of class I cytokine receptor family. In human and mouse, several isoforms of leptin receptor are expressed in a variety of peripheral tissues including circulating leukocytes, suggesting for leptin a role in the regulation of immune response. The aim of the study was to evaluate, by flow cytometry, the LEPR expression on buffalo leukocytes. Two multicolor panels of antibodies were designed and optimized for the identification of subsets of buffalo leukocytes expressing LEPR. The first panel was designed for the identification of monocytes and B lymphocyte and included the anti-LEPR polyclonal antibody, the anti-human CD14 (clone-TÜK4) and the anti-bovine CD21 (Clone-CC21). The second panel was designed for the identification of T lymphocytes and Natural Killer cells, and included the anti-LEPR polyclonal antibody, the anti-bovine CD4 (clone ILA11A), the anti-bovine CD8 (clone CC63), and anti-bovine CD335 (clone AKS1). LEPR expression on polymorphonuclear cells was made evident based on the light scatter characteristics (FSC vs SSC). The staining of cells from ten buffaloes was carried out in 96-well plates with 5x105 cells/well/50 μL of PBS and 1% BSA. Analysis was performed on Cytomics FC500 Flow Cytometry Analyzer (Beckman Coulter Inc.). The proportion of cell populations expressing LEPR was determined by comparison with the isotype control of the corresponding cell population and the staining of LEPR antibody. The data were analysed for both percentage of cells expressing LEPR as well as for molecule density (MFI) obtained by the Kaluza Analysis 1.3 Software (Beckman Coulter, Inc). In our results monocytes and B lymphocytes showed the same percentage of cells expressing LEPR, 66.9% ± 12.2 and 66.8 % ± 7.2 respectively. The 27.0 % ± 7.9 of Natural Killer cells expressed LEPR, while lower values were expressed by T cells (1.1 ± 0.69), neutrophils (4.6 %± 3.8) and eosinophils (2.2 %± 1.9). The observed higher expression of LEPR in monocytes and in B lymphocytes is in agreement with LEPR expression in human peripheral blood leukocytes. To confirm the differential LEPR expression in leucocytes, an antibody-free FACS-sorting on MoFlo XDP cell sorter instrument (Beckman Coulter, Inc) was performed to amplify the LEPR cDNA from total RNA extracted from each specific population. To further confirm LEPR expression on monocytes, immunofluorescence microscopy on freshly purified PBMC, was performed to assess the localization of LEPR staining on surface of CD14+ cells. In conclusion, results of this study encourages the realization of further research which may be help to elucidating the relationships between leptin and immune functions in bovine and buffaloes species. Acknowledgements: This research was funded by the Italian Ministry of Agriculture in the frame of GENZOOT-project.

C-8

PHYLOGENETIC ANALYSIS OF THE CD EPITOPES RECOGNIZED BY MONOCLONAL ANTIBODIES ON BOVINE, SWINE AND OVINE LEUKOCYTES CELLS

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Cell surface markers, defined in the CD Workshop by monoclonal antibodies (mAbs), have changed the diagnostic and clinical potential of medicine and therapy. However, outside of human models, the panel of surface molecules that can be used as reference is very limited. The purpose of our study is to see whether the epitopes recognized in human by mAbs differ from those characterizing bovine, swine and ovine cells obtained from peripheral blood. On one side, the potential of the present study is immediately utilitarian, allowing a rapid

translation to veterinary medicine. Secondly, the same results may be useful in phylogeny to monitor the determinant domains as seen by the murine immune system. Depending of the animals tested, an existing panel of mAbs with different specificities (approximately 60 mAbs directed against HLA Class I, HLA Class II, $\beta_2$microglobulin, CD1a, CD2, CD3, CD5, CD8, CD10, CD11a, CD11b, CD14, CD16, CD18, CD19, CD25, CD26, CD27, CD28, CD29, CD31, CD34, CD36, CD38, CD40, CD42a, CD43, CD45, CD48, CD49e, CD56, CD57, CD71, CD73, CD81, CD100, CD157, CD229, PC-1, Transferrin receptor 2, Estrogen receptor, HER-2, Tenascin, IFNβ, PSMA, α-fetoprotein, Osteopontin, Oxitocin receptor, hCG receptor, CEA, hLDR, Purinergic receptor, PSA, TNFα, IFNα and IL-18 binding protein) displayed reactivities characterized by different intensities. More specifically, an expression profile similar to that found in humans was found for $\beta_2$microglobulin, CD18 and CD43 in bovine PBMC, similar results for $\beta_2$microglobulin, CD5, CD14 (with different antibodies), CD19, CD25, CD29, CD31 and CD43 were obtained for swine and HLA Class I, CD14 and CD71 were detected in sheep PBMC. One mAb specific for the human HLA Class II molecule showed a transversal reactivity for bovine, swine and ovine cells. This last result suggests that the epitope recognized by this antibody maintain a strong phylogenetic stability, as expected when an immunoglobulin domain is the molecular target. Other reactivities are variably conserved among the different species analyzed. These results support a possible application of these mAbs to categorize blood cells of veterinary interest.

C-9

CHARACTERIZATION OF VASCULAR WALL PROGENITORS CELLS IN PIG POSTNATAL AORTA

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The dynamic nature of blood vessels has been nowadays demonstrated. Increasing evidences indicate the presence of multipotent vascular progenitor cells inside the mural layers of embryonic and postnatal blood vessels. Considering that pig is an excellent model for the translational medicine, we have recently isolated a MSCs population with pericyte-like properties from porcine aorta. The aim of the present research was to characterize this population for the typical MSCs markers following guidelines of International Society for Cell Therapy (ISCT). pAVPCs have been isolated from 3-month-old pigs, euthanized for other experimental purposes. Cells were isolated and cultured to passage sixth as previously described. Phenotypical characterization was carried out through flow cytometry analysis for the expression of CD105, CD90, CD56, CD44, CD45, CD34. The mesenchymal trilineage differentiation potential was assessed through adipogenic, osteogenic and chondrogenic differentiation followed by Alizarin Red, Alcian Blue and OilRedO stain and qPCR analysis of specific differentiation markers. Flow cytometry analysis revealed the presence of a homogeneous population. Furthermore immunophenotypization showed that cells were positivity to CD105 (85,97 ± 0.5 %), CD90 (99.5 ± 0.2 %), CD44 (99.6 ± 0.3 %) CD56 (99.9 ± 0.1 %) and less than 2% to CD45 (1.4 ± 0.4 %) and CD34 (1.3 ± 0.1 %). This immunophenotype was well maintained through the different passages. Istological stain and PCR analysis confirmed that pAVPCs were able to differentiate towards adipo-, osteo- and chondrocyte phenotypes. In conclusion, data obtained showed that pAVPCs present mesenchymal immunophenotype and property, confirming the presence of a vascular wall progenitor in the post-natal aorta of the swine species.

R-5

L'IMMUNOFENOTIPO NELLA MALATTIA MINIMA RESIDUA DEL MIELOMA MULTIPLO

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Un crescente numero di ricerche indica che la malattia minima residua (MRD) negativa di pazienti affetti da mieloma multiplo (MM) si traduce in un miglioramento della sopravvivenza libera da progressione e generale. Il fattore fondamentale associato a tale miglioramento è il raggiungimento di una remissione completa (CR) al termine della terapia, documentata come assenza di componente monoclonale all'immunofissazione sierica e urinaria (sCR) e assenza di plasmacellule patologiche midollari.

La probabilità di ottenere una CR nel paziente affetto da MM è estremamente variabile e dipende, non solo dall' età del paziente, ma anche dalla possibilità di utilizzare nuovi farmaci con meccanismi di azione differenti che agiscono sinergicamente sulle plasmacellule patologiche. Nella maggior parte dei pazienti in sCR è, comunque, dimostrabile una MRD midollare identificabile con tecniche molecolari e/o citometriche avanzate (1).

La citometria a flusso consente di distinguere con massima precisione ed elevata sensibilità le plasmacellule normali da quelle patologiche e quindi stabilire con ottima probabilità la presenza di eventuale MRD. Tale distinzione risulta dall'applicazione di linee guida che descrivono il corretto utilizzo di combinazioni di numerosi marcatori immunologici, l'applicazione di "gates" logici, analisi multiparametriche, (effettuate anche mediante nuovi software di analisi, per esempio infinicyt) e l'acquisizione di un elevato numero di eventi. I marcatori consigliati per la discriminazione delle plasmacellule normali da quelle patologiche, entrambe

accomunate dall' espressione del CD38 o CD138 sono il CD19, il CD56; risultano raccomandatati CD117, CD28, CD27, CD20 e CD81 (2).
La citometria a flusso offre numerosi vantaggi clinici, tra cui il fatto che i risultati finali possono essere generati in laboratori locali, entro poche ore dalla raccolta del campione risultando di ampia applicabilità e producendo risposte rapide. La possibilità di poter determinare con una più elevata sensibilità la CR mediante tecniche di citometria a flusso permetterà di poter monitorare l'efficacia delle strategie di risposta per i pazienti affetti da MM potendo decidere, per esempio, che i pazienti che rimangono MMR positivi durante il trattamento iniziale potrebbero richiedere l'intensificazione della dose o un cambiamento di terapia di induzione e fornire, inoltre, una guida per l'identificazione dei pazienti che potrebbero beneficiare di cessazione della terapia di mantenimento.

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C-10
THE USE OF MULTIPARAMETER FLOW CYTOMETRY FOR DETECTING MINIMAL RESIDUAL DISEASE IN PATIENTS WITH ACUTE MYELOID LEUKEMIA LEADS TO THE IDENTIFICATION OF PATIENTS WITH HIGHER RISK OF RELAPSE

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Background: Multiparameter flow cytometry (MFC) is an effective method for assessing minimal residual disease (MRD) in several malignancies, including Acute Myeloid Leukemia (AML), thus providing independent prognostic information in addition to molecular-based approaches. AIM To define a potential MRD/MFC-based signature to differentiate AML patients among the intermediate(Int)-I and Int-II ELN risk groups. Methods: MFC was performed on bone marrow (BM) aspirates of 148 pts. At disease onset, a panel of 22 monoclonal antibodies combined in four 8-color tubes was employed to detect a leukemia associated immunophenotype (LAIP). Patients were treated according to NILG AML study, irrespective of the MRD results. The following time points were identified: TP1=post 1 or 2 courses of induction therapy; TP2=post 1st consolidation course; TP3=end of treatment program. MRD was studied with 6-color MFC using the cut-off values of 0.1% and 0.035% at TP1, and 0.035% at TP2 and TP3. The pts median age was 56yr (18-75yr), the F/M 60/88; de-novo AML were 86.4% and secondary AML 13.6%. Relapse-free survival (RFS) was analyzed using Kaplan-Meier curves and the log-rank test. Results: A LAIP was detected in 121/148 pts (81.8%). ELN 2010 risk groups were distributed as follow: 44 favorable (36.4%), 40 int-I (33.1%), 17 int-II (14.0%), and 20 adverse (16.5%). Ten pts were excluded because they did not reach complete response and 4 pts due to sample inadequacy. Pts were analyzed at the different TPs as follows: TP1 (n: 107), TP2 (n: 102), TP3 (n: 66). MRD-positivity was detected in 63.6% and 85.1% of the patients at TP1 (threshold of 0.1% and 0.035%, respectively); being 71.6% and 75.8%, at TP2 and TP3, respectively. At TP1, MRD positivity by MFC predicted relapse with the 0.1% threshold (p=0.022). The median RFS was 15.7mo for MRD-pos and not reached for MRD-neg pts. Moreover, MFC-based MRD predicted relapse in ELN Int-I and Int-II pts (51/107), at TP1, using either the 0.1% (p=0.002) or the 0.035% threshold (p=0.038). The median RFS was 11.9mo (0.1% threshold) and 14.4mo (0.035% threshold) in MRD-pos vs not reached in MRD-neg pts with either threshold. Conclusion: As for today, there is a lack of prospective data guiding towards the best MRD-based therapeutic approach in AML: we report on the evidence for stratifying ELN-defined intermediate risk patients by using MFC-based MRD, thus leading to the most appropriate, tailored post-remission therapeutic strategy.

C-11
FLOW CYTOMETRY EVALUATION OF MRD STATUS IN LONG TERM RESPONSE MULTIPLE MYELOMA PATIENTS CAN IDENTIFY MM PATIENTS DISEASE FREE/CURED

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In the era of novel agents, many Multiple Myeloma (MM) patients can achieve stringent complete remission (sCR), however most of these patients still will relapse, therefore minimal residual disease (MRD) status play a crucial role in the management of these patients. We here evaluate the value of flow cytometry in measuring the MRD status of MM patients. Between February 2016 and February 2017 we studied 31 MM patients (M/F= 17/14) at a median of 36 months after therapy (range 2-186). The MDR status was assessed using OneFlow™ PCST and PCD (BD, Biosciences), in all cases >2x10^6 cells were analyzed by Diva 8 software (BD, Bioscience). At study time 15/31 (48%) patients were in very good partial response (VGPR) and 16/31 (52%) in sCR. Our results show that MRD+ status was detected overall in 10/31 (32%) patients of which 2/10 (20%) were in sCR with the median duration of 45 months (range 6-84) while

8/10 (80%) were in VGPR with a median duration of 10 months (range 3-186). Plasma cells were detected at median of 0,35% (range 0,1-5,4) of bone marrow cells? with median abnormal plasma cells (APC) of 46% (range 16-93). No differences in terms of percentage of MRD+ cases were recorded between patients with response lasting >2 years, 4 MRD+/18 (22%) or > 5 years 2 MRD+/9 (22%). Albeit still preliminary, the results of this study show that MM patients in long term response display a high proportion MDR- cases or display an MGUS–like phenotype. Therefore, it is warranted to study flow cytometry MRD status in larger series of patients to better distinguish who need continued therapy from those that may stop treatment, with consequent important social and economic benefit.

C-12

FLOW-CYTOMETRIC ASSESMENT OF LYMPH NODE SUSPENSIONS WITH CLINCAL SUSPICION OF NON-HODGKIN LYMPHOMA. A SINGLE CENTER EXPERIENCE

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Background. Few studies have addressed the utility of flow cytometry on tissue samples of lymph nodes. A high concordance between results of flow cytometry and immunohistochemistry has been reported in a single large series of patients with non-Hodgkin lymphoma (NHL). AIM. We report our experience with a flow-cytometric diagnostic approach to lymph node tissue in patients with a clinical suspicion of lymphoproliferative disorders. Methods. Lymph node suspensions were prepared by mechanical disaggregation of the of solid tissue using the Medimachine system (Becton Dickinson). The screening panel included CD19, CD20, CD10, Kappa, Lambda, CD5, CD3, CD4 and CD8. Results of immunophenotyping were compared with immunohistochemical diagnoses. Results. We assessed 33 lymph node suspensions by flow cytometry. Immunohisotchemical diagnoses were diffuse large B-cell lymphoma (DLBCL) in 10 patients, follicular lymphoma (FL) in 4 patients, marginal zone lymphoma (MZL) in 1 patient, Hodgkin lymphoma (HL) in 8 patients, T-cell lymphoblastic lymphoma (T-ALL) in 1 patient, mycosis fungoides (MF) in 1 patient, metastatic carcinoma in 4 patients, reactive lymph node changes of various types in 4 patients. All B-subtype non-Hodgkin lymphomas (B-NHL) were correctly identified (100% sensitivity) by flow-cytometric analysis. B-cell pathological populations were CD20 positive and presented a clear clonal light chain restriction in 9 cases out of 15 (6 DLBCL and 3 FL), while 6 cases (4 DLBCL, 1 FL and 1 MZL) did not show any light chain expression. All DLBCL cases displayed an aberrant population with high forward and side scatters, while the 4 FL and the MZL cases showed low scatter parameters. CD10 was expressed in all FL cases and in 2 out of 10 DLBCL (20%). CD5 was present in 1 case of DLBCL (10%). In non B-NHL samples, we observed a physiological B-cell population with a normal kappa/lambda ratio. We detected a pathological T-cell population with co-expression of CD4 and CD8 and CD3 dim expression in the sample of T-ALL patient. Moreover, lymph node suspension of MF presented the typical phenotype CD4+/CD26-/CD7±. Conclusions: In our limited series of patients, flow-cytometric assessment of lymph node suspensions had a high sensitivity and specificity for B-NHL. It could be useful to provide the clinician and pathologist with rapid information to guide further diagnostic work-up and direct therapy in symptomatic patients with bulky disease.

R-6

LA CITOFLUORIMETRIA NELLA DIAGNOSI DEI LINFOMI DEL CANE

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L'analisi citofluorimetrica in caso di sospetto linfoma nel cane si sta affermando come test di routine complementare all'esame citologico. Essa viene infatti eseguita su materiale prelevato dal linfonodo megalico tramite aspirazione con ago di siringa. È quindi un prelievo minimamente invasivo che può essere eseguito contestualmente alla preparazione dello striscio per la valutazione citologica. I pannelli di base per la tipizzazione citofluorimetrica comprendono CD45 (panleucocitario), sCD3, cyCD3, CD5, CD4, CD8 (linfociti T), CD21, cyCD79a/b (linfociti B) e CD34 (precursori). A differenza di quanto accade nell'uomo, il rapporto κ/λ non è indicativo a fini diagnostici nel cane. La diagnosi di neoplasia è affidata alla valutazione delle caratteristiche di scattering delle cellule (indicativa in caso di consistente aumento di elementi di grandi dimensioni), alla presenza di una popolazione con immunofenotipo univoco (indicativo di proliferazione clonale) e/o alla presenza di popolazioni con immunofenotipo aberrante rispetto alla controparte non neoplastica. L'esposizione di CD34 è spesso il segno di una forma leucemica infiltrante il linfonodo, anche se è possibile il raro riscontro di linfomi CD34+. La definizione dell'immunofenotipo, oltre a contribuire alla corretta classificazione secondo gli schemi di riferimento (Kiel aggiornato in citologia; WHO in istopatologia), permette di riconoscere gli elementi neoplastici in altri tessuti (in particolare sangue e midollo) consentendo la stadiazione della patologia.

Il solo quadro immunofenotipico consente quindi una diagnosi di linfoma B o T a piccole, medie o grandi cellule e la definizione di stadio V. Dati preliminari

ottenuti nel nostro laboratorio indicano un possibile ruolo dei valori di Ki67 e di attività apoptotica nell'identificazione di alcuni sottotipi di linfoma secondo le classificazioni di riferimento. Attualmente tuttavia, l'identificazione attendibile di tipo istologico è possibile solo per il linfoma T-zone, una forma indolente peculiare del cane: tale linfoma T è infatti caratterizzato da elementi di piccole dimensioni che appaiono negativi per CD45 ed espongono spesso CD21 a bassa intensità (1). Linea cellulare (B e T) e stadiazione hanno impatto prognostico riconosciuto. In aggiunta a questi, recenti lavori hanno affidato un ruolo alla determinazione citofluorimetrica di Ki67 (distinzione di forme alto e basso grado e valutazione prognostica nei linfomi B a grandi cellule) (2-3) e della MFI di MHC-II nei linfomi B (4).
Merita infine una menzione l'efficace impiego dell'analisi citofluorimetrica per la diagnosi differenziale tra timoma e linfoma in caso di massa mediastinica basata sulla valutazione della prevalente presenza di una popolazione CD4+ o CD8+ rispetto agli elementi CD4+CD8+ (5).

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C-14

STUDY OF CELL-MEDIATED IMMUNE RESPONSE IN MICE STIMULATED WITH CLOSTRIDIUM PERFRINGENS BETA2 ATYPICAL RECOMBINANT TOXIN

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Beta2 (β2) toxin is a plasmidic secondary enterotoxin, produced by Clostridium perfringens and encoded by cpb2 gene that works in synergy with major toxins. Purpose of the study is to check whether the recombinant protein produced is able to stimulate a cell mediated immune response and to evaluate the intensity. 48 male BALB/c mice were involved and subdivided in group: A stimulated with β2 protein; B with β2 protein and adjuvant; C and D are the respectively control groups. Samples were harvested at three different times (3, 10 and 30 days), and marked with fluorophores of lymphocyte lines and macrophages. Data were detected by BD FACSCalibur. The immunophenotypic analysis showed an increase in the percentage of T and B lymphocyte populations, NK cells and macrophages 3 days after inoculation with a peak at tenth. At T3 (30 days after inoculation) the observed values are comparable to those of their respective controls.. We compared the groups A and B with the respective controls to assess the immunogenic properties of the toxin and the group A with B to check whether the presence of adjuvant is able to influence the immune response. The processing of the data showed statistically significant difference in CD3+CD4+, CD3+CD8+ and F4/80+ 3 days after stimulation (T1) in comparison between A and D. At the same time, we observed a difference with regard to F4/80+ and CD45+ populations between B and C. Comparing A with B, we observed an increase in the percentage of CD3+CD4+ T cells of group A. Ten days after stimulation (T2) there was no significant difference between A and D in the CD45+ population while among the mice in groups B and C, we observed an increase in the percentage of CD45+ and CD86+ cells. In conclusion β2 toxin shows good immunogenicity because it can stimulate the production of a good percentage of cells belonging to the immune system. Data showed that the adjuvant does not significantly affect in the intensity of the immune response; for this reason, in future tests, we will not take this variable into account. The next step of the research involves the evaluation of antibody production in mice and subsequently both studies will be repeated on species of choice animals to determine the actual effect of the vaccine in the field. This study was supported by a research grant from the Italian Ministry of Health (IZSUM 07/13 RC).

C-15

POSITIVE SELECTION OF MACROPHAGES FROM OVINE MILK: ROLE OF FLOW CYTOMETRY

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Milk quality and udder health are strictly connected milk somatic cells that contain lysosomes that release active proteolytic enzymes in milk, so contributing to milk protein breakdown. Milk macrophages can convert plasminogen to plasmin, which, together with lysosomial enzymes, is the main native proteolytic enzyme in milk. However, there is a lack of available immunological kits for ovine cells separation. The present study describes an immunomagnetic procedure for positive selection of milk macrophages. This technique could be easily applied to the study of the role of macrophages in relation to i) defence mechanisms of the mammary gland and ii) the release of proteolytic enzymes affecting milk quality. A simple immunomagnetic procedure to select macrophages from ovine milk was developed, using a non specific magnetic positive separation technique. Samples of ewe bulk milk were collected during early, mid- and late lactation; milk samples were centrifuged at

2000 x g for 30 min at 4 °C; the fatty fraction and supernatant were removed, and each pellet was dissolved in 500 μL of pH 7.4 Phosphate Buffer Saline (PBS) + 0.02% NaN_3. Cells were targeted for selection using mouse-IgG anti-ovine macrophages. Several trials were performed to evaluate purity of samples by flow cytometry, testing two different fluorochrome-conjugated antibodies, i.e. mouse anti-human CD14: RPE (MCA1568PE, Serotec) and F(ab')2 rabbit anti-mouse IgG: RPE (STAR12A, Serotec), and three different labelling procedures. A morphological test was carried out by direct microscopic count in enriched fraction smears stained with May-Grünwald-Giemsa to confirm the presence of macrophages. The method described in the present technical note, can be considered an innovative application to obtain, rapidly and easily, a single cell population of high purity selected from the whole somatic cells of milk, and to prevent the risk of contamination from other non investigating cells. Thus, the present technical note can be useful to the monitoring of changes occurring in the somatic cells of mammary gland throughout lactation. Moreover, the positive selection of macrophages allowed us to achieve new knowledge about the behavior of ovine milk macrophages in plasmin-plasminogen complex.

C-16

A MULTI-STEP APPROACH TO THE FLOW CYTOMETRIC BRONCHOALVEOLAR LAVAGE ANALYSIS

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Introduction: The current practice of limiting the analysis of immune system cells in bronchoalveolar lavage (BAL) to T CD4+ and CD8+ lymphocytes by flow cytometry may limit the range of clinically valuable information retrievable. For instance, extending the analysis to B Cells, granulocytes, eosinophils and macrophages can provide evidence of occult disorders or activation mechanisms that may affect the underlying disease outcome. However, an idea on the cell composition in each BAL aspirate may consolidate only after the first analytical step, i.e. when the combined evaluation of absolute cellular count, CD45 expression and physical parameters become evident. Methods: BAL aspirates referred to our laboratory for routine analytical assessments are processed stepwise following this internal guideline: 1) If Lymphocytes are >10% and CD3+ T cells are <90%, then B cells are also analyzed by CD19, CD20, CD5 and sKappa/sLambda expression. 2) If Polymorphonuclears are >5% the evaluation of CD9- CD13++ CD15++ Granulocytes and CD9+ CD13+ CD15+ Eosinophils is also performed. 3) If Granulocytes prevail, their activation status is further studied by quantitative CD64 expression. 4) In selected cases of marked lymphocytosis in patients with autoimmune diseases the evaluation of TH1- and TH2-like T cells is performed using CXCR3 and CCR6. 5) In case of large mediastinal masses the differential diagnosis between sarcoidosis and Hodgkin's lymphoma (HL) may benefit from the detection of an increased CD7++ expression on CD4+T cells in HL. Results: This approach proved flexible and informative, since it can be quickly adapted according to the patient's clinical history and the first step cytometric findings, avoiding bulky, costly and complex analyses on unselected samples. Lymphoproliferative disorders with lung or mediastinal involvement may be also detected, provided certain prerequisites. Conclusion: A multi-step approach to the multicolor flow cytometric analysis of BAL is proposed here, with specific indications and a decision tree, which can be tailored on a patient-to-patient basis, for a cost-effective data collection and the generation of biological data that can be useful in differential analysis processes.

C-17

MURINE THYMIC NK CELLS ARE DISTINCT FROM ILC1s AND HAVE UNIQUE TRANSCRIPTION FACTOR REQUIREMENTS

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The recent discovery of a number of other lymphoid subsets involved in the innate immune response, has questioned part of the notions already acquired. Group 1 innate lymphoid cells include natural killer (NK) cells and ILC1s, a group of cells that mediate the response to intracellular pathogens. ILC1 and conventional (c)NK cells share many characteristics but they have different developmental origins [Robinette et al., 2015], with ILC1 arising from a common progenitor of all ILC and NK cells arising from a distinct progenitor that has different transcriptional requirement. The adult thymus has been reported to have a unique subset of NK cells that share many characteristics attributed to ILC1 like the dependence on the transcription factor GATA3, the expression of CD127 and the absence of CD11b and Ly49 receptors, whose expression characterizes mature cNK cells [Vossenrich et al., 2006]. Thymic NK (tNK) cells have been described with hybrid features of immature NK cells and ILC1 but whether these cells are related to NK cells or ILC1 has not been fully investigated. The aim of this work was to characterize the thymic Group 1 ILCs, focusing on their transcriptional requirements and phenotypic characteristics, to better define tNK cells developmental relationships with other subsets of Group 1 ILCs. We used thymus of mice lacking some genes known to be involved in the

development and differentiation of NK cells and ILC1, and we have analysed thymocytes through flow cytometric analysis. We found that murine thymic Group 1 ILCs are a heterogeneous population composed primarily of "naive" cNK cells that require the transcription factor NFIL3 and expressed EOMES, the NK cell-associated transcription factor; they also developed independent of the essential ILC1 transcription factor TBET, confirming their placement within the NK lineage. Moreover, tNK cells resemble NK cells rather than ILC1 in their requirements for the E protein transcription factor inhibitor ID2, and they are largely EST1-independent, even though this transcription factor prevented tNK cell from the acquisition of the conventional NK cell maturation markers CD11b and KLRG1. Our data demonstrate that tNK cells have developmental requirements consistent with the NK cell lineage and are not ILC1; however, they have a requirement for the transcription factor ETS1 that only partially overlaps with cNK cells.

C-18

HYPERGLYCEMIA AND CD31 EXPRESSION ON LYMPHOCYTES NEGATIVELY IMPACT DIABETIC WOUND HEALING

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Introduction: Type 2 diabetes mellitus is a chronic disease that often causes ulcers or wounds in legs and foot with a poor prognosis. An intact and finely regulated immune system is necessary to guarantee a complete wound healing, and many signals are involved in controlling the correct behaviour of cells. One of this molecules is CD31 (PECAM-1), an inhibitory motif expressed on the surface of many blood cells, that could be implicated in the regulation of immune response, but with unclear functions in diabetes. Aim: This preliminary study aimed to evaluate the expression and the possible role of CD31 on in vivo and in vitro peripheral blood (PB) T helper lymphocytes in neuropathic diabetic patients with skin lesions. Methods: PB from 6 healthy controls (C) and 22 diabetic neuropathic patients with foot lesions (N) was collected and analyzed with flow cytometry with anti-CD31 antibody. PB mononuclear cells were separated and $2*10^6$ cells (5C and 5N) were cultured for 24 hours in RPMI 1640 with 10%FBS, 1% Glutammine, 1% antibiotic and/or 30 mM glucose (GLU) or 1 ug/mL LPS. Cells were then analyzed at time 0 and 24 h for the expression of CD31, CD3, CD4, CD8 (BD FACS Canto II; Diva Software). Results: Total lymphocytes and CD31 lymphocytes percentages significantly decreased in C versus N (37,8% vs 23,8% $p<0,001$) (32,1% vs 23,9% $p<0,05$). The two parameters showed a direct correlation ($p<0,05$, $R^2=0,223$). We found an interesting decrease in CD4+ T cell number(C 1073 vs N 769 cells/mm 3, p=0,077) and a tendence to correlation with CD31 lymphocyte number(p=0,098, $R^2=0,106$). In vitro data showed a reduced number of total cells at T0 in N and after 24h treatment with GLU and GLU+LPS in C. Only in C, but not in N, CD4+ cell number decreased significantly ($p<0,05$ vs T0) with GLU and GLU+LPS. CD31 on CD4+ increased at 24h after LPS and GLU+LPS treatment in C but not in N. Conclusions: Our results show a correlation between CD31 and lymphocytes, suggesting a possible role of the molecule in regulating immunity. CD4+ T cells seems to be more influenced by GLU, that in vitro reduces the number of cells in C with and without LPS mimicking the diabetic in vivo data. In N, the population seems to be unresponsive, maybe because already damaged by hyperglycemia. High blood glucose concentration could alter CD31 expression of thymic-derived CD4+ T cells, reducing numbers and functionality in diabetes and resulting in difficult wound healing.

R-7

GROUP 3 INNATE LYMPHOID CELLS IN ANTI-CANCER IMMUNE RESPONSE

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The recent appreciation of novel subsets of innate lymphoid cells (ILCs) as important regulators of tissue homeostasis, inflammation and repair, raise questions regarding the presence and role of these cells in cancer tissues. In addition to natural killer and fetal lymphoid tissue inducer (LTi) cells, the ILC family comprises non-cytolytic, cytokine-producing cells that are classified into ILC1, ILC2 and ILC3 based on phenotypic and functional characteristics.

Differently from natural killer cells, which are the prototypical members of ILC1 and whose role in tumors is better established, the involvement of other ILC subsets in cancer progression or resistance is still fuzzy

and in several instances controversial.
We recently analyzed ILC subsets in the context of non-small-lung cancer (NSCLC) tissues, showing that NKp44+ ILC3 cells are present in the lymphocytic infiltrate of human NSCLC and mainly locate at the edge of tertiary lymphoid structures (TLS).
TLS are a common finding in NSCLC and are predictors of favorable clinical outcome. This tumor-associated NKp44+ILC3 subset is endowed with lymphoid tissue-inducing properties and, upon activation, produces IL-22, TNF-α, IL-8, IL-2 and activates endothelial cells. Tumor-NKp44+ILC3 can be activated by lung tumor cells and tumor-associated fibroblasts, primarily via NKp44 receptor. The frequency of NKp44+ILC3 cells is significantly higher in stage I/II NSCLC than in more advanced tumor stages.
Our results indicate that NKp44+ILC3 locate in human NSCLC and might contribute to the formation of protective tumor-associated TLS.

C-19

BONE MARROW INVOLVEMENT MIMICKING ACUTE LEUKEMIA IN SMALL CELL LUNG CANCER: A CASE REPORT

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A 73-yo female presented severe dyspnea, pallor and hepatomegaly. Laboratory studies showed anemia (Hb, 96 g/L), leukocytosis (WBC, 13.62x10^9/L), neutrophilia (ANC, 9.54x10^9/L), and thrombocytopenia (PLT, 25x10^9/L). The differential WBC count showed: 73.5% neutrophils, 17.6% lymphocytes, 6.2% monocytes, 0.9% eosinophils and 6.2% basophils; total bilirubin 2.4 mg/dL (normal 0.3-1.2), direct bilirubin 1.8 mg/dL (normal <0.3), alkaline phosphatase 415 UI/L (normal 35-104), and γGT 1.122 UI/L (normal 5-36).
PB smear documented the presence of erythroblasts (8/100 WBC) and immature granulocytes (13%). The BM aspiration showed hypercellularity with 100% of blast cells, sometimes vacuolated, with basophilic cytoplasm and negative to myeloperoxidase (MPO).
The flow cytometric analysis on BM aspirate revealed the presence of a small and low complex population with a negative CD45 and a bright expression of CD117 and CD56. This population resulted negative for CD34, CD13, CD33, HLA-DR, MPO, CD36, CD19, CD38, CD7, CD3 (surface and intracytoplasmic), CD71, CD105. The observed immunophenotype did not match any WHO classification of hematological malignancies.
BM biopsy confirmed the neoplastic involvement by small cell lung cancer (SCLC) cells. Chest X-rays and total body CT scan documented the presence of a mass of neoplastic nature in hilar and para-hilar upper left lung. Some nodular hypodense areas are present in liver. Enolase were augmented: > 200 ng/mL (normal < 16.5). The poor clinical conditions of the patient did not permits a lesion biopsy rather they rapidly deteriorated and she died 10 days after hospitalization.
SCLC is a neuroendocrine carcinoma with aggressive behavior with early spread to distant sites (common sites: brain, bones, liver, adrenal glands, and bone marrow). BM involvement by metastatic tumor has anemia as most common finding.
Due to that bone marrow metastasis of SCLC (range 10 to 20%) had found to be an indicator of a bad prognosis, BM biopsy can be performed on selected patients who have changes of routine laboratory tests (elevated LDH, elevated AP, elevated CA and anemia, thrombocytopenia and leucopenia) suggesting bone marrow invasion.
In this case the BM aspirate with morphological and cytometric studies discovered the right diagnosis of an unknown tumor, confirming that a high specialization center is able to put a complex diagnosis from first level exams.

C-20

SYSMEX XN-9000 BODY FLUIDS MODE: PERFORMANCE OF NUCLEATED CELL COUNTING IN PERICARDIAL FLUID

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Introduction: Cytometric, biochemistry and immunometric analysis of pericardial fluid (PF) play a decisive role in defining the etiology of the effusion, such as in pleural and ascitic fluid. Pericardial effusion is present in a variety of pathologic conditions and often the etiology of the effusion is uncertain and cannot be clearly defined on the basis of clinical assessment. The light microscopy (LM) analysis is still considered the reference technique for the cytometyric analysis. Although it is plagued by notorious drawbacks such as high imprecision, need for skilled personnel and long turnaround time (TAT). In many clinical laboratories, to avoid the difficulties of microscopic counting, body fluids analysis is performed using hematological analyzers. Sysmex XN-9000 is an automated hematology analyzer with a dedicated module for body fluid analysis (XN-BF) that provides the following parameters: total nucleated cells (TC-BF), leukocytes (WBC-BF) polymorphonuclear (PMN #; PMN%) and mononuclear cells (MN#; MN%). It has shown good counting performances on ascitic, pleural and cerebrospinal fluids. Interestingly, no data are currently available about the performances on PF. Aim of this study was to evaluate the application of XN-BF in PF analysis, according to CLSI document H56-A, 2006 (1).

Methods: A total of 70 consecutive PF samples with TC-BF ranging from 47 to 38287x10^6 cell/L collected in K_3EDTA tubes (Becton Dickinson) were simultaneously assessed by XN-BF and LM. TC-BF performed by XN-BF were compared to those obtained with LM after staining with Turk reagents and counting in Nageotte chamber (dilutions 1:200). The agreement between XN-BF and OM was assessed with Pearson's correlation coefficient, Passing Bablok regression and Bland-Altman analysis. Statistical analysis was carried out with Analyse-it software 3.90.5 (Leeds, UK).
Results: The agreement between manual and automated TC count was good; it showed a Pearson correlation of r=0.99 (p<0.0001), Passing Bablok regression of y=1.0x-34.1 and an absolute bias of -39.1x10^6 cell/L.
Conclusion: XN-BF offers excellent performance, rapid and accurate total and differential counts in clinically relevant concentration ranges in PF, replacing the counting chamber for most samples.

Reference

1. Body Fluid Analysis for Cellular Composition. CLSI document H56-A (2006).

C-21

FLOW CYTOMETRIC CHARACTERIZATION OF NON-HEMATOLOGICAL CELLS IN PLEURAL EFFUSION

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Background: Flow cytometry (FC) is largely used in the diagnosis of hematological diseases for characterization of lymphoid cells, whereas it's not usually employed in characterization of non-hematological cells. We are testing a FC antibody panel in pleural effusion specimens, for identifying and counting epithelial cells.
Aim: The aim of our study is the evaluation of FC non-hematological cell detection in suspected malignant effusion specimens as an ancillary method, close to the classical immunoistochemistry.
Methods: 44 samples of pleural effusion, collected from patients with suspected malignancy, have been analyzed in our laboratory by flow cytometry using the following monoclonal antibodies (moAb): EMA- FITC (Ber-EP4 clone), pan- Cytok-FITC, CD66abce-FITC, CD71-APCAlexa700, CD45-PB, CD14-EDC, CD15-KO, 7AAD. Last three antibodies were used for cell gating and 7AAD for debris removal. We evaluated neoplastic cells MFI normalized on lymphocyte MFI (MFI ratio), for each moAb. All samples were also tested in immunocytochemistry (ICC) for diagnosis.
Results: ICC analysis on cell block preparations of pleural effusions allowed to split our samples in four groups: 19 lung cancers, 3 malignant mesotheliomas, 6 reactive mesothelial hyperplasia and 16 lymphocytic pleurisies. Our results show that MFI ratio of EMA and CD66abce antigens is at least ten fold greater in lung cancer than the other three groups (T Student <0.05); conversely MFI ratios of panCytok and CD71 don't show any statistically significant difference.
Conclusions: FC applied to pleural effusions adds quantitative informations affecting neoplastic cells and other cell populations usually found in effusions (lymphocytes, machrophages, mesothelial and epithelial cells) and may be rapidly applied to viable cells in fresh specimens avoiding fixation artifacts. Knowledge of cell immunophenotypes is essential for an exact classification of normal and pathological cells. We found statistically significant differences in MFI ratio of EMA and CD66abce on neoplastic specimens. Our preliminary results indicate that FC could help the pathologist in the diagnosis of pleural diseases, but to reach definitive conclusions more experiments are needed.

C-22

SKELETAL MUSCLE CELL FATE: FROM MICROSCOPY TO CYTOMETRY FOR IDENTIFYING CELL DEATH

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Apoptosis plays a crucial role in muscle pathology, in denervation and disuse. On the other hand, autophagy is a physiological mechanism, responsible for cell homeostasis, aimed to remove damaged organelles and unfolded proteins.
In this work, we studied in vitro muscle cell response to chemical triggers, all leading to final cell death. C2C12 murine myoblasts and myotubes were exposed to etoposide, H_2O_2 or staurosporine and cell response was investigated by means of a variety of morpho-functional and cyto-fluorimetric approaches. Myotubes appeared more resistant than myoblasts to apoptotic induction. In particular, etoposide- or H_2O_2-treated myoblasts showed characteristic apoptotic features, even if in the absence of the classic cup-shaped dense patches. Apoptotic cell death could be observed in etoposide-treated myotubes, confirmed also by a diffuse DNA cleavage presence. After H_2O_2 exposure, myotubes, differently from myoblasts, showed a poor sensitivity to cell death. Cells exposed to staurosporine, evidenced late apoptotic features and secondary necrosis.
In our experimental conditions, the coexistence of normal and apoptotic nuclei within the same fiber has been demonstrated, in particular in the case of etoposide and staurosporine treatments. The deletion of a single nucleus can occur without the death of the entire myotube, evidencing that a multinucleated cell dies 'more slowly'.
Confocal and flow-cytometry assays revealed, for each

conditions and with a lesser extent for myotubes, the mitochondrial membrane integrity loss, by monitoring the stability of cardiolipin, a phospholipid strictly correlated to cytochrome c release, which finally induces apoptosis activation (Salucci et al., 2016).
After the majority of stimuli, autophagic granules could be diffusely revealed in myotube cytoplasm. We hypothesize that they could preserve muscle cell integrity, counteracting chemical treatments, some of which could activate death pathways involving mitochondria. It is the case of etoposide drug, which induced skeletal muscle apoptosis in the presence of an autophagic flux impairment; in this experimental condition, LC-3 positive complex vacuoles and a reduced stability of lysosomal compartment could be detected.
These findings reveal that apoptosis and autophagy play a central role in muscle biology and, ultrastructural and flow cytometric approaches appear useful for highlighting these processes.

C-23

COMPARISON BETWEEN SYSMEX XN-9000 AND MINDRAY BC-6800 IN THE DETECTION OF MALIGNANT CELLS IN BODY FLUIDS

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Introduction: Analysis of body fluids (BFs) is an essential tool for the diagnosis and classification of several human disorders. The presence of malignant cells in BF is an important finding with therapeutic implications. Laboratories should be able to detect these cells, even if not suspected or requested by the clinician. The gold standard for analysis in BFs is optical microscopy (OM) using counting chamber, combined with differential cell count on cytospin. As this procedure have several disadvantages like high cost and imprecision, requirement of skilled personnel, there is a tendency to perform BF analysis on automated cell counters. However, BF often contain other cell types than typical blood cells (e.g. mesothelial cells, macrophages, and malignant cells),which might not be recognized by analyzers. Sysmex XN-9000 (Sysmex, inc. Kobe Japan) (XN-BF) and Mindray BC-6800 (Mindray Medical International Ltd., Shenzhen-China) (BC-BF) are two new generation hematology analyzers with a platform for BF analysis that provide the total (TC) count, leukocyte (WBC) and differential count, included high fluorescent cell count (HF), in which are included the malignant cells. The aim of our study was to evaluate the correlation between two analyzers for TC, WBC and HF parameters. METHODS: A total of 348 samples (99 ascitic [AS], 45 pleural [PL], 129 cerebrospinal [CSF] and 75 synovial [SY] fluid) were collected and analyzed both with BC-BF and XN-BF. The diagnostic concordance between the two analyzers was evaluated. RESULTS: Comparison of TC, WBC and HF parameters showed, respectively, Passing–Bablok regression (PB) y=1.0x-1.0, y=1.0x-0.0 and y=0.2x +0, Bias (B) of -20.6x10^{6} cell/L, 8.5x10^{6} cell/L and -41.1x10^{6} cell/L in all kind of body fluids; PB y=1.0x-5.2, y=1.0x+9.4 and y=0.2x+0.3, B of 5.1x10^{6} cell/L, 46.3x10^{6} cell/L and -48.8x10^{6} cell/L in AS; PB y=1.0x - 8.3, y=1.0x+3.0 and y=0.3x and, B of 28.2x10^{6} cell/L, 96.6x10^{6} cell/L and -71.1x10^{6} cell/L in PL; PB y=0.9x +0.6, y=0.9x-0.5 and y=0.3x+0, B of -19.2x10^{6} cell/L, -16.8x10^{6} cell/L and -5.8x10^{6} cell/L in CSF; PB y=1.0x-2.7, y=1.0x-21.5 and y=0.1x+2.3, B of -132.3x10^{6} cell/L, -94.8x10^{6} cell/L and -37.5x10^{6} cell/L in SY. Conclusions: XN-9000 and BC-6800 showed a good correlation for TC and WBC parameter, but they showed no correlation for HF parameter. It is, therefore,very important to know and to evaluate the performance of the automated hematology technologies that are using.

C-24

IMMUNOMODULATION AND APOPTOSIS INDUCED BY CATIONIC POLYSTYRENE NANOPARTICLES IN MARINE BIVALVE MYTILUS GALLOPROVINCIALIS

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The development of nanotechnology will inevitably lead to the release of consistent amounts of nanomaterials (NMs) and nanoparticles (NPs) into environment.
In particular, nanoplastic debris, resulted from runoff and weathering breakdown of macro- and microplastics, represents an emerging concern for marine ecosystems. The bivalve Mytilus galloprovincialis has proven as a suitable model invertebrate for evaluating the potential impact of nanoparticles (NPs) in the marine environment.
Polystyrene nanoparticles (PS NPs) can be considered as a model for studying the effects of nanoplastics in marine organisms: recent data on sea urchin embryos indicate that marine invertebrates are vulnerable to amino-modified PS NPs (PS-NH2), as observed for mammalian cell lines where they can induce apoptotic processes. However, no information is available on their effects and mechanisms of action in the cells of marine organisms. In this work, the effects of 50 nm PSNH2 were investigated in the hemocytes of the marine bivalve Mytilus galloprovincialis. Hemocytes were exposed to different concentrations of PS-NH2 suspension in ASW.

At the highest concentrations (50 μg/ml) these nanoparticles induced cytotoxic effects. Polystyrene nanoparticles induce a decrease in phagocytic activity in dose dependent manner and increase lysozyme activity. PS-NH2 NPs also stimulated increase in extracellular ROS (reactive oxygen species) and NO (nitric oxide) production, with maximal effects at lower concentrations. Moreover, at the highest concentration tested, PS-NH2 NPs induced apoptotic process, as evaluated by Flow cytometry (Annexin V binding and mitochondrial parameters).
The results indicate that bivalve immune system represents a sensitive target for toxicity of nanoplastics in marine invertebrates.
Further research is necessary on specific mechanisms of toxicity and cellular uptake of nanoplastics; in particular, the knowledge of their interactions with cells within the physiological environment of model species represents the basis for understanding their fate and impact on marine biota.

R-8

IMMUNOSUPPRESSIVE NEUTROPHILS IN B-CELL LYMPHOPROLIFERATIVE DISORDERS

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Neutrophils have been traditionally considered as simple components of the innate immune system with a limited number of pro-inflammatory functions. Recent findings In in tumor biology showed that neutrophils are capable of a wide range of specialized functions, also related to osteoclastogenesis and angiogenesis.
In cancer, several subpopulations of neutrophils could be detect based on their physical properties and sedimentation rate, e.g low and high-density neutrophils (respectively LDN and HDN). Our group evaluated the contribution of LDN and HDN in multiple myeloma and Hodgkin lymphoma, showing that in both settings HDN are dysfunctional, with reduced phagocytosis and aberrant expression of surface markers CD64 and CD11b.
Moreover, HDN (and to a lesser extend LDN) are immunosuppressive due to production Arginase-1 that depletes arginine in the environment and make T-cells anergic, with lack of expression of activation markers CD69, CD71 and HLA-DR upon PHA stimulation.
Gene expression profile of HDN confirmed in MM patients increased amounts of Arginase-1, HCK and BV-8 suggesting a wide change in HDN transcriptome in patients with active disease.

R9

MOBILIZZAZIONE DI CELLULE STAMINALI E COMPOSIZIONE CELLULARE DEL GRAFT : RUOLO DEI NUOVI AGENTI MOBILIZZANTI

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La chemioterapia ad alte dosi e il trapianto autologo di cellule staminali (ASCT) rappresentano un'importante opportunità di cura per i pazienti con patologie onco-ematologiche. La raccolta di cellule staminali emopoietiche (CSE) per trapianto autologo si esegue preferenzialmente dal sangue venoso periferico mediante separatore cellulare, dopo adeguato stimolo mobilizzante con somministrazione di fattori di crescita granulocitari ("Granulocyte Colony Stimulating Factor", G-CSF) associata o meno a chemioterapia "mobilizzante" che consenta di garantire una raccolta efficace di CSE ai fini di autotrapianto. La quantità di CSE CD34+ da raccogliere dipende dal numero di trapianti che si intendono effettuare, dall'intensità della chemioterapia di condizionamento e dalla patologia da trattare. Generalmente, la dose minima di CD34+ da raccogliere per assicurare al paziente un'adeguata ricostituzione midollare (entro 3 settimane dal trapianto) è $>2.0x10^6$/kg, con inizio della raccolta leucoaferetica in presenza di CD34+ >20/uL. E' necessario sottolineare, tuttavia, che ~10-25% dei pazienti sottoposti a mobilizzazione, non è in grado di raggiungere un adeguato numero di CSE CD34+ nel sangue venoso periferico e quindi fallisce il tentativo di raccolta. Recentemente si sono resi disponibili nuovi farmaci "mobilizzanti" da associare al G-CSF e alla chemioterapia, utili proprio in questi pazienti noti come "poor mobilizers". Il principale di questi farmaci è il Plerixafor. In base ai farmaci impiegati per la mobilizzazione CSE, la composizione del graft cambia in maniera significativa, e nel presente lavoro verranno discusse queste modificazioni anche alla luce dei recenti progressi in ambito medico e biotecnologico che hanno portato allo sviluppo di prodotti medicali altamente innovativi basati su cellule e tessuti. I prodotti contenenti CSE possono essere infusi senza alcuna manipolazione o essere sottoposti a diversi tipi di trattamento quali rimozione di plasma/eritrociti in caso d'incompatibilità ABO, criopreservazione in caso di utilizzo differito, procedure d'immunoselezione (rimozione di possibili componenti neoplastiche o di specifiche componenti cellulari, es.: T-linfociti). Tutte queste metodologie sono considerate manipolazioni minime e devono essere effettuate in laboratori adeguati, di classe D, in base alla normativa attuale. La sinergia tra diverse branche scientifiche quali medicina, biologia cellulare e ingegneria finalizzata al riparo, sostituzione o rigenerazione di tessuti umani (la cosiddetta medicina rigenerativa) ha dato avvio a un settore in rapida evoluzione denominato ingegneria tissutale. Queste nuove strategie terapeutiche hanno dimostrato numerose applicazioni cliniche ad uso umano.

C-25

CD26 EXPRESSION ON LEUKEMIC STEM CELLS IN PERIPHERAL BLOOD: A SIMPLY AND RAPID NEW TOOL FOR DIAGNOSIS OF CHRONIC MYELOID LEUKEMIA

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Diagnosis of Chronic Myeloid Leukemia (CML) implies documenting in bone marrow (BM) or in peripheral blood (PB) Philadelphia (Ph) chromosome by cytogenetics and BCR-ABL1 fusion by FISH or RT-PCR. Lately, a specific co-expression of dipeptidylpeptidaseIV (CD26) within the CD34+/CD38–/Lin– stem cell fraction appeared a robust biomarker for identifying CML LSCs in BM. We recently demonstrated that CD34+/CD38-/CD26+ LSCs can be easily identified by flow-cytometry also in PB during TKI therapy. We here investigated accuracy and specificity of CD34+/CD38-/CD26+ assessment in PB as a new diagnostic tool in 107 pts with clinical suspicion of CML. All pts were evaluated for PB CD26+LSCs, cytogenetics,
FISH and/or BCR-ABL1 RT-PCR analysis; in 53/107 pts CD26+LSCs were tested also in BM. We used a flow-cytometry 4-color staining procedure. 2.0×10^6 leucocytes were incubated with CD45V500 (c.2D1), CD34FITC (c.581), CD38APC (c.HIT2), CD26PE (c.M-A261) and negative controls (BD Pharmigen). Acquisition and analysis of at least 1.0×10^6 CD45+ cells were done by FACSCanto II with DIVA 8 software (BD, Biosciences). In 83/107 pts we showed CD34+/CD38-/CD26+ LSCs in PB and in all of them CML was confirmed by cytogenetics, FISH and RT-PCR analysis. Median value of circulating PB CD26/μL was 14 (range 0,27-698) and a positive correlation with leukocyte count ($p < 0.01$) was found. All CD26+ PB-BM matched pairs (49/53) showed superimposable results in terms of absolute number of CD26+LSCs/μL (19,18 and 18,73 respectively) while the percentage of CD26+ cells within the CD34+/CD38- fraction appeared lower in BM than in PB samples (median 28,18 and 37,33; range 0,87-77,14 and 5,59-99,57 respectively). In 24/107 (22.5%) PB samples and in 4/53 BM samples CD26+ LSCs were not detected and no one was found Ph or BCR-ABL1 positive. Pts with CD26 neg PB/BM samples were subsequently diagnosed as Idiopathic Myelofibrosis, Myelodysplastic/ Myeloproliferative disorders benign neutrophilia and Ph+ acute lymphoblastic leukemia. Flow-cytometry evaluation of PB CD34+/CD38-/CD26+ LSCs is a feasible, very rapid and highly specific alternative/ complementary diagnostic tool for CML. To validate these data in a larger cohort of patients we are developing a pre-titrated lyophilized antibody mixture (lyotube, BD Biosciences) to maximize sensitivity and to optimize standardization and working time, with the further aim to monitor stem cells minimal residual disease in CML patients.

C-26

MULTICENTER VALIDATION OF A SIMPLIFIED METHOD FOR PAROXYSMAL NOCTURNAL HEMOGLOBINURIA SCREENING

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Background: Established flow cytometric Paroxysmal Nocturnal Hemoglobinuria (PNH) diagnostic guidelines recommend single-tube 5/6-color or 2-tube 4-color assays. PNH clones may be detected in only a fraction of the tested samples in patients at risk, and screening for new PNH cases can be complex and expensive. Cost-restriction policies may hamper the identification of new PNH patients. In this multicenter study we have validated a simplified, cheap one-tube 2-color FLAER-based assay to be used for PNH screening.
Methods: Six laboratories received 10 fresh samples containing spiked PNH leucocyte clones from 0% to 35%, to be analyzed in parallel with a centrally prepared 6-color cocktail (FLAER/CD24/CD45/CD64/CD15/ CD14) and a simplified 2- color mixture (FLAER/CD15), along with a shared calibration procedure and a common analysis protocol. Precision and sensitivity tests were performed with the simplified panel on PNH patients with replicates, from undiluted to 1:10,000. Additional specificity tests were performed on normal donors in order to identify all the possible sources of artifacts.
Results: The performance comparison between 6-color and 2-color assays showed an excellent agreement, especially for granulocyte PNH clones. Dilution experiments showed an accurate and specific detectability down to 0.01% sensitivity level for granulocyte PNH clones and to 1% for monocytes. Specificity experiments also disclosed that basophils and platelets can contaminate the monocyte gate and may generate false type-II-like PNH events.
Conclusions: A simplified 2-color (FLAER/CD15) PNH

screening test has been validated in a highly standardized multicenter study and proved feasible and effective in ongoing regional programs. Precision, sensitivity and specificity of the simplified test for granulocytes were comparable to the more complex and expensive 6-color assay and proved suitable for screening purposes also in small peripheral laboratories. The diagnostic confirmation of PNH should be in any instance performed by a reference center using the state of the art technique on at least two cell lineages and red cells.

R10

NUOVE FRONTIERE DELLA CITOMETRIA A FLUSSO NELL'IDENTIFICAZIONE DELLE MICROVESCICOLE CIRCOLANTI

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Lo scambio di informazioni nei sistemi biologici complessi è basato su meccanismi particolarmente sofisticati che utilizzano mediatori molecolari e cellulari. In questo contesto sta emergendo il ruolo delle vescicole extracellulari, rilevabili nei liquidi biologici, che sono associate ad importanti funzioni di regolazione (Montoro-García, Shantsila, Marín, Blann, & Lip, 2011). Tra le vescicole extracellulari spicca il ruolo delle microvescicole (MV), vescicole circolanti rilasciate per "budding" da molti tipi cellulari a seguito di stimoli di diversa natura. Le MV possono inoltre essere considerate degli effettori paracrini extracellulari, in grado di veicolare messaggi biologici (mRNA, miRNA, proteine, molecole di superficie) a specifiche cellule target. La loro origine, concentrazione e composizione biochimica, possono fornire preziose informazioni eziopatologiche e fisiopatologiche in numerose patologie (es. variazioni del numero di MV in ambito cardiovascolare, oncologico o neurologico) (György, Módos, et al., 2011).
La citometria a flusso multiparametrica rappresenta il metodo di elezione per l'identificazione e la conta delle MV nei liquidi biologici e, in particolare, nel sangue periferico (Horstman, Jy, Jimenez, & Ahn, 2004). Tuttavia, poiché le MV sono caratterizzate da dimensioni che si pongono al limite di risoluzione degli strumenti ad oggi disponibili, la loro identificazione, mediante i parametri di scatter, è particolarmente difficile e poco affidabile. La nuova frontiera nello studio delle MV in citometria a flusso è, secondo molti, lo sviluppo di protocolli che permettano di evidenziare il comparto delle MV circolanti mediante l'utilizzo di marcatori fluorescenti. Discuteremo delle tecniche di analisi ad oggi considerate il "gold standard" per l'identificazione delle MV in citometria a flusso e delle potenzialità e dei limiti applicativi delle stesse. Analizzeremo inoltre nuove metodologie di analisi citometrica e di sorting strumentale, attualmente in fase di messa a punto, che potranno permettere di ottenere nuove informazioni utilizzabili sia nell'ambito della ricerca sia nella clinica.

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C-27

AUTOPHAGY, LIPOPHAGY AND MITOCHONDRIAL DYSFUNCTIONS IN NIEMANN-PICK TYPE B LYMPHOCYTES

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Lysosomal storage disorders (LSD) are a group of more than 60 genetic diseases in which a deficiency of specific lysosomal enzymes produce an accumulation of undegraded substrates in lysosomes. Niemann-Pick disease (NPD) type A and B are LSD resulting from the sphingomyelin accumulation in lysosomes relying on reduced or absent acid sphingomyelinase (ASM). Recently, there has been an increased in investigating the autophagic pathway in lysosomal storage disease, based on the hypothesis that accumulation of undegraded substrates in lysosomes (due to deficiency of specific lysosomal enzymes), may impairs the autophagic process.
In the present study, we examined this hypothesis in a cellular model of Niemann-Pick disease type B, in which autophagy has never been studied. The basal autophagic pathway was first examined in order to evaluate its functionality using several autophagy-modulating substances such as rapamycin and nocodazole. We found that human NP-B B lymphocytes display considerable alteration in their autophagic vacuole accumulation and mitochondrial fragmentation that bring to mitophagy induction for damaged mitochondria clearance. Moreover, intra and extra-cellular lipids analyses shows lipid accumulation in NP-B B lymphocytes and also reveals their peculiar trafficking/management, culminating in lipid microparticle

extrusion, by lysosomal exocytosis mechanisms, or lipophagy. All of these features point to the presence of a deep autophagy/mitophagy alteration revealing autophagic stress and defective mitochondrial clearance. Hence, rapamycin might be used to regulate autophagy/ mitophagy (at least in part) and to contribute to the clearance of lysosomal aberrant lipid storage.

C-28

CAMPYLOBACTER JEJUNI CELL LYSATES INDUCE MODIFICATIONS AT DIFFERENT CELLULAR LEVELS ON HeLa CELLS

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The Gram-negative bacterium C. jejuni is one of the most common causative agents of food-borne infectious illnesses within humans. The cytolethal distending toxin (CDT), a genotoxin produced by C. jejuni, is a cyclomodulin that leads to cell cycle arrest and apoptosis in a wide variety of cell types. In this work we evaluated the sequence of lethal events in epithelial HeLa cells exposed to cell lysates of two human wild type strains, C. jejuni ATCC 33291 and C. jejuni ISS 3, and one C. jejuni 11168H cdtA mutant strain. Lysates were added to HeLa cell monolayers which were analysed after 24, 48, 72 and 96 hours in confocal microscopy and flow cytometry to detect DNA content, death features, bcl-2 and p53 status, mitochondria/lysosomes network and finally, CD54 and CD59 alterations. Data showed that mitochondria and lysosomes were differently targeted by these bacterial lysates. In fact, the C. jejuni ATCC 33291 lysate induced an apoptotic cell death mitochondria-mediated (mainly via a caspase-dependent mechanism) although a p53 lysosomal pathway (also caspase-independent) seems to appear partially activated. Otherwise, in cells treated with C. jejuni ISS 3 lysate lysosomal alterations were detected and the p53-mediated oxidative degradation of mitochondrial components seemed to be lost.
Moreover, our data show lysates caused a downregulation of CD59 due to the degradation or internalisation of the endocytic pathway and an increase in CD54 surface expression because of the presence of virulence factors in the lysate. The absence of these modifications in cells preincubated with the mutant strain meant that CDT toxin might have a pivotal role in these processes. Preliminary studies, in epithelial intestinal cells (T84) and immune cells U937 and monocytes, also suggest that C. jejuni wild type strains, compared with the cdtA mutant strain, are able to induce lethal and sub lethal events, mainly involving mitochondria/lysosome networks and endoplasmic reticulum alterations.

C-29

6-(METHYLSULFINYL) HEXYL ISOTHIOCYANATE AS POTENTIAL CHEMOPREVENTIVE AGENT: MOLECULAR AND CELLULAR PROFILE IN LEUKEMIA CELL LINES

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Background: numerous laboratory and epidemiological studies show that the risk of developing several types of cancer can be reduced with the employment of natural substances that act by multiple mechanisms. In this context an important role is played by the isothiocyanates. Recently, the 6-(methylsulfinyl) hexyl isothiocyanate (6-MITC), present in the root of Wasabia Japonica has stimulated the interest of researchers due to the demonstrated anti-inflammatory, antioxidants and neuroprotective properties, which permit to hypothesize its potential use as a chemopreventive agent. Aim: this work is focused on the evaluation of 6-MITC cytotoxic, cytostatic, cytodifferentianting activities and pro-apototic potential in vitro.
Methods: these effects were investigated by flow cytometric analysis on Jurkat and HL-60 cells and in parallel, on healthy lymphocytes extracted from the blood of AVIS donors, in order to verify a potential selectivity of action. Results: the results demonstrate that 6-MITC exerts a stronger cytotoxic effect on tumor cells than on healthy cells. The apoptosis induction exerted by the 6-MITC on transformed cells is triggered by extrinsic pathway, as demonstrated by the statistically significant increase in the percentage of cells with activated caspase 8, while the intrinsic pathway does not seem to be involved, as demonstrated by the number of cells with depolarized mitochondrial membrane potential cells, the bax/bcl2 ratio and the level of cytochrome c that remain comparable to those found in the controls. Furthermore, the apoptosis induction resulted in tumor cells is dose- and time-related, p53 indipendent and statistically significant at lower concentrations than those required for exert the same effect on non-transformed cells. In addition, it was observed that 6-MITC is able to limit tumor growth by slowing down and blocking the cell cycle of Jurkat and HL-60 cells respectively, in a dose- and time-related manner, while it does not exert any kind of activity on replication of healthy cells. Finally, by measuring the expression levels of CD14 and CD15, 6-MITC showed the ability to induce cytodifferentiation of HL-60 into the macrophage and granulocytic phenotype. Conclusions: It is possible to conjecture for 6-MITC a potential use as chemopreventive agent and a range of concentrations to act selectively.

P-1

UN METODO BASATO SULLA CITOMETRIA A FLUSSO PER LA RILEVAZIONE DI CONTAMINAZIONI BATTERICHE DELLE COLTURE CELLULARI

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Le contaminazioni microbiche delle colture cellulari derivano da fonti multiple e comprendono batteri, funghi (muffe e lieviti), micoplasmi e virus. Tra queste, le contaminazioni batteriche vengono rilevate mediante ispezione visiva delle colture cellulari e/o procedure batteriologiche. La sensibilità di queste non è ottimale, il che può dar luogo a ritardi deprecabili nella rilevazione di culture contaminate e a conseguenti gravi perdite. In particolare, vi è evidenza che i mezzi batteriologici non possono fornire condizioni di crescita idonee per alcune specie di batteri a crescita lenta e per fasi intracellulari di batteri quali gli stafilococchi. Inoltre, diversi batteri patogeni subiscono mutazioni nel loro ambiente, al fine di sopravvivere e stabilire un'infezione. Molti fattori di stress sono noti per influenzare le dimensioni, la crescita, la divisione e il metabolismo batterico, il che rende necessarie nuove procedure di controllo. La citometria a flusso è utilizzata da lungo tempo per rilevare batteri, lieviti e funghi. Sulla base di questi risultati, abbiamo sviluppato una procedura citometrica per colture cellulari e relativi terreni. Il protocollo si basa su due coloranti che si legano, rispettivamente, ad acidi nucleici di cellule batteriche vitali e morte. I nostri risultati hanno dimostrato che è possibile discriminare i profili di scatter e di fluorescenza dei batteri da quelli delle particelle di nucleoproteina rilasciate dalle cellule necrotiche e apoptotiche (colorazione non specifica).
Pertanto, un "gate" di contaminazione batterica è stato definito sulla base sia di forward scatter che di fluorescenza. E' stato inoltre definito un livello soglia di eventi nel "gate" dopo aver esaminato diverse colture cellulari non-contaminate di diversa origine, tipo (fibroblastiche, epiteliali, mesenchimali) e specie. La nostra procedura è stata in grado di rilevare contaminazioni batteriche sperimentali entro 4-5 ore dall'inoculazione. Inoltre, fatto ancora più importante, il saggio ha rivelato contaminazioni batteriche anche prima di un test batteriologico. Sulla base di questi elementi, questa nuova procedura di test rapido ha una notevole potenzialità per applicazioni di routine, essendo alla base di controlli di sterilità "robusti" delle colture cellulari e di prodotti immunologici, quali i vaccini.

P-2

EARLY T-CELL PRECURSOR LEUKEMIA: A PEDIATRIC CASE REPORT OF A NEW 2016 WHO PROVISIONAL ENTITY

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Early T-cell precursor leukemia (ETP-ALL) is a rare WHO provisional entity characterized by blasts originating from the most primitive progenitor T cells. Diagnosis is challenging, due to the unusual morphology and immune phenotype: absence of CD1a and CD8 T-lineage markers, weak or absent CD5 expression, and expression of at least one myeloid or hematopoietic stem cell markers such as CD13, CD33, CD34, CD11b, CD65, HLA-DR or CD117.
Here we report the case of a 7-year-old child referring to the pediatric emergency room for a persisting fever and recurring skin pustules scarcely responding to antimicrobial therapy. On admission, peripheral blood counting detected $424x10^3$ WBC cells/µL, a severe anemia (Hb=5.9 g/dL), and low platelets ($75x10^3$/µL). Bone marrow aspirate showed 80% of large-sized multi-nucleolated blasts (with scant cytoplasm resembling cells of lymphoid origin, but also displaying features of myeloid lineage. Immunephenotyping was performed using a BD FACs Canto II cytometer with the new Euroflow Acute Leukemia Orientation Tube (ALOT), which contains a combination of 8 antibodies (CyCD3, CD45, CyMPO, CyCD79a, CD34, CD19, CD7, SmCD3) allowing for an orientation of suspected acute leukemias towards the most appropriate characterization panel. The blasts were CD34+cyCD3+CD2+CD7+HLADR+, but also expressed CD117 and CD13 myeloid antigens, thus fulfilling the criteria for an ETP-ALL diagnosis.
Features of both lymphoid and myeloid neoplasms were also indicated by the detection of the clonal TCR DD2-DD3 gene rearrangement, WT-1 mRNA overexpression, and FLT3-ITD mutation.
Minimal residual disease (MRD), detected by flow cytometry at day +15 after therapy start, included the patient in the high-risk group of the AIEOP-BFM LLA 2009 protocol, which is characterized by an intensified consolidation. This regimen successfully cleared the disease: the MRD switched from positive at day +33 to below the cut off at day +78, and bone marrow aspirate morphology was negative. The patient is now in the reinduction phase and in complete remission.
While originally described as a poor prognosis entity, improved outcomes were recently reported for ETP-ALL using the same protocol. Our data appear in keeping with these observations, and considering the cumbersome morphology and the importance of MRD, enforce the need for a precise immune phenotyping. In this context, the ALOT approach can be helpful and is effective.

P-3

DETERMINATION OF BRONCHOALVEOLAR LAVAGE LEUKOCYTE POPULATIONS BY FCM

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Introduction: Broncho alveolar lavage (BAL) moderately invasive technique is procedure for the diagnosis of respiratory. Characteristic changes in the proportions of leukocyte populations in BAL reflect different disease states in the lung. The standard method for examination of BAL leukocytes is by microscopy of cytospin preparations. We hypothesized that flow cytometry (FCM) may be a more precise tool for investigating BAL.
Study objective: Performing FCM investigations interconnected to diagnostic information in order to perform targeted insights for the best diagnostic classification.
Materials and methods: 313 BALs were performed on 313 patients, received at Bologna Laboratory Metropolitan (LUM), in 2016, were subjected to evaluation of total cellularity using automatic blood cell counts determination the white cell differential with microscopy slides contained and leuco-lymphocyte immunophenotyping with FCM (BD). All samples were stained using the pan-leukocyte marker CD45/SSA in combination with CD3, CD33, CD15, CD13, CD9 and CD3, CD19, CD4, CD8, CD56+CD16 allowed the study of lymphocyte subpopulations.
Results: 143 BAL / 313 samples had the medical history information and was therefore possible to make specific investigations.
The evaluation of all samples examined showed: 26% of samples had prevalence of granulocyte population and were associated with the inflammatory pathologies;23% had the prevalence of the lymphocyte population, were associated to oncological diseases, or viral etiology and pathology. In the group with predominantly lymphocytic 11% of the patients showed an increase of CD4+CD103+ population, but only in one case it was possible to have diagnostic indications of sarcoidosis.
Conclusion: we have described a simple FCM technique for distinguishing the BAL leukocyte populations that avoids the potential complications recorded by previous investigators, furthermore, the use of the CFM for the evaluation of leukocyte populations in the BAL and the lymphocyte characterization has been shown to correlate with the diagnostic suspicion.
These features, combined with its speed and the ability to perform simple additional lymphocyte phenotyping panels, argue strongly in favor of FCM being adopted as a standard method for BAL analysis.

P-4

APPLICATIONS OF BASOPHIL ACTIVATION TESTS IN THE DIAGNOSIS OF DRUG HYPERSENSITIVITY: OUR EXPERIENCE

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Background: Adverse reactions to drugs are important and frequent complications in clinical practice. IgE-mediated reactions to beta-lactam antibiotics are the most frequent drug allergic reactions and Amoxicillin is the antibiotic more often involved in allergic reactions. The basophil activation test (BAT) is a useful tool in the in-vitro diagnosis of immediate hypersensitivity reactions to drugs, whether these are caused by IgE-dependent mechanisms or other mechanisms leading to the release of histamine and other mediators of immediate hypersensitivity reactions. In our Laboratory (LUM) of Bologna, we have applied the technique of BAT for the identification of allergic reactions to some allergens including drugs and in particular beta lactam.
Aim: The purpose of this study was to confirm the applicability and advantages of the BAT for in vitro diagnosis of allergic reactions to medications.
Method: 253 requests, received in the last two years, for the BAT survey with Amoxicillin (AM) and Amoxicillin + Clavulanic Ac (AM+CL) in combination or individually were included in the study. BAT was done according to method Flow-Kit CAST Bühlmann, based on double staining with anti CCR3 and anti-CD63 antibodies and subsequent determination of the % of activated basophils by flow cytometry. Results are expressed as the % of CD63+ basophils; a stimulation index (SI) ≥2 and an absolute activated basophil %≥5 were considered positive BAT responses.
Results: We tested 220 patients for AM or AM+CL. 4% had an insufficient number of Basophils to obtain statistically reliable results. 0.5% were high responders, 6% no responders (PC1 negative) and 4% PC2 negative. One patient was PC1 and PC2 negative. 211 patients were eligible to perform BAT test. 119 patients with the only request for AM, were positive in 3 cases (3%).20 patients were tested only for CL+AM and one was positive(1%); 72 patients with requests of AM and CL +AM were positive in 7 cases (10%).
Discussion: The use of the combination of AM and CL+AM, is useful for the discrimination between reaction to CL or AM, in addition to being a control of the result obtained. However, we believe that this simple and immediate rapid test is a great aid for screening patients at potential risk of adverse reaction and that it is important to perform combined assessments. Fundamental is to have a good clinical history to proceed with focused investigations and interpretation of results is performed by a specialist.

P-5

FLOW CYTOMETRIC EVALUATION OF PERIPHERAL BLOOD LYMPHOID ANTIGENS IN CALVES TREATED WITH GROWTH PROMOTERS

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The use of growth promoters (GPs) in bovine livestock is banned in the EU. The detection of the abuse of GPs is largely based on the direct chemical identification of specific residues in several matrices (1). These methods are highly sensitive but expensive and time-consuming. Moreover, chemical methods are limited to few known molecules, whereas many new drugs are introduced every year on the black market. In addition, GPs are often used in cocktails and at low-dosage in order to evade official controls. Recently indirect methods to identify biomarkers of illegal use of GPs have been developed (2, 3). Among these, the histological test has been included in the Italian National Residues Monitoring Plan as a screening method (4, 5). Unfortunately all current indirect assays can be run only after slaughtering.

Aim: Evaluate if CDs expression in peripheral blood lymphocytes can be a marker of illegal treatment in calves before the end of the breeding cycle.

Materials and methods: Eighteen veal calves were allocated in 3 groups: controls (no treatment); dexamethasone (0,4 mg/die OS for 20 days); 17β-estradiol (5 mg/week IM, for 4 weeks). Blood was collected at 3 time points: T0, T10 and 22 (days of treatment). Lymphocytes were labelled with CD18, CD62L, MHC-II, WC1, CD4, CD8, CD335, CD25 e GR (glucocorticoids receptor) and both percentage and MFI of each antigen were recorded. Fluorescent microbeads were added to each tube, and antigen fluorescence intensity was reported as MFI ratio (beads*100/CD). The effect of group (treatment), time and group*time CD percentage and MFI was evaluated.

Results: 17β-estradiol was significantly associated with a down regulation of CD18 MFI. Percentage of CD25+ cells was significantly increased at T10 in dexamethasone treated calves.

Conclusions: Dexamethasone and 17β-estradiol can influence the expression of some CD in circulating lymphocytes. These preliminary results indicate flow cytometry as a possible tool to identify illegal treatment of calves before the end of the breeding cycle.

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