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The role of aquatic exercises in maintaining the musculo-skeletal status in haemophiliacs

Luminiţa Rusen[1], Laura Izabella Ionescu [2], Nicolae Oniga[3]

1. Central Reference Laboratory for Diagnosis and Treatment of Haemorrhagic Diatheses, National Institute for Transfusion Haematology „Prof. Dr.C.T. Nicolau", Bucharest
2. Atlantis Sports Association
3. The Romanian National Association of Haemophiliacs

Summary

Haemophilia is a blood disease with muscles and joints complications. The joint destruction most frequently determines morbidity in haemophilia. The chronic pain and functional disability caused by chronic arthropathy decrease the quality of patient's life. In the past, physical activity was not indicated to haemophiliacs. Today, it is recommended and even deemed to be an adjuvant for substitute therapy. Two groups of patients with haemophilia, a group of adults and a group of children have attended a kinetotherapy program both in the sport room and in the pool. The mobility assessments and the questionnaires received have been analyzed annually. A normalization of the range of joint motion and a growth of the muscle strength have been noticed in the children group, while in the adults, a slight improvement of these parameters have been noticed. A program of physical exercises adequate to each patient reduces the destructions caused by inactivity. It is necessary that aquatic physical exercises be integrated as treatment in the management of haemophilia.

Keywords: haemophilia, aquatic exercise, haemophilic arthropathy, musculo-skeletal

INTRODUCTION

Haemophilia is the most common hereditary hemorrhagic disease caused by the deficit of either factor VIII (FVIII) – haemophilia type A, or of factor IX (FIX) – haemophilia type B. They are clinically expressed by typical hemorrhagic syndrome associating haemarthrosis and muscle hematomas in severe forms. The determination of the type of haemophilia, A or B, is an essential stage of the biologic diagnostic. The disease manifests only in boys. There are several degrees of severity, depending on the clotting factor, classified as mild, moderate and severe forms. Bleedings may appear anywhere in the body. The joints and muscles are the most frequent sites where the haemorrhages occur. As the blood fills the joint (knees, ankles, elbows) it becomes painful and it doesn't allow the patient to move. It is accompanied by atrocious pains and the patient remains bedridden until the treatment is provided. Without immediate treatment, the accumulation of blood in the joint leads to the destruction of joints and the patient moves increasingly difficult, then he needs crutches or wheelchair in order to move.2 Such situations are frequent in older patients who have not beneficiated of treatment with clotting factor concentrate.

The destruction of joints most frequently determines morbidity in haemophilia, 90% of the haemophiliacs with severe forms of disease have recurrent bleeding in one or more joints evolving towards degenerative arthropathy. 5 The chronic pain and functional disability caused by chronic arthropathy reduce the quality of patient's life and lead to expensive surgeries. 7 Many authors have tried to study the mechanisms and especially the factors favouring chronic arthropathy characterized by joint destruction, pain, deformity and disability. It has been demonstrated that, in the persons predisposed to arthropathy, it sets after few intra-joint bleedings.(4) The goals of therapy consisted in the prevention and control of hemorrhagic accidents, the prevention and improvement of sequelae in order to provide physical, psychological and social comfort for each patient. The substitute treatment with clotting factor concentrate products is by far the most important in stopping the bleeding but not enough, because it must be associated later, between the hemorrhagic episodes, with locomotor recovery measures. These target the osteoarticular and musculo-ligamentous systems. The joint contracture in patients with haemophilia occurs as a result of intra-joints and intramuscular hemorrhagic episodes. It has been noticed that the first changes in the joints occur in childhood and final changes set slowly. 7

It has been found that a progressive limitation of motility occurs in time with functional disability caused by painful flares, improper posture as well as over use or under use of various muscle groups. The musculature has the most important role in causing movement, and its maintenance in normal parameters can be done by physical exercises only.1 Performing continuous motor activities, which shouldn't cause pain and fatigue, leads to obvious improvements. The importance of physiotherapy as integral part of the acute bleeding management is universally recognized. At international

level, hydrotherapy is a method unanimously accepted by haemophiliacs and their physicians.

MATERIALS AND METHODS

Two groups of patients with severe haemophilia A and B have been selected: a group of 15 adults with ages between 25 and 55 years and a group of 15 children with ages between 7 and 17 years. Both groups have done physical exercises in water and in the sport room, with different programs, for 3 years consecutively. The adults have done 2 sessions a week, for 8 months a year, while children have worked in sessions of 14 successive days, quarterly. O session had minimum 45 min. Assessments of musculo-skeletal system have been made at every 6 months and satisfaction questionnaires have been filled yearly. The articular status have been examined by using Orthopaedic Joint Score, which contains the clinic score (swelling, muscle atrophy, range of motion, etc.), pain score and bleeding score. The purpose of kinetotherapy in children is to minimize the musculoskeletal effects on long term.

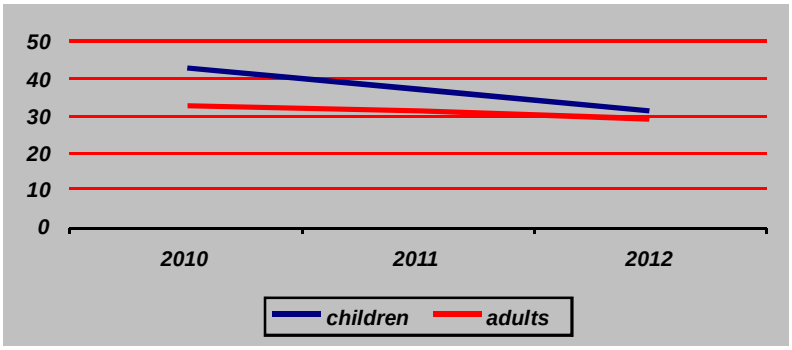
RESULTS

The measurement of articular scores in the group of children has shown a normalization of the range of motion and a strengthening of muscles. In the group of adults, the articular scores have improved slightly after those three years. The compliance rate of the attendance to the hydrotherapy sessions and execution of exercises was 90% for children and 65% for adults.

The characteristics of patients

Characteristics	Children Mean ± SD	Adults Mean ± SD
Age (year)	12 ± 5	33 ± 12
Height (cm)	134 ± 14	169 ± 7
Weight (kg)	29.5 ± 8.95	68.7 ± 12.49
BMI –body mass index(kg/m^2)	17.27 ± 1.84	21.32 ±2.35

In other countries, the existence of prophylactic treatment is of great help and is performed before starting the session. Physical activity must be controlled and protection measures must be taken. If, during the first sessions for children, small doses of preventive factor concentrate have been administrated, subsequently the substitute treatment was made only immediately after occurring the bleeding and it has been noticed that the number of hemorrhagic accidents has decreased with more than 50% in the second year and in the third year, even though the session time has increased. A decrease in the average annual number of the hemorrhagic episodes, especially in the group of children, after they attended to the kinetotherapy sessions, has also been noticed.



The average annual number of haemorrhages

Regarding the satisfaction questionnaires, the data show a 27.3% trust in the efficiency and use of kinetotherapy of the attending children's parents in the beginning of the action, and a 41.2% trust of the adults. In the third year, the satisfaction rate was of 98.7% for the group of children and 61.5% for the group of adults.

DISCUSSIONS

Haemophilia is a blood disease with complications in muscles and joints. By pain and disability, arthropathy is the most frequent complication of the haemophilia. For decades, this category of patients was forbidden to practice sport and a maximum caution was required. Since the 70's, the therapeutic conduct of the haemophiliac was not to do any kind of sport due to the traumatic risk. An inadequate substitutive treatment associated with sedentarism, the fear that any physical activity causes a new bleeding, the interdiction to practice any sport can be seen in nowadays adults. They have many chronic arthropathies with semi-ankylosis and ankylosis of the big joints associated with muscular atrophy in the superior and inferior members. The most affected joints are elbows, knees and ankles, meaning exactly the joints which are the most used daily.[5]
For many patients, doing sport was synonym to doing professional sport, forgetting that physical activity was not the same thing.

An American study has demonstrated that 36% of the patients with severe forms of haemophilia needed mobility assistance and 30% missed school as a consequence of problems related to limb joints. [8]
Kurz et al sustain that articular degradation starts since the young age. This was the reason for which many countries have introduced prophylaxis since very young ages, evidencing that primary prophylaxis was the best method to prevent the arthropathy and to improve the quality of life.[2]

Czepa et al analyzed two groups of subjects: one of haemophiliacs and one of healthy persons, of the same age and physical activity, and asked them to subjectively assess their physical performance. Then, they tried to perform an assessment correlated with the objective data. It was noticed that the haemophiliacs under-evaluate their physical performances comparing to healthy persons. [3]

All started from the idea that the haemophiliac shouldn't live closed in a shell. Sport is allowed and even recommended, even in a country where prophylaxis is only a desideratum. With only one condition: to learn to know his/her limits. The research conducted in the last years demonstrated that the active persons with haemophilia had less bleeding problems comparing to the persons who didn't practice physical activities and who were overweight as well. The physical activity and sport activity just need to be permanently adapted to the severity of illness. It's very important that the haemophiliac should practice exercises that he likes and that keep him motivated. If the sport allows children to develop their muscles and to protect themselves from the orthopaedic degradations, in adults it represents a precious tool for stopping or correcting them. Among the sport activities allowing the harmonious musculo-skeletal development of a haemophiliac, swimming is the most recommended. This discipline has very few risks of injury. In addition, swimming is a complete sport by excellence. The time when it was deemed that the physical activity was responsible for bleedings is long gone. Today, it is demonstrated that the joints surrounded by strong muscles have lower risk of bleeding comparing to the joints with atrophied muscles.

The optimal age for starting sport activities is 6 - 7 years, and especially 8-13 y.o., when learning capacity is better. Parents have a primordial function: they should encourage the children with haemophilia to take exercises. Even at school, they can participate together with their colleagues, provided that the sport teacher is told and he should know in what degree he can work with the child. The physical activity influences in a positive way the growth and development of children by strengthening the bones and muscles. To develop the muscles, one needs time in the first place, and the physical activity should be adapted to each patient and made under the kinetotherapist supervision.

A special therapeutic alternative to swimming is hydrotherapy, which has many advantages: it eases pain (movements in water are less painful), by flotation it protects the joints from the pressure exercised by the body weight allowing walk training, it may support in or offer resistance to physical activities when the increase of muscle strength is envisaged, it prevents or corrects joint deformities, it increases exercise tolerance and, the most important aspect, it represents a pleasant work environment especially for children.

Hydrotherapy is excellent for the haemophiliac's health because the entire body participates to movement with minimum of joint pressure. It is highly recommended especially for those who benefit of prophylaxis as it allows to young people to grow with healthy muscles and joints. Even in the absence of prophylaxis it was demonstrated to cause:

- **stronger** and harmonious **muscles** allowing protecting the joints against traumatisms, thus reducing articular bleedings;
- it maintains a **good mobility** of the joints due to high amplitude movements favouring cartilage lubrication and preventing stiffness;
- it improves the balance, coordination and reflexes thus allowing the decrease of the incidence of joint sprain responsible for bleeding and it decreases the risk of falls;
- general development of the body;
- it prevents sedentarism and it decreases the risk of obesity – “the no. 1 enemy” of the joints;
- it generally increases muscle tonus;

- it allows the decrease of musculo-skeletal disorders related to arthropathy;
- it preserves the joints as the activity is practically in water, therefore with a minimum pressure;
- it allows maintain a optimal body weight;
- it improves self confidence.

The benefits of the aquatic gymnastics

- involving all major muscle groups in the body without over exhausting them (it has a massage effect);
- strengthening tissues, improving breathing, correcting digestion;
- reducing joint pressure;
- it doesn't mechanically stress the joints, tendons, ligaments;
- it is not tiring;
- it increases muscle strength faster than the exercises on land;
- it reduces the intra-joints pressure and the pains;
- it improves the relaxation capacity and it helps moving easily.

Apart from the benefits of physical activity in water, we must also mention the "wellness" that children have after practicing such a program. Water represents an excellent relaxation modality.

Aquatic gymnastics may be exercised all year long.

CONCLUSIONS

A well dosed physical activity, under experienced kinetotherapist supervision, reduces the incidence of articular bleedings and muscle bleedings. A decrease of articular complications on medium and long term has been noticed. It is recommended to undergo evaluations of musculo-skeletal system at least half-yearly.

The conclusions, following the three years of experience, are clear: physical activity in water reduces musculo-skeletal disorders related to arthropathy; it improves the balance sense, thus decreasing the risk of falls. It improves self confidence.

Apart from medical benefits, the physical activity and sport activity favour the social insertion of children and teenagers with haemophilia. Exercising these activities allows them to develop more harmoniously and to feel better not only in their bodies, but in their mind as well. That's why it is important they and their parents understand well the benefits of a physical activity on maintaining a good articular condition. The exercise of physical activities must continue in adulthood and must become, in a way, a rule of life.

Acknowledgements:

The Romanian National Association of Haemophiliacs has developed the program "Be in shape! – Take exercises! – Swim!" since the last quarter of 2010, in order to make the haemophiliacs aware of the importance of physical activity. The project was able to come to life due to the broad support of Atlantis Sports Association (Asociaţia Sportiva Atlantis) and of coach and physical education teacher, Laura Ionescu. It was first a shy experience, it then became an objective, and today we can say it is an actual philosophy of ANHR (Romanian National Association of Haemophiliacs). The pharmaceutical company, Novo Nordisk, has a special merit, as by its financial support turned the project "Be in shape! – Take exercises! – Swim!" into reality.

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The porphyrias – part I

Gabriel Găman*, Amelia Găman**
* University of Medicine and Pharmacy of Craiova
** Clinic of Hematology, Filantropia City Hospital Craiova

Abstract

Traditionally, the porphyrias have been classified as either hepatic or erytropoiethic, depending on the primary site of the overproduction and accumulation of porphyrin and porphyrin precursors, although some porphyrias have overlapping features. For simplicity, we have classified the eight major porphyrias into three groups: (1) the four acute hepatic porphyrias, (2) the single hepatic cutaneous porphyria (PCT) and (3) the three erythropoietic cutaneous porphyrias.
The acute hepatic porphyrias includes AIP,HCP,VP (AIP is the most common). The major manifestations of these disorders are acute neurological attacks, abdominal pain, cramps, constipation, abdominal distension, increased bowel sounds, nausea, vomitting, tachicardia, hypertension, menthal symptoms, chest pain, headache, muscle weakness, tremors,disuria, bladder distension. Once a biochemical diagnosis is established , mutation analysis of the genes for AIP (HMBS), HCP (CPOX),VP (PPOX) and then ADP (ALAD) should be undertaken.

DEFINITION

The porphyrias are metabolic, heterogenous, either hereditary or acquired disorders characterised by malfunctions in the synthesis and accumulations of precursors of porphyrins, porphyrins or both, followed by their excretion through urine or stool. Clinically, porphyrias appear as cutaneous (photodermatitis), gastrointestinal or neuropsychic disorders (1).

FUNDAMENTAL PRINCIPLES

Each group of porphyrias is produced by a specific enzymatic deficiency in the synthesis of heme. The enzymatic deficiency can be autosomal dominant, recessive or X-linked (exception- PCT with sporadic transmission).

Their rarity is rather elusory than real (underdiagnosed). Some cases in literature have been incorectly classified as dermatologic, gastrointestinal, neuropsychic diseases. The majority of cases are in a latent phase, at carriers of the specific illnesses.
A series of endo- or exogenous factors are able to trigger clinical manifestations with a dramatic evolution, sometimes even lethal (2).

CLASSIFICATION

For simplicity, we have classified the eight major porphyrias into three major groups:
- GROUP 1: the four types of acute hepatic porphyrias;
- GROUP 2: the single hepatic cutaneous porphyria - PCT;
- GROUP 3: the three cutaneous erythropoietic porphyrias;

The porphyias have been classified as either hepatic or erythropoietic depending on the primary site of overproduction and accumulation of porphyrin or porphyrin precursors.

Therefore, the hepatic porphyrias are characterised by an overproduction and initial accumulation of porphyrin precursors (ALA and PBG) and/or of porphyrins primarily in the liver, whereas, in the erythropoietic porphyrias, the overproduction and initial accumulation of the pathway intermediates occur primarily in B.M. erythroid cells. The hepatic porphyrias consist of three types of porphyria autosomal-dominant transmitted, one autosomal-recessive transmitted and one with ALA-dehydratase deficiency.

HUMAN PORPHYRIAS: MAJOR CLINICAL AND LABORATORY FEATURES (3)

Type	Deficient enzyme	Inheritance	Enzyme activity, % of normal	Increased porphyrin / porphyrin precursors		
				Erythrocytes	Urine	Stool
Acute hepatic porphyrias (AHP)						
ADP	ALA-dehydratase	AR	5%	Zn-proto-porphyrin	ALA, Coproporphyrin III	-
AIP	HMB – synthase	AD	50%	-	ALA, PBG Uroporphyrin	-
HCP	COPRO – oxidase	AD	50%	-	ALA, PGB Coproporphyrin III	Coproporphyrin III
VP	PROTO – Oxidase	AD	50%	-	ALA, PGB Coproporphyrin III	Coproporphyrin III Protoporphyrin
Hepatic cutaneous porphyrias						
PCT	URO - decarboxylase	Sporadic or AD	20%	-	Uroporphyrin 7-carboxylate-porphyrin	Isocoproporphyrin

CLINICAL MANIFESTATIONS

The most common acute hepatic porphyria is AIP; ADP has been reported in <10 patients until December 2012.

NEUROLOGICAL MANIFESTATIONS
- Occur after puberty;
- Acute neurological attacks are life-threatening;
- Menthal disorders;
- Sensory disorders;

ABDOMINAL MANIFESTATIONS

- Abdominal pain is the most common symptom, poorly localised, may appear as cramps;
- Constipation;
- Abdominal distension;
- Bowel sounds;
- Diarrhea (less common);
- Nausea, vomitting;

OTHER MANIFESTATIONS (possible):
- Tachycardia, hypertension;
- Pain of the extremities of the neck, head and muscles;
- Dysuria;
- Vesical distension;

PARACLINICAL DIAGNOSIS
BIOCHEMISTRY
- PBG levels are significantly increased in AIP, HCP and VP. The urinary PBG level can be rapidly determined through a PBG kit;
- From the same specimen spot of urine, which must be saved, ALA and PBG levels can be determined to confirm the qualitative presence of PBG and to detect the elevated ALA levels in the rare cases of ADP.
- In order to diagnose AHP, subsequent urinary and fecal porphyrin tests must be run.

MUTATIONAL ANALYSIS

- Molecular diagnosis studies for the AIP (HMBS), HCP (C-POX), VP (PPOX) and ADP (ALAD) genes;
- More than 375 mutations for AIP, 60 for HCP and 165 for VP have been identified;
- ADP is rareand only 12 ALAD mutations have been detected;

THE BIOCHEMICAL AND MOLECULAR DIAGNOSIS is useful to identify the risk of attacks for the heterozygous, asymptomatic patients if certain hormones, drugs or medicine are consumed;
- With the exception of AIP, VP and HCP (and its mutational-specific variant form, HARDEROPORPHYRIA) there have been no genotype/phenotype correlations at homozygous children (dominant forms) in hepatic porphyrias;
- Genomical and exomical studies are focusing on identifying predisposing genes at patients exposed to chronic forms or acute attacks of porphyria (5).

PATHOGENESIS
HYPOTHESIS:

1. Elevated levels of ALA and PBG, one or both being neurotoxic;
2. Heme deficiency in CNS.
3. Both mechanisms.

The triggering of acute attacks is furthered by environmental and hormonal factors, drugs, diets that induce ALAS I, resulting in the overproduction of ALA and/or PBG. The acute attacks can result from the hepatic production of a neurotoxic substance, presumably ALA (a gamma-amnobutyric acid analogue) or/and PBG that may interact with GAMMA-aminobutyric acid or glutamate receptors (6).

HEPATIC CUTANEOUS PORPHYRIAS
CLASSIFICATION

I) SPORADIC, type 1
The patients have no URO –decarboxylase mutations, URO -decarboxylase levels are normal and the patients are asymptomatic.
II) FAMILIAL, type 2
The patients are heterozygous for URO –decarboxylase mutations and have URO –decarboxylase (UROD) levels that reach 50% of the normal value. The disease is autosomal-dominant, with incomplete penetrance. For clinical symptoms to manifest in either type, the hepatic UROD activity must be of less than 20% of the normal value.

Although a diagnosis of PCT is usually made by a family physician, internist or dermatologist, the most common treatment for this disease are repetitive phlebotomies, so these patients typically refer to hematologists. Phlebotomies are performed to decrease the hepatic iron load. An alternative oral treatment is a low dose of chloroquine.

CLINICAL MANIFESTATIONS

- Blistering skin lesions that appear most commonly on the back of the hands;
- The tearing of the crusts is followed by the appearance of atrophic regions on the forearms, face, fingers, legs;
- Hypertrichosisand hyperpigmentation, particularly on the face and especially at women;
- If the skin in heavily exposed to sunlight, these areas become severely exfoliated and calcifications occur, resembling systemic sclerosis;.
- Small white papulae named ”MILIA” are common, especially on the back of the hands and fingers ;
- Neurological features are absent (7).

PARACLINICAL DIAGNOSIS

- Increased urinary ALA level;
- Normal PBG level;
- Increased uroporphyrins - UROPORPHYRIN and HEPTACARBOXYLATE-PORPHYRIN;
- Increased plasmatic porphyrin levels;
- Increased isocoproporphyrin primarily in faeces (diagnosis of URO-decarboxylase deficiency);
- URO-decarboxylase activity in erythrocytes is half the normal value in PCT type 2 and normal in type 1;
- UROD gene mutation analysis is recommended for diagnosis for whom the family history is lacking and who may be predisposed to UROD-mutations, being reclassified as type 2 PCT.

PATHOGENESIS

- URO-decarboxylase activity is reduced to < 20 % in types 1 and 2 when the cutaneous lesions are present;
- UROPORPHOMETHENE, an oxidized form of uroporphyrinogen, the substrate of uro-decarboxylase, is regarded as the enzyme inhibitor;
- Inherited UROD-mutations and multiple susceptibility factors (alcohol abuse, chronic hepatitis C virus, oestrogens) seem to act synergistically, causing oxidative stress and iron overload, generating the inhibitor and causing clinical expression;
- The excess of hepatic iron increases the prevalance of common hemochromatosis, causing C28Y and H63D mutations at patients with types 1 and 2 of PCT;
- Susceptibility factors (alcohol abuse, chronic hepatitis C virus and oestrogens) decrease HEPCIDIN production in hepatocytes, leading to increased intestinal iron absorbtion;
- The increased hepatic iron and oxidative stress lead to the formation of the enzyme inhibitor and the oxidation of porphyrinogen to porphyrins (8).

THERAPEUTICAL PRINCIPLES
- Avoiding susceptibility factors;
- Repeated phlebotomies - aprox. The equivalent of one unity of total blood – every two weeks (five-six phlebotomies are generally sufficient);
- The gradual reduction of pheritine levels to <25ng/ml;
- Periodical tests for the values of Hb and Ht as to avoid anemia;
- At patients with PCT, phlebotomies should be interrupted if the pheritine level is once again normal. The treatment will be continued with small doses of chloroquinine or hydroxychloroquinine every two weeks, especially at those at whom the therapeutical manoeuvre is badly tolerated or not suggested.

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Myelodysplastic Syndrome- A Case Report of Refractory Anemia With Ringed Sideroblasts

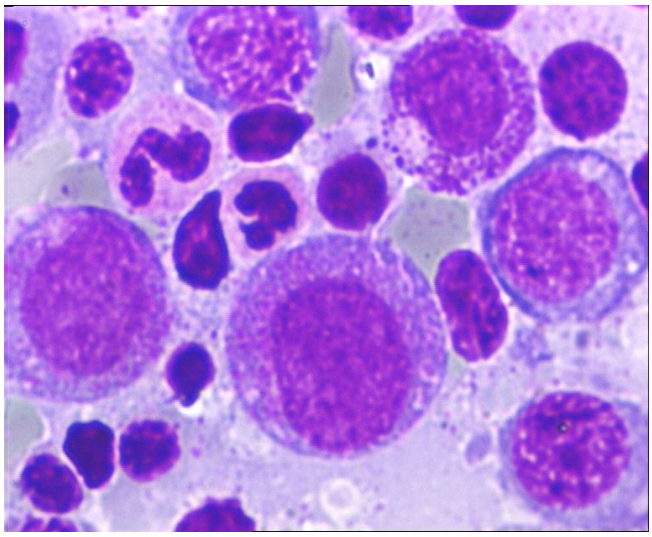
Diana Agatinei, Aurelia Tatic
Fundeni Clinical Institute, Bucharest

1. Introduction

The myelodysplastic syndromes (MDS) compose a heterogeneous group of clonal stem cell disorders, characterized by ineffective hematopoiesis in one or more cell lineages, and an ability to progress to acute myeloid leukemia (AML). The risk of transformation to leukemia is determined in part by the degree of morphologic atypia, blast percentage in the bone marrow, and cytogenetics of the MDS clone.

Often, MDS patients are asymptomatic, and the diagnosis is made at the time of routine laboratory screening tests that reveal cytopenias in one or more lines or dysplasia on the blood smear. Typical disease manifestations include weakness, fatigue from anemia, infections due to neutropenia, or bleeding caused by thrombocytopenia or platelet dysfunction.

50% of MDS cases have cytogenetic abnormalities at presentation (most frequenly: gain or loss of major segments of chromosomes (-5/del(5q), -7/del(7q), +8, +9, +11, del(11q),del(12p), del(17p), -18, +19, del(20q), +21).



Img. 1.

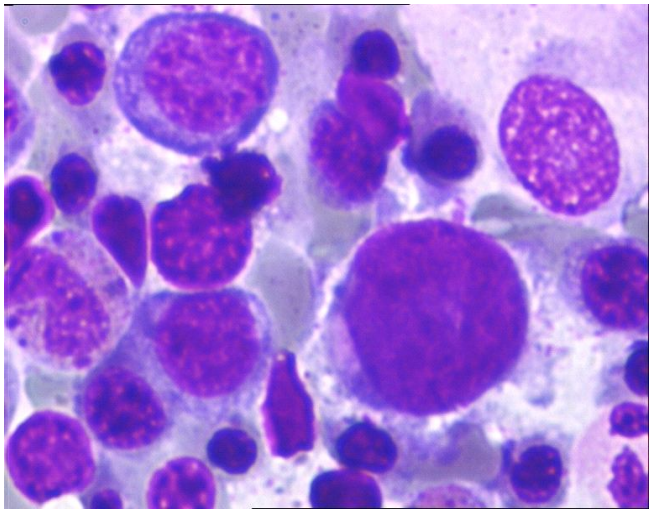
2. Case Report

A 71-year-old man, with a history of hypertension and empfysema, presented with 3 months of asthenia, fatigue, pallor, headache, vertigo, palpitations and a 3kilos weight loss over 3 months.

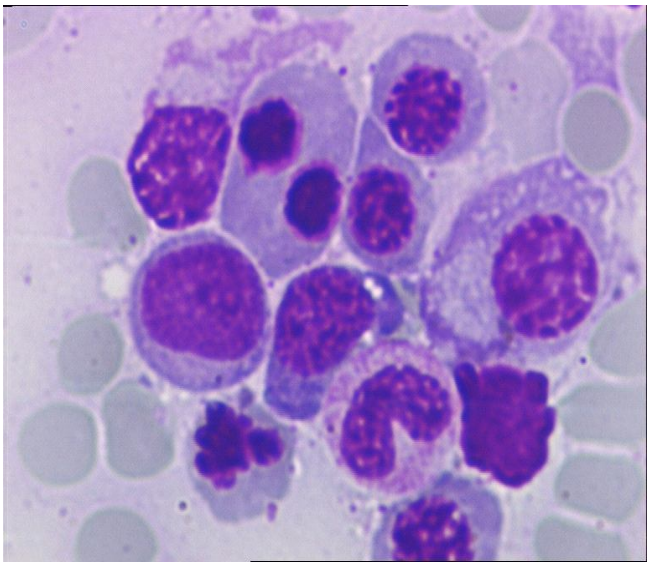
Complete blood count revealed a macrocytic anemia Hb 5g/dl, with a mean corpuscular volume (MCV) as high as 95.8, normal leucocytes 5510/mmc, normal trombocytes 259000/mmc, no blast cells in peripheral blood, with normal cobalamin and red blood cell folate, high ferritin level. Coombs testing was negative, and his level of lactate dehydrogenase (LDH) was high, and unconjugated bilirubin was normal.

The bone marrow aspirate showed dysplastic changes in hematopoietic lineages including 2%blasts, megaloblastoid looking erythroblasts, erythroblasts in mitosis, erythroblasts with jolly bodies, micro megakaryocytes (<10%). Hemosiderin was present, but higher level in bone marrow, 45% sideroblasts with 30% ringed sideroblasts.

Cytogenetic exam: **46,XY [11], 43,X -7-10[1]; 43,X-7-10-11[1]; Fish exam for -7 was not done.**



Img. 2



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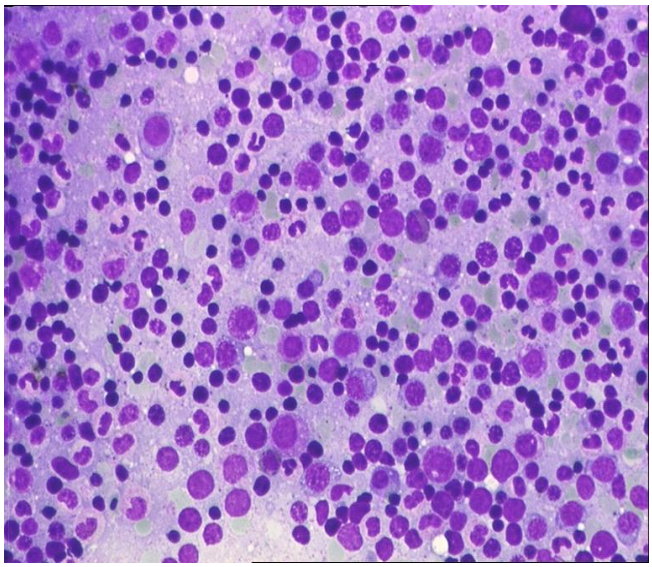
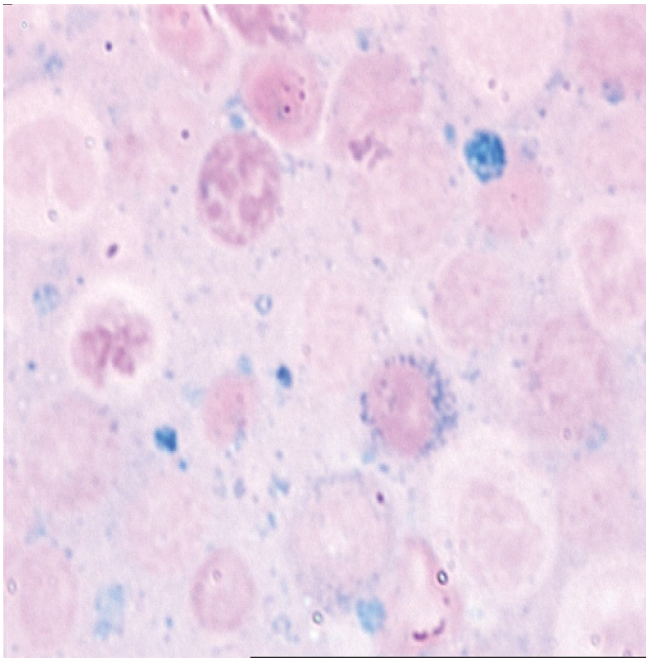
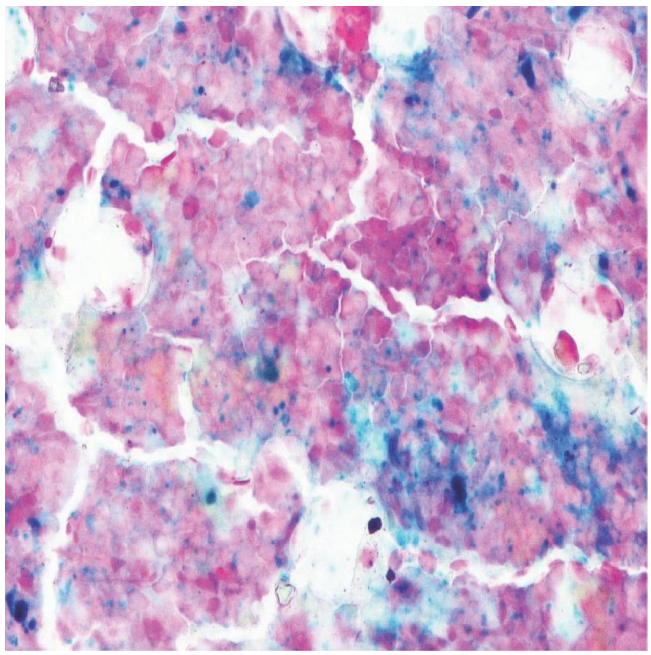


Img. 4.

MGG Stein (Img 1,2,3,4): Erithroblasts with irregular shaped nuclei, eritroblasts with 2 nuclei, hypercellular bone marrow, micromegakariocits.





Perls Stein: iron is deposited around the nucleus.

Differential Diagnosis:

Other causes of anemia: nonclonal: hemolysis, renal failure, B12 or folate deficiency, blood loss;

Other causes of myelodysplasia : HIV infection, alcoholism, recent cytotoxic therapy, severe intercurrent infections, etc..

Prognostic factors:

Diagnossis: RARS, IPSS score 1; risk lower (score intermediate 1) (poor karyotype, less than 5% blasts in the periphery, one ciytopenia, the mean survival of 3.5 years.

Goals:

1) Maintaining a good quality of life by increasing Hb levels;

2) Recombinant human erythropoietin to decrease transfusion requirements;

3) Substitution treatment - avoiding iron overload and adverse effects of iron burden.

Follow up of the patient:

Patient required transfusion support for symptomatic anemia and was red blood cell transfusion dependent from the begining.

Check ups at 6 months: complete blood count, biochemistry, ferritin level, urinalysis, bone marrow aspirate, karyotype, cardiac ultrasound, FEV, ophthalmological and ENT exam (for patients treated with deferasiroxum), abdominal ultrasound.

Year	01.2012	02.2012	06.2012 (6luni)	09.2013 (15luni)
Hb (g/dl)	4.7→ 9,3	5.0→8.1	7.5 → 8.9	6 →
Ferritin	Not done	835	**936**	1090
Iron seric level	165	169.8	159	182.5
Transfusion	2 MER	2MER	3MER	3MER
Epoetinum	**No** erythropoietin	Patient begins administration of erythropoietin: Epoietinum alfa 40000ui/ week	Epoietinum alfa 60000ui/ week	Deferasiroxum, for iron chelation:

3. Discussion

The patient presented is a typical case of MDS-RARS. An old patient with heart disease diagnosed with lower risk myelodysplastic syndrome.
Because of his age and his comorbidities, the patient was given packed red blood cells to maintain hemoglobin levels above 8g/dl, which proved to be sufficient to decrease symptoms .
The patient was transfusion dependent shortly after diagnosis, requesting 2-3 blood units/month. He had no response to recombinant human EPO at 6 months and 12 months , and the ferritin level increased shortly after diagnossis over 1000ng/ml.
Chelation therapy was needed after only 3 months after diagnosis, to avoid iron overload.
The patient had the presence of monosomy 7 that appeared in two metaphases, and is associated with poor prognosis.
The response to iron chelation was very good, and ferritin levels maintained under 1000 ng/ml, although patient continued to receive blood transfusions.

References:

Images are from Didona Vasilache MD, Laboratory of Citology and Morphology.
http://www.ncbi.nlm.nih.gov/pubmed/23796988 - Revised International Prognostic Scoring System (IPSS) predicts survival and leukemic evolution of myelodysplastic syndromes significantly better than IPSS and WHO Prognostic Scoring System: validation by the Gruppo Romano Mielodisplasie Italian Regional Database.
www.nccn.org -The National Comprehensive Cancer Network (NCCN) **http://www.qxmd.com/calculate-online/hematology/myelodysplastic-syndrome-prognosis-ipss**

THE 9th ROMANIAN CONGRESS OF CYTOMETRY
Bucharest, May 16-18, 2013

Abstracts

Dear collegues,

The Organizing Committee of the Romanian Association of Cytometry is glad to welcome you to the ***9th Romanian Congress of Cytometry*** *which will be held on May 16-18, 2013 in Bucharest. Cytometry has been strongly developed in recent years. It has become indispensable in many laboratories by the multiplication and the diversity of its applications. Mastering the concepts of this technique is now essential in basic or applied research as well as in routine diagnosis. However this is becoming increasingly difficult due to the complexity of the new cytometers and of new softwares. The cytometrists must now consider two important points: the standardization of their technique and the necessity of continuous training to enhance their knowledge and develop new skills as this technique is constantly changing. During these two and a half days the Romanian Congress of Cytometry will feature a rich scientific program with plenary lectures, poster sessions and round tables.*

An EuroFlow Workshop chaired by Alberto Orfao will focus on "Leukemia/Lymphoma case studies: analysis of FCS data files".

I hope that the meeting will be very interesting and you will enjoy the friendly atmosphere of our annual meeting.

I am looking forward seeing you in Bucharest.

Sincerely yours,

Pr. Lydia Campos - Laboratoire d'Hématologie, Hôpital Nord, CHU de Saint Etienne, France
Honorary President of Romanian Association of Cytometry

C1. **Exploring Primary Immunodeficiencies by flow cytometry**

Claude Lambert
Immunology Lab Univ Hospital Saint-Etienne, France

Flow cytometry (FCM) gives the possibility to explore a large number of cells individually, on quantitative level, with a very high precision using multiple parameters. This makes FCM of particular interest in exploring cellular primary immune deficiencies (PID). However, more than 180 different PID syndromes have been described so far and most of the time for only a very low number of patients. This may be why there is not much protocol developed to diagnose and identify PID using the most recent tools and even less any international standardization. The aim of this report is to review the most frequent PID and look for their respective immunological features that could make flowcytometry helpful in their diagnosis. We will focus on lymphocytes related PID.

Severe Combined immunodeficiencies (SCID) are usually induced by a mutation in a single gene involved is a crucial step of lymphocytes development. Nine mutations have been described that lead to total defect of one to three lineages according to the step concerned in lymphopoiesis.

The full immune response result of a number of intercellular cooperation using mediators that could also de genetically deleted or inactivated. More PID are then linked to the loss of one of these mediators including cytokines, cytokine receptors or coactivation molecules with as a consequence a defect in cell maturation.

Finally, the immune regulation also play a crucial role in the immune response, eliminating unadapted response (apoptosis through Fas family) or limitating the level of the reaction (through Fox p3 nuclear factor). This leads to PID with inadapted immune response (hypersensitivity, auto-immunity).

However there are still clinical cases of recurrent or opportunistic infections that do not correspond to these clearly identified cases and need to be first elucidated on a functional level in order to orient toward a genetic mutation. Theses explorations can include maturation, or functional tests. Proliferation tests were usually performed using radionucleotides far less reproducible and precise compared to what can be analysed using FCM but standardized tests are still in need.

In conclusion, PID is still a large field that needs to be explored through FCM. Patients at any age have repeated infection / immune disorders for which a primary dysfunction needs to be identified for more focused genetic analysis

C2. **BD Accuri™ C6: Flow Cytometry within Reach**
Julia Warneboldt
BD Biosciences, Heidelberg, Germany

The BD Accuri™ C6 is a personal flow cytometer that brings flow cytometry within reach by being easy to use, simple to maintain, and affordable. The analytical power and versatility of today's laser-based flow cytometry systems have unlocked the mysteries of cell biology and empowered entirely new fields of research.

As a result, flow cytometry has become a staple of modern laboratories around the world. Innovations in ease of use reflected in the BD Accuri C6 make these powerful capabilities more accessible to a new generation of flow cytometry users. The presentation will give you an overview of the unique features of the instrument and explains the advantages, the full power and the simplicity of the system.

C3. **AQUIOS: the first Load & Go Flow Cytometer to improve workflow process**
Andreas Boehmler
Beckman Coulter, Krefeld, Germany

Abstract not available

C4. **Cellular analysis by microcapillary flow technology**
Cornelia Roessler
Merck Millipore, Schwalbach, Germany

The Muse™ Cell Analyzer is a unique instrument based on microcapillary cytometry that greatly simplifies cytometric analysis by using a guided touch screen interface and dedicated software modules to provide quantitative cellular data. We will discuss the novel miniaturized flow technology utilized in the Muse™ Cell Analyzer, and also showcase data on the expanding range of optimized Muse™ assays available. A wide range of cell health assays allows for the rapid, single-step measurement of cell count and viability, provide information on cell cycle distribution and allow for characterization of apoptosis and death using Annexin V binding, caspase activity or mitochondrial depolarization. Immunology assays on the Muse™ platform allow for the identification and enumeration of CD4 T cells, CD8 T cells or B cells in whole blood or PBMC samples and also allow for obtaining information on activation status of lymphocytes based on CD69 or CD25 expression levels. Data from recent Cell Signalling assays on the platform will also be discussed. The combination of easy to perform assays with the simple, dynamic interface of the Muse™ Cell Analyzer can greatly empower researchers to obtain cytometric data with ease

C5. **Thermo Scientific Cyto-Cal beads - Instrument Independent Control for Flow Cytometry**
Arie van der Marel
Leusden, The Netherlands

Abstract not available

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Cornelia Roessler
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C5. **Thermo Scientific Cyto-Cal beads - Instrument Independent Control for Flow Cytometry**
Arie van der Marel
Leusden, The Netherlands

Abstract not available

C6. **Identification of subpopulations within the immature myeloid cells**
Lydia Campos[1], Carmen Mariana Aanei[1],
Denis Guyotat[2]
(1) Laboratoire d'Hématologie, Hôpital Nord, CHU Saint Etienne, France
(2) Département d'Hématologie, Institut Cancérologie Lucien Neuwirth, Saint Etienne, France

Hematopoietic stem cells (HSCs) are a very primitive and rare population capable of self-renew,

extensive proliferation, and multilineage differentiation into myeloid cells. The strategies for identifying normal stem cells include flow cytometry using cell surface markers such CD34, CD133, CD38 and functional approaches including SP analysis, Aldefluor assay. The enriched and sorted population is further tested for the capacity of lymphomyeloid engraftment into immunodeficient mice.

Because of the facility to access hematopoietic tissue and the development of functional assays to measure their activity, the HSC is perhaps the most well-characterized stem cell on both the biological and molecular levels. However, HSC studies remain quite difficult because of their rarity within the total population of hematopoietic cells (<1 in 50 000).

The stem cell population is not only rare but also heterogeneous containing a mixture of true stem cells and more mature progenitors. Primitive stem cells expressed surface markers which help to their characterization. The HSC phenotypic has being studied by different authors who propose that the HSC is Lin-CD34+CD38-CD45RA-. The CD34+CD38- defines a quiescent subpopulation in adult bone marrow that can be distinguished functionally from the CD34+CD38+ population by the capacity of repopulating the bone marrow in NOD/SCID mice.

The Lin−CD38−CD45RA− population is composed of a stem/progenitor cell pool in which several subsets of cells preferentially expressing surface markers, such as CD34 and/or CD90, are hierarchically organized. The Lin−CD34+CD38−CD45RA−CD90+ subpopulation has only ~5% cells possessing long-term hematopoiesis-reconstituting activity compared to ~1% Lin−CD34+CD38−CD45RA−CD90− cells having such activity. The HSC activity can be also enriched using the.
Lin−CD34+CD38−CD45RA−CD90+CD49f+ marker profile with 9.5% cells possessing the long-term repopulating property compared to 0.9%
Lin−CD34+CD38−CD45RA−CD90+CD49f− cells having such activity. The HSC activity can be further enriched using the 7-marker Lin− CD34+ CD38− CD45RA− CD90+ Rholo CD49f+ profile with 28% bone marrow marker-sorted cells possessing the long-term repopulating property in NOD-scid-IL2Rγc−/− or NSG mice. Janssen et al found that normal stem cells have the CD34/CD45 low FSC/ SSC low CD38- CD90 low-HLA-DR-CD7-CD11b-CD56- profile.
Considerable progress has been made studying HSC function, based on the high efflux of fluorescent dyes. The ABCB1 gene product P-glycoprotein (Pgp) is expressed in many HSC, which are mainly in the CD34+ compartment.

Increased dye efflux in HSC is associated with long-term repopulating ability, and the highest levels of Pgp expression are found in pluripotent. ABCG2 transporters are also expressed in CD34 negative cells, and they are assimilated as Side Population (SP).

Th e significance of CD34 negative HSC for human bone marrow repopulation has not yet been established. Most primitive progenitors reside within the G0/G1 phase of the cell cycle. The repopulating activity of HSC decreases from G0 to the G1 phase following stimulation; stem cells lose engraftment potential and do not reenter G0. Recent data have suggested that human CD34 negative HSCs exist, challenging the concept that HSCs necessarily and exclusively express the CD34 antigen. The Hoechst 33342 dye efflux property identifies a distinct side population. SP cells are mostly CD34 negative, highly enriched for long-term repopulating cells, and durably engraft in sub-lethally irradiated non-obese diabetic/severe combined immunodeficient mice.

The objective of our study is to evaluate the immature myeloid cells in the CD34 positive compartment.

Material and methods: We have studied normal bone marrow (healthy donors), regenerating marrows from acute myeloid leukemia (AML) patients.
Flow cytometry and gating strategy: We have gated the CD34 population, by intersection of two gates SSC low CD34+ and CD45 low CD34+. Thereafter the different subpopulations were gated using the following markers: CD38, CD90, CD133, CD123, HLA-DR, and CD117.
Analysis was performed with a CantoII BD cytometer and analysis with Infinicyt software (Cytognos).

Results: We have defined 5 different steps of maturation in the CD34+ compartment that are different between normal and CR AML bone marrow.

Conclusion: The five stages identified in the CD34 compartment allow drawing a maturation profile. A largest number of normal bone marrows must be evaluated in order to define the normal phenotype, which could be useful to interpret the aberrant phenotypes with impact on AML-minimal residual disease evaluation.

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C7. **Minimal residual disease analysis – clinical impact in hematology**

Horia Bumbea
„Carol Davila" University of Medicine and Pharmacy Bucharest, Romania,
Department of Hematology, Emergency Universitary Hospital, Bucharest, Romania

Background. Target therapy in hematological malignancies is eliminating malignant hematopoietic cells.

Morphological analysis was classically used to search for residual malignant cells, but this technique was known to have a very low sensitivity and specificity. Standard criteria used were: lack of blast cells per field groups or aspirated, and less than 5% blasts in 200-500 cells.

Methods currently used are based on high performance techniques:
flow cytometry
Cytogenetics (FISH)
PCR techniques

Flow cytometry in MRD analysis has several advantages: it can be used in over 90% of ALL, 30-80% of AML, has strong correlation with relapse, especially when clone frequency is 0.01-5%, and the sensitivity can be increased by the number of cells analyzed. But also disadvantages: subjective phenotypic criteria of malignancy, phenotypic instability, overlap with normal.

Cytogenetics (FISH) in MRD analysis has the following advantages: it is more specific if there are changes and cell culture stage selects viable tumor and disadvantages: it requires the presence of significant structural aberrations and is less sensitive as other methods.

PCR has the advantages of being the most sensitive (0.0001%) and highly specific and being an automatic method leads to less subjectivity, but it has disadvantages: it requires a large panel of specific primers spanning more than 50% AML / ALL, coverage for ALL can be extended, but it requires synthesis of specific primers for the patient.

Analysis of minimal residual disease (MRD) is primarily used in clinical trials after chemotherapy to assess the duration and intensity of treatment, the time of stem cell transplantation and remission follow-up.

Analysis of MRD in precursor B ALL is extremely important and standardized: immunophenotypic criteria that allow differentiation of the hematogenic precursors. Published studies clearly show the superiority of survival for patients with negative MRD compared with MRD negative 0.1% and with MRD negative 0.01% at the end of induction.

MRD in AML by immunophenotyping analysis allows the approach of all cases, and is addressed to at least 50% of cases in which molecular aberrations are not detected. The principle is to identify aberrant immunophenotype precursors (LAIP). Also MRD detection by flow cytometry has been reported in several studies to be extremely useful in decision making regarding secondary induction and stem cell transplantation.

MRD analysis in patients with stem cell transplantation has superior sensitivity and specificity for chimerism detection, therefore immunophenotyping analysis is becoming the method of choice in these cases.

MRD analysis in chronic lymphoproliferative disorders is generally used in clinical trials, although standardized protocols were developed in some lymphoproliferative disorders, such as chronic lymphocytic leukemia, multiple myeloma. Studies have shown superior survival of MRD negative cases. Decision making regarding stem cell transplantation is a very useful application of MRD analysis, particularly in multiple myeloma, where immunophenotyping detection has proved to be superior to molecular techniques.

In conclusion, the analysis of minimal residual disease in hematologic malignancies is required by one or more methods, and multiparametric immunophenotyping by flow cytometry is a rapid, sensitive and specific, which has applications in almost all hematological malignancies.

C8. **The contribution of multiparametric flow cytometry to the detection of distinct malignant clones in the same leukemia/ lymphoma cases – six case studies (2012 - 2013)**

Mihaela Zlei[1], Georgiana Grigore[1,2], Iuliu Cristian Ivanov[1,2], Daniela Constantinescu[2], Angela Dăscalescu[1,2], Cătălin Dănăila[1,2], Cristina Burcoveanu[1], Eugen Carasevici[1]
1 Regional Institute of Oncology, Laboratory of Molecular Biology, Iași, Romania
2 University of Medicine and Pharmacy Grigore T. Popa of Iași, Romania

Introduction. The presence of more than one population of malignant cells identified in the same patient has been thought to occur exceptionally rarely and to be specific for bilineage or biclonal hematological neoplasias. These cells may be either of different lineages (bilineage) or of different clonality (biclonal), while sharing the same lineage markers. Aim. We report here six different cases with multiple malignant clones recently identified by multiparametric flow cytometry in our department.

Materials and methods. Bone marrow (BM, n=1) and peripheral blood (PB, n=5) samples from six patients with suspicion of hematological malignancies (five suspicions of chronic lympho-proliferative disorders and one suspicion of acute leukemia) were investigated by flow cytometry using a FACSCantoII (Becton Dickinson) cytometer and combinations of up to 6 colors. All patients were enrolled within the Hematology Department of Regional Institute of Oncology, Iasi, Romania in 2012-2013.

Results.

Case 1: (CL, F, 71 y); PB (500 000 cells/ µL) - 91% T lymphocytes (CD45+ CD3+ CD4- CD8+ CD2+ CD5+ CD7+high (atipic) CD45RA+ CD45RO- TCRab+ CD56+ CD25- HLA/DR+/-) + 1,2% (6000/ µL) monoclonal B lymphocytes (CD45+ CD19+ CD5- CD10- CD22+ CD20+ CD103- FMC.7+ CD23- CD38- CD43- kappa+ lambda- IgM+ IgD+ IgG-).

Case 2: (SA, F, 91 y); PB (30 400 celule/ µl) – 42% B-CLL lymphocytes (CD45+ CD19+ CD5+ CD10- CD22+low CD23+ FMC7- CD43+ CD103- CD25+low CD11c- CD38- IgM+ IgD+ IgG-.) with a kappa+/ lambda+ ratio of 1,3, which suggests the presence of two distinct CLL clones.

Case 3: (BA, M, 64 y); BM, low cell count (4 400 cells/ L) – 22% HCL B cells (lambda restricted), + 5% B lymphocytes with a kappa monoclonal excess (kappa+/lambda+ ratio = 5,2).

Case 4: (UE, F, 72 y), PB (7 000 cells/ l) - 8,4% monoclonal B cells (CD45+ CD19+ CD5- CD10- CD20+high CD22+ high CD23- FMC7+ CD43- CD103+low CD25+low CD38- IgM+ high IgG-kappa+high lambda-) + 4% limfocite B CD45+ CD19+ CD5+/- CD10- CD20+int CD22+ CD23+ FMC7- CD43+ CD103- CD25- CD38- IgM+int, IgG-) with a lambda monoclonal excess (kappa+/lambda+ ratio = 0,4).

Case 5: (LA, F, 60 y), PB (90 000 cells/ L) - 70% monoclonal B CLL cells (CD45+ CD19+ CD5+ CD10- CD23+ CD20+low CD22+low FMC7- CD38- CD43+ IgM+ IgD+ IgG- kappa+low lambda-) + 6% monoclonal, imature/ prenaive B cells (CD19+ CD5+ CD20+ CD22+low HLA/DR+high CD38+high kappa+).

Case 6: (SAD, F, 3 months), PB (47 000 cells/L) - 81% monocytoid cells with aberant phenotype + -5,4% B cell precursors (CD45+low CD19+ CD34+ CD10- CD20+low CD22+int CD58+ CD38+ HLA/DR+ CD79a+ TdT+ IgM(s+ic)-.

Conclusions. The most frequent explanation for the co-occurrence of these apparently distinct malignant populations was attributed to the malignant transformation of a common precursor with potential to differentiate into any of the two clones/ lineages. While the prognostic significance of these pathologies are rather unpredictable, cases presented here are intended to offer an image on the major contribution of multiparametric flow cytometry for their accurate diagnosis.

C9. **The BD Accuri™ C6 flow cytometer and BD™ Cytometric Bead Array reagent sets: A powerful flow cytometry toolset for functional cell analysis**

Matthias Engele
BD Bioscience, Heidelberg, Germany

With BD™ Cytometric Bead Array (CBA) reagents, the BD Accuri C6 flow cytometer, and FCAP Array™ analysis software, users can quantify multiple proteins simultaneously, using the broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads. This method significantly reduces sample volume requirements and time to results in comparison with traditional ELISA and Western blot techniques. BD CBA Kits take advantage of the standard BD Accuri C6 configuration. The optional Selectable Laser Module available for the BD Accuri C6 introduces flexibility, enabling detection of two parameters from the red laser, a requirement for BD CBA Flex Sets.

C10. **Contribution of flow cytometry to the selection**

of new antimicrobials

Carmen Chifiriuc[1], Coralia Bleotu[2],
Luminița Mărutescu[1], Veronica Lazăr[1]
1. University of Bucharest, Faculty of Biology, Bucharest, Romania
2. Virology Institute Ștefan S. Nicolau, Bucharest, Romania

Infectious diseases remain as a major world health problem due to the rapid development of resistance to the existing antimicrobial drugs, by different mechanisms, either encoded by different genetic elements or by phenotypic or behavioral changes occurred when bacteria and fungi are developed in monospecific or polyspecific biofilms. The development of new antimicrobial drugs through the discovery of new drug classes as well as exploring possible new drugs from existing classes of antibiotics is presently encouraged. For this purpose, different synthetic or natural compounds are screened in vitro using different standard assays and the search for compounds with new mechanisms of action, inhibiting either planktonic or biofilm associated cells is desired, in order to enlarge the time interval between the release of a new antibiotic on the marker and the moment of the emergence of resistance mechanisms. This oral presentation will discuss the contribution of flow cytometry method to the development of the in vitro methodology for the selection of new antimicrobials.

C11. **Aspects of flow cytometryc characterization of platelet derived microparticles**

Andra Costache[1], Doina Barbu[2], Florentina Grădinaru[2]
Anca Roxana Lupu[1,2]
1 "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
2 "Colțea" Clinical Hospital, Hematology Department, Bucharest, Romania

Microparticles represent submicrometer fragments resulting from the remodeling of the plasma membrane in response to numerous conditions including activation and apoptosis. All cell types shed microparticles, therefore they are present in various body fluids. Platelet derived microparticles are, by far, the most abundant in the circulation, constituting between 70% and 90% of the total number of microparticles.

The level of microparticles increases in various diseases and have been proposed to play various roles in many biologic processes such as blood coagulation, inflammation, atherogenesis, angiogenesis, cancer metastasis, possesing pathophysiologic significance. Therefore, the evaluation of microparticles may represent a possible investigation and diagnostic tool.

Flow cytometry remains the most widely used method for microparticles analysis in clinical samples, although because of their small size and increased sensitivity to pre-analytical conditions, their enumeration and characterization is challenging.

C12. **Effect of flavonoids on cell cycle and apoptosis in tumor cell lines overexpressing ErbB proteins**

Maria-Magdalena Mocanu[1], Eva Katona[1],
Mihaela Surcel[3], Laura Georgescu[1], Timea Varadi[2],
Irina Baran[1], Peter Nagy[2], Cornel Ursaciuc[3],
Janos Szöllősi[2], Constanta Ganea[1]
1"Carol Davila" University of Medicine and Pharmacy, Faculty of Medicine, Department of Biophysics, Bucharest, Romania
2Department of Biophysics and Cell Biology, University of Debrecen, Debrecen, Hungary
3"Victor Babeș" National Institute of Pathology, Department of Immunology, Bucharest, Romania.

Introduction: ErbB proteins are a family of receptor tyrozin kinases and their overexpression was frequently associated with poor prognosis in cancer. Epigallocatechin-3-O-gallate (EGCG) and genistein are small flavonoid molecules found in green tea leaves (Camellia sinensis) and respectively, soy plants. Their anti-proliferative, anti-metastatic and pro-apoptotic effects were correlated to chemoprevention and therapy of various types of cancer. The aim of this study was to investigate the effect of EGCG and genistein on cell proliferation, cell cycle and apoptosis in case of mammary and epidermoid cancer cell lines which overexpressed ErbB proteins. Methods: SK-BR-3, mammary cancer cell line which overexpress ErbB2 protein and A-431, epidermoid cancer cell line which overexpress ErbB1 protein were grown in standard conditions. Spectrophotometry for 96 well plates (WST-1 kit) was applied in case of IC50 evaluation. Cell cycle progression (propidium iodide staining) and apoptosis (Annexin V-FITC/ 7-AAD staining) were visualized by flow cytometry. The treatments were carried out in complete medium for different concentrations of the flavonoids after the starvation in medium with 0.1% FBS. Results: Both cell lines were more sensitive to EGCG compared to genistein, since they displayed a pronounced resistance to genistein according to IC50 values higher than 100 μM. The effects on the cell cycle progression were evident after 24 h of treatment in case of genistein, pointing out the arrest of the tumor cells in G2/M phase. EGCG was more effective in driving the tumor cells overexpressing ErbB proteins to late apoptosis compared to genistein. Conclusions: Our data support the anti-proliferative and pro-apoptotic effects of small flavonoid molecules on breast and epidermoid cancer cell lines with higher expression of ErbB proteins. Acknowledgments: This work was supported

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C13. **Flow Cytometry Detection of Ca2+ Overload in Normal and Treated Gingival Fibroblasts**

Ancuţa Goriuc[1,3], Eugen Carasevici[1], Marcel Costuleanu[2]
1. Laboratory of Molecular Biology, Regional Oncology Institute Iaşi
2. Department of General and oro-maxillo-facial pathology, Faculty of Dental Medicine, University of Medicine and Pharmacy „Gr. T. Popa" Iaşi
3. Departament of General and Oral Biochemistry, Faculty of Dental Medicine, University of Medicine and Pharmacy „Gr. T. Popa" Iaşi

Introduction: Ca2+ is the key body regulating factor not only for various cytosolic enzymes, but also for multiple metabolic pathways developing in the intracellular organelles. Several data suggest that, apoptosis plays an important role in gingival overgrowth controlling and that involves a cascade of biochemical steps that require an increase of intracellular Ca2+. Initiation phase of apoptosis, including apoptosis induced by Ca2+ overload is represented by opening of mitochondrial permeability transition pore (MPTP).

Materials and Methods: To obtain fibroblasts we used untreated gum rat and explant technique. Fibroblasts thus obtained were grown on specific culture media with and without cyclosporine A (1µg/ml). After about 14 days we followed mitochondrial permeability transition pore functioning by flow cytometry and calcein-AM as a marker for opening of this pore (MPTP). For this, some control fibroblasts and treated with CsA in culture medium were subjected to the action of CaCl2 (1 mM) and calcium ionophore like ionomycine (1 mM) and A23187 (10 µM).

Results and discussion: Statistical analysis of normal and treated fibroblasts under the action of Ca2+ ionophore A23187 and ionomycine showed a significant difference for using the ionophore A23187. Fibroblasts treated before with CsA in culture medium has a strong dissipation of mitochondrial membrane potential under the action of this ionophore. For ionomycine, statistical analysis did not show significant differences for opening of MPTP any normal fibroblasts or those treated with CsA.

Conclusions: Mitochondrial permeability transition pore opening under Ca2+ overload was observed using ionophore A23187 both in normal fibroblasts and especially in those treated with CsA in culture medium.

On the other side, Ionomycine, hasn't significant effects on mitochondrial calcein load in normal or treated fibroblasts.

C14. **Flow cytometry standardization and accreditation: current problems and future perspectives**

Katherina Psarra
Dept of Immunology–Histocompatibility, Evangelismos Hospital, Athens, Greece, President of the Hellenic Cytometry Society

Accreditation according to EN ISO 15189 leads to the implementation of elements of an integrated system of quality management in clinical laboratories. Accreditation is put into practice by national accreditation bodies that have national monopoly. Clinical flow cytometry labs present special problems during the accreditation process in most steps of the quality cycle, including quality assurance, assay validation and regulatory compliance

Quality assurance in flow cytometry consists of standardized processes and practices, assays validation, instruments maintenance, monitoring and finally improvement. Standardized processes in flow cytometry are very few. There is an international effort for more standardization, but at the moment it seems really difficult with the exception of immunologically defined lymphocyte subpopulations and CD34+ hematopoietic stem cells counting, for which validated IVD CE assays are provided by the manufacturers. Regarding other flow cytometry assays and most importantly hematological malignancies immunophenotyping (of the main applications in clinical laboratories throughout the world) great variability is found even within the borders of one country, because of instruments, reagents and "possibilities" diversity. In fact harmonization and not standardization seems the most feasible goal at this moment.

Guidelines by scientific societies such as ESCCA may help towards homogenization of panels and protocols. ESCCA accreditation committee has prepared a paper in order to inform the flow cytometry community about the situation in several European Countries regarding acceditation, external quality assessment, and education requirements of people working in flow cytometry labs. This paper was accepted for publication in Cytometry. Another one concerning guidelines and ESCCA opinion on accreditation is under preparation. It will be really helpful for lab personnel as well as for inspectors and experts working for national accreditation bodies to have guidelines, because it is difficult to implement

accreditation rules prepared mainly for automatic validated analysers in flow cytometry procedures. In addition ESCCA regards basic as well as advanced and continuous education as key elements of any accreditation process and is already organising and helping national societies in organising all levels of education leading possibly to a certification for flow cytometrists in several aspects of involvement in flow cytometry work.

A pivotal and integral part of accreditation is the external proficiency testing schemes. These schemes for flow cyometry, provided mainly by UK NEQAS in Europe, use fixed samples unlike the everyday samples of our labs, so they cannot be treated in the same way routine samples are treated, an important rule of accreditation. In addition there are not EPT schemes for all flow cytometry assays. Some countries have their own EPT schemes, using fresh samples similar to everyday routine ones with very good results.

It is well known that accreditation is not yet obligatory in most European countries, but it is really desirable. We are all hoping to be able to proceed to acquire accreditation for as many as possible flow cytometry assays for the patients and flow cytometrists benefit and for better communication and understanding between labs in Europe and all over the world.

C15. **Standardization of diagnostic protocols by immunophenotyping in the working groups of Romanian Society of Hematology**

Horia Bumbea
„Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
Department of Hematology, Emergency Universitary Hospital, Bucharest, Romania

Diagnosis by immunophenotyping is a current method of diagnosis in hematologic diseases. Working groups within the Romanian Society of Hematology (RSH) aims addressing diagnosis and treatment of hematological diseases and develop protocols for the management of these disorders Romania.

Standardization of protocols involved standardization of diagnosis by immunophenotyping in various biological products (peripheral blood, bone marrow aspirate, serous, cerebrospinal fluid).

Are currently proposed minimal diagnosis and monitoring protocols as recommended by the European Leukemia Net (ELN) adopted by the Romanian Association of Cytometry (RAC) group standards that are applicable especially in the diagnosis of acute leukemia and chronic lymphoproliferation.

In CLL is generally applied standardized diagnosis protocols recommended by ESMO and IWCLL, even with standardized MRD analysis.

In the working group of monoclonal gamapathias are proposed EMN protocols for diagnosis and monitoring.

Procedures for developing protocols SRH in diagnosis and treatment are necessary for harmonized of hematologic therapeutic approach to these diseases and standardization of these protocols enable them to impose the recommended practice of RSH.

C16. **Validation and quality assurance in multiparametric clinical flow cytometry**

Mihaela Zlei
Regional Institute of Oncology, Laboratory of Molecular Biology, Iași, Romania

The aim of this presentation is to deliver and discuss guidelines for laboratory's validation and quality control documentation in polychromatic clinical flow cytometry, particularly in hematopathology. Based on regulatory issues as they apply in Romania, all flow cytometry units country-wide are responsible for designing and maintaining their own laboratory performance standards. Therefore, each laboratory should validate their own panels for sensitivity, specificity, and correlation with morphology and clinical findings, based on documentation for instrument validation, QC, QA, and troubleshooting, sample handling, preparation, processing, data analysis and final interpretation.

Regardless the source of guidelines, all should include the following items: all laboratories must be enrolled in a proficiency testing (external control scheme)

-establish specimen requirements, recommended transport conditions and criteria for acceptability/ rejection

-assess the analytical accuracy (by comparison to previously characterized cells, morphology, cytogenetics and molecular biology; each laboratory should establish its own expected rate of discrepancy between flow and morphology)

-asses the qualitative staining pattern used to identify cell lineages, descriptions of dim, moderate, and bright staining patterns

-assess the specificity of monoclonal reagents, by how well the antibody recognizes the correct antigenic target

-assess the reagent sensitivity defined by the minimum staining intensity above non-specific or negative staining

-determine the instrument precision, accuracy and sensitivity of the instrument and validate the instrument performance by instrument setup and daily qualification of both light scatter and fluorescence measurements, and cross-instrument performance using relevant clinical specimens

-validate the panel (antibody combinations and proper compensations)
-validate their own strategies and tools for data analysis and interpretation

There is no world-wide consistency in terms of how the integration of this technology into diagnostic hematopathology should be applied in a unitary model, although multinational organizations, especially the Euroflow consortium, are currently involved in creating a certain level of uniformity.

C17. **Implementation of EuroFlow Protocol in the immunophenotypic diagnosis of the hemopathias in Romania**

Mihaela Baica[1], Mihaela Zlei[2], Adriana Dumitrescu[3]
1„Louis Turcanu" Hospital, Timisoara, Romania
2 Regional Institute of Oncology, Iași, Romania
3 Fundeni Clinical Institute, Bucuresti, Romania

In 2005 a large group of specialists with experience in flow cytometry and molecular diagnosis initiated the EuroFlow project due to the need for a new approach in hematological malignancies immunophenotyping. Work performed within six years has materialized in a standard operating protocol that allows diagnosis and monitoring of these diseases. Practically, the goals achieved are: 1. development of new software based on a new concept to allow fast and easy handling of large data sets and for integration in a single data file; 2. development of standardized 8-color antibody panels for fast and easy flow cytometric diagnosis and classification of hematological malignancies as well as for sensitive monitoring of patients and for evaluation of treatment effectiveness; 3. development of assays for detection of fusion proteins to be used for characterization of acute leukemia; 4. development of software for automated recognition of normal, reactive, and malignant leukocyte populations in blood and bone marrow and 5. creating of a large data base with hundred sets of normal, reactive and malignant cells, which can be used as ready-to-use template for fully automated comparison with the new cases sets of cells. EuroFlow protocol availability will make possible hematological malignancies immunophenotyping standardization with direct consequences on diagnosis, treatment and monitoring of these diseases. EuroFlow protocols implementation in all flow cytometry departments from our country that are working in onco-hematology diagnosis field is impossible for the moment because of the higher costs required. Through the perspective of cost /efficiency it is possible to conceive a unified strategy in the country based on the current situation. In a first step EuroFlow protocol could be implemented in some pilot centers – thus a high proportion of leukemia and lymphoma will be diagnosed according to standard procedures. Functioning to the appropriate parameters of these laboratories involve to ensure the equipment (BD FACS Canto II cytometers with three lasers and the rhythmic supply with reagents) as well as human resources (at least two physicians/biologists and two technicians in each laboratory to make possible a continuous activity) well trained. Centers location on the map must ensure a balanced coverage of our country. Practical implementation requires a close collaboration between all the involved factors: cytometry laboratories and onco-hematology clinics (for adults and children), working groups of the scientific societies (Romanian Hematology Society and Romanian Cytometry Association), ministry of Health. It is a problem that can be solved only in a centralized manner. Romanian cytometrists involved in hematological malignancies diagnosis hope to benefit and in the future from scientific support provided with generosity by professor Alberto Orfao, vice-coordinator of EuroFlow Consortium, attending to our cytometry congresses since eight years.

C18. **The role of flow cytometry in the characterization of monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia**

Julia Almeida[1], Wendy Nieto[1],
Arancha Rodríguez Caballero[1], Cristina Teodosio[1], Santiago Muñoz Criado[2], Alfonso Romero[3],
Paulino Fernández Navarro[4], Ignacio Criado[1],
Alberto Orfao[1], y el Grupo de Estudio de la "Linfocitosis B Monoclonal" de Atención Primaria de Salud de Salamanca
1Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer/IBMCC (CSIC-USAL), IBSAL, Departamento de Medicina and Servicio de Citometría, Universidad de Salamanca, Salamanca.
2Servicio de Microbiología, Hospital Universitario de Salamanca, Salamanca
3Centro de Atención Primaria de Salud Miguel Armijo Salamanca, Sanidad de Castilla y León (SACYL), Castilla y León
4 Centro de Atención Primaria de Salud de Ledesma, Salamanca, Sanidad de Castilla y León (SACYL), Castilla y León

For decades now, the diagnosis of B-cell chronic lymphoproliferative disorders (BCLPD) has mainly relied on the presence of symptoms and signs characteristic of the disease. However, in the 80's this situation has changed since the diagnosis of some leukemic BCLPD cases, particularly of chronic lymphocytic leukemia (CLL), more and more frequently started to be established on the basis of a

clonal lymphocytosis detected in a routine blood analysis. This placed the diagnosis of a significant fraction of these disorders, particularly of CLL, in an asymptomatic phase of the disease prior to the emergence of characteristic clinical symptoms. In turn, this has also lead to a significant reduction of treatment requirement at diagnosis in a significant proportion of cases. In addition, the diagnosis of the disease became much more precise, the immunophenotypic analysis of tumor B-cells becoming an essential part of the diagnostic information required, in addition to conventional cytomorphology, histopathology, cytogenetics and molecular data (1).

From the 90's on, the availability of multiparameter flow cytometry techniques of an increasingly higher sensitivity, facilitated the identification in peripheral blood samples from asymptomatic subjects of cells which phenotypically resemble those of CLL patients, although such cells were frequently present in the blood of these individuals at very low frequencies, such as 1 cell in 10,000 leucocytes. In recent years, the sensitivity of multiparameter flow cytometry has even increased more to reach the detection of <100/mL, i healthy adult subjects. Because of the benign character of most of such clones, this situation was given the name of Monoclonal B-cell Lymphocytosis or MBL, allowing its distinction from the typical BCLPD disease categories (2). Therefore, MBL is currently defined by the presence of small populations of clonal B-lymphocytes (<5x109/L) in peripheral blood of otherwise healthy adults; in most cases MBL clones are phenotypically similar to those of CLL patients (CLL-like MBL).

Early studies investigated the presence of MBL is those individuals presenting with lymphocytosis; however, such studies also rapidly demonstrated that similar clones could also be detected at lower numbers in adults presenting without lymphocytosis, among the general population (3-5). More recent studies (3-5), including our own series (6), have shown that the frequency of such CLL-like MBL clones in adults over 40 years of age is rather high, ranging between 3.5% and 12% of the general population over 40 years, depending on the sensitivity of the technique used for their investigation and detection. Such prevalence increases with increasing age (3-6) and it could even reach frequencies close to 100% among those individuals from the general population who are older than 70 years, if a relatively large blood volume would be screened (e.g. > 50mL of peripheral blood). These latter findings suggest that in such cases, the MBL cells could more likely represent the normal counterpart of CLL cells, than a real leukemic precursor cell (7). Despite this, it should be noted that clonal CLL-like MBL cells from the general population, may already display genetic alterations which are characteristic of CLL cells -e.g. del(13q)- as revealed by interphase FISH analysis of highly-purified MBL cells, although such cytogenetic alterations are found at lower frequencies than those described among CLL patients at the same time they are restricted to good-prognosis cytogenetic changes (6). In contrast, CLL-like MBL cells from the general population typically show a IgH(VDJ) BCR repertoire which differs significantly from that of CLL patients (8). Furthermore, recent results from our group suggest that some specific cytogenetic/molecular profiles are associated with specific IGVH BCR repertoires in MBL cases with low vs high lymphocyte counts as well as CLL (Henriques et al, submitted for publication).

Independent of the potential practical interest of these findings which derive from the demonstration of the high prevalence of MBL clones in the general population and the availability of a flow cytometry tool for the early diagnosis of the disease, this brings us close the the onset of the disease (e.g. CLL). In this regard, an increasing number of publications have addressed the potential of MBL cases to undergo malignant transformation to e.g. CLL. Two recent papers support this hypothesis by showing that: (i) almost 100% CLL cases are preceded by an MBL (9), and; (ii) the rate of transformation of MBL (with lymphocytosis) to CLL is of around 1% per year (4). Currently, the rate of progression of low count MBL from the general population to high-count MBL and CLL remains unknown; despite this, in our series based on the general population of Salamanca (Spain) after 5 years of follow-up, we could not see disappearance of the MBL clone in any of the cases analysed, at the same time no case showed progression to a high-count MBL or CLL.

Based on the observation that almost all CLL cases are preceded by an MBL (9), raises the possibility that a better understanding and knowledge of those events that favour the development and emergence of MBL clones in otherwise healthy adults could contribute to the understanding of the ontogeny of BCLPD ingeneral, and CLL in particular. Most recent hypothesis suggest that chronic and maintained stimulation of the immune system could be a basic determinant of the B-cell expansion observed in these MBL subjects, as a first step in the development of symptomatic disease. In line with this hypothesis, we have recently investigated the potential risk factors associated with the presence of MBL clones in the general population through an epidemiological study. Accordingly, we found that the frequency of MBL was two-times lower among those individuals who had been vaccinated against neumococcus and influenza, vs. non-vaccinated subjects of the same age and geographical area. In addition cases with MBL referred more frequently having suffered from pneumonia, meningitis and flu

compared to non-MBL controls. These findings were further supported by a greater frequency of respiratory infeccions among the children of MBL vs. non-MBL cases. Therefore, our results suggest that chronic exposure to infection could be on the basis of the development of MBL in the general population and potentially also among high-count MBL and CLL (10). Further studies are now ongoing to try to dissect the specific factors and antigenic stimuli involved in the ontogeny and transformation of MBL to BCLPD.
In this presentation we will focus on the flow cytometry approaches and techniques used to: 1) detect MBL clones, 2) characterize them phenotypically, as well as 3) on genetic/molecular grounds, and 4) investigate the role of chronic antigen stimulation on the development of MBL.

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C19. **Applicability of ESMO guidelines in diagnosis and monitoring CLL in Romania**

Horia Bumbea
„Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
Department of Hematology, Emergency Universitary Hospital, Bucharest, Romania
On behalf of Roche Romania

Chronic lymphocytic leukemia is the most common form of leukemia, with an incidence that increases exponentially after age 80 years.

Diagnosis LLC is now directly related to immunophenotyping by flowcytometry method, and has been continuously improved.

ESMO criteria are commonly used in clinical practice of CLL management. Thus, detection in peripheral blood of clonal B cells with specific immunophenotype CD19+ CD5+ CD23+ CD43+/- CD79b+ CD20 low and restriction of immunoglobulin light chains is a diagnostic criterion, with the threshold over 5000/mmc.

It is important to note that biopsy is not required for diagnosis, so use immunophenotypic criteria are the primary means of diagnosis.

Also flow cytometry is the only method to identify cases of benign monoclonal lymphocytosis (MBL).

Prognosis analysis allows identification of immunophenotypic markers of prognosis, and in ESMO recommendations are mentioned the analyses of CD38 and ZAP-70 expression, which is undergoing standardization by ERIC group.

Analysis of minimal residual disease has proven prognostic impact in CLL, on standardized method 4 colors.

These recommendations were used in current diagnosis of CLL in Romania by analysis of several smples from patients from centers around the country, and allowed the correct identification of cases of CLL and exclusion of other chronic lymphoproliferative disorders, or even identifying of cases of MBL.

In conclusion, ESMO guidelines establish clear guidelines for diagnosis and treatment in CLL, and immunophenotyping by flowcytometry analysis is the main method used.

C20. **Therapy effects over normal residual immune cells in B-cell chronic lymphoid leukemia patients**

Georgiana Emilia Grigore[1,2], Susana Barrena[1],

Martín Pérez-Andrés[1], Rosa Ana Rivas[1],
Julia Almeida[1], Alberto Orfao[1]
1. Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer/IBMCC (CSIC-USAL), IBSAL, Departamento de Medicina and Servicio de Citometría, Universidad de Salamanca, Salamanca, Spain; 2"Gr. T. Popa" University of Medicine and Pharmacy & Regional Institute of Oncology, Iasi, Romania.

Introduction: Advanced stages B-CLL associates with important defects in both humoral and cellular mediated immune responses, that can be worsen by treatment. The clinical course and therapy response are modulated not only by the heterogenous molecular features of the clonal expanded B cells, but also by the normal immune cells that support/fight the leukemic clone. Although we know the benefits of combined therapies targeting the malignant clone, it is unclear if these have also an immunomodulatory effect as for functionally preserving the normal and reactive, innate and adaptive, immune cellular compartment.
Aim: To evaluate the immune cell defects related to CLL treatment, as compare to those inherent to the disease.

Material and method: A total 108 peripheral blood samples from B-CLL patients Binet stages B and C were evaluated: 55 pts untreated- prior to 1st line of treatment, 31 pts at the beginning of the 2nd line and 22 pts at the beginning of the 3rd or more lines of treatment (up to 7th line). Two combinations for multiparameter, 8 color flow cytometry were used: a) CD8+LAMBDA FITC, CD56+KAPPA PE, CD5 PerCP CY5.5, CD3 APC, CD19 PC7, CD38 APC-H7, CD45 PO, CD4+CD20PB and b) IgM+IgA FITC, IgA+IgG PE, CD20 PerCP CY5.5, CD19 PC7, CD5 APC, CD38APC-H7, CD45 PO, CD27 BV421.

Results: When compared to untreated, patients treated with more than 1 line showed decreased numbers of neutrophils (5824±3433 vs. 3772±3818 cells/uL; p=0.03), basophils (64±51 vs 40±39; p=0.05), monocytes (724±707 vs 444±450; p=0.04) and NK cells (680±650 vs 338±380; p=0.03). Same, total T-cells were reduced (4041±3407 vs. 2464±2213; p=0.04), due to decreased numbers of CD4+ T cells (2095±1428 vs 1045±804; p=0.002). Normal residual memory B-cells were also decreased in total (77±107 vs 19±31; p=0.02) and subsets (IgM p=0.007, IgG p=0.03, IgA p=0.04), but no differences (p>0.05) were found for immature (9±18 vs 11±21), naïve (18±68 vs 33±139) and plasma cells (4±22 vs 2±6). A similar trend was observed when comparing untreated vs 1 line treated patients for total and subsets of memory B cells (p>0.05 and <0.1).

Conclusion: B-CLL therapies significantly act by decreasing absolute counts of circulating innate immune cells, CD4+ T and memory B cells, without a consistent effect on cytotoxic T and pre-germinal center B cells, or the newly generated plasma cells. These preliminary results show the need for the analysis of circulating subsets according to different therapeutic options in order to reveal their specific immunomodulatory effect.

C21. **Hepatosplenic T-cell lymphoma – a rare type of T-cell lymphoma. Case report**

Köpeczi Judit Beáta, Benedek Lázár Erzsébet, Kakucs Enikő, Tunyogi Aliz, Horváth Emőke, Benedek István
Clinical Hematology and BMT Unit Tg-Mures, Romania

Introduction. Hepatosplenic T-cell lymphoma (HSTCL) is a rare form of peripheral T-cell lymphoma, comprising <1% of all non-Hodgkin lymphomas with predominant occurrence in young male adults. HSTCL is characterized by marked sinusoidal infiltration of spleen and liver without lymphadenopathy. Bone marrow is involved in approximately 2/3 of patients at diagnosis. The clinical course is aggressive and the prognosis is poor with a median survival <2 years. Case report. We present a case of 25 year old male patient with an evolution of 9 month from diagnosis. The disease started with organomegaly respectively the enlargement of the liver and spleen without lymphadenopathy and the presence of B symptoms (fever, night sweats, fatigue). The blood count showed slightly elevated white blood cell count and thrombocytopenia: WBC: 11.5x106/l, Hgb: 14.6g/dl, Tr: 125x106/l. The routine laboratory tests showed abnormal liver function and elevated value of the lactate-dehydrogenase.

The immunophenotyping by flow cytometry performed from the bone marrow shows 20% atypical T-cell with the following immunophenotype: CD45+(bright), CD3+(downregulated), CD7+, CD2+, CD4-, CD8-, CD5-, CD1a-, CD45RO+, CD45RA-, CD25-. Several natural killer cell-asociated antigens had variable expression CD56+ (50%), CD16+ (51%), CD11c+ (40%), CD11b+ (85%) and CD57-. The immature cell antigens were negative: CD34-, TdT-, CD99-, HLA-DR negative, and CD38-.

Immunohistochemistry from the bone marrow biopsy showed about 20% of all atypical cell infiltrate with CD3 and CD7 positivity and partially positive for CD56. Most of the cells were double negative for CD4/CD8 and CD5. Endothelial view by CD34 emphasized the intrasinusoidal component of the infiltrate. Immunostain for Granzyme B was negative.

The results of immunophenotyping and immunohistochemistry pleaded for the diagnosis of mature T-cell lymphoma, hepatosplenic T-cell

lymphoma.

Despite of multiple courses of polychemotherapy: 4 courses with CHOP and one course of DHAP and granulocyte colony stimulating factor G-CSF followed by stem cell recoltation, the spleen enlarged and thromocytopenia persisted. The spleen was surgically removed before autologous stem cell transplantation. Microscopic findings were similar to BM involvement, showing the characteristic pattern of hepatosplenic T-cell lymphoma. But in one month post-surgery fever reappeared and the hematological examination revealed a high white blood count 65x109/l with low platelet count and anemia. Immunophenotyping from the peripheral blood showed 25% of malignant T cells which lead us to the conclusion of leukemic evolution. The patient received the induction treatment for T cell leukemia. The therapeutic response was partial with the 4% of atypical cells in the peripheral blood identified by flow cytometry. Due to the lack of a compatible HLA identical sibling donor the standard Bu-Cy high dose conditioning regimen and autologous stem cell transplant was performed. Signs of disease progression (thrombocytopenia with severe episodes of melena, hepatomegaly) appeared in very short time (2 month) after autologous stem cell transplantation. Search for unrelated donor for allogeneic stem cell transplant was started.

Conclusion. HSTCL is a rare entity of T-cell lymphoma. Immunophenotyping by flow cytometry is essential in diagnosing rapidly and should additionally be confirmed by bone marrow examination and spleen and/or liver immunohistochemistry.
Immunophenotyping is important in monitoring the evolution of disease. The clinical course is aggressive and the prognosis is poor.

P1. **Unusual relapse as hypogranular morphologic subtype in classic acute promyelocytic leukemia**

Cristina Enache, Daniela Vasile, Madalina Begu, Ana-Maria Vlădăreanu, Horia Bumbea
„Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
Department of Hematology, Emergency Universitary Hospital, Bucharest, Romania

Background. Acute promielocytic leukemia is a rare leukemia, usually described with hypergranular morphologycal type, and rarely, with hypogranular variant.

We present a 64 year old pacient admitted for pancytopenia: WBC = 1200/uL, HGB = 9.4g/dl, PLT = 75.000/uL. PBS: about 57% atypical promyelocites. BMA: 60% atypical promyelocites of medium-large size, thin cromatine, nucleols, basophilic cytoplasm, with multiple granulations and large number of Auer rods. These findings meet the criteria for acute promyelocytic leukemia – classical hypergranular subtype. Immunohistochemistry: 55% MPOX + promyelocytes. Immunophenotyping analysis described promyelocytes with medium SSC, medium CD45+, CD34-, CD34-, CD33+, CD117-/+, CD9+, CD2-, CD56-, CD13+, CD11c-. PCR analysis: positive PML/RAR transcript. Pacient received specific treatment with ATRA.
Pacient obtained hematological remission with immunophenotyping control followed by one consolidation treatment with Idarubicin and ATRA.
7 months after diagnosis, pacient relapses with pancytopenia: WBC: 900/uL, HGB = 9.7g/dl, PLT = 75.000/uL and PBS: aprox. 75% atypical promyelocytes and BMA: 70% promyelocytes of medium-large size, bilobed nuclear shape with thin cromatine, nucleols, agranular basophilic cytoplasm, very rare granulated cells, no Auer rods, typical aspect for Acute promyelocytic leukemia – microgranular subtype. Citochemical staining: MPOX +. Immunophenotyping of BMA describes specific phenotype for atypical promyelocytes: medium SSC, medium CD45+, CD34-/+, CD33+, CD117-/+, CD9+, CD2+, CD56-, CD13+, CD11c-.
Conclusion. Morphological transformation of acute promyelocytic leukemia from hypergranular to microgranular subtype has never been described before in literature, this case raising the posibility of an additional genetical instability.

P2. **Copy Number Alteration Detected by MLPA in B-ALL Patients– Correlations with the Presence of Fusion Gene Transcripts and Immunophenotype**

Iuliu Cristian Ivanov[1,2], Roxana Popescu[1], Georgiana Grigore[1,2], Angela Dăscalescu[1], Anca-Viorica Ivanov[1,3], Mihaela Zlei[2], Ingrith-Crenguța Miron[1,3], Eugen Carasevici[2]
1. University of Medicine and Pharmacy Grigore T. Popa of Iași, Romania
2. Regional Institute of Oncology, Iași, Laboratory of Molecular Biology
3. Pediatric Hematology and Oncology Unit of St. Mary Clinical Emergency Hospital for Children Iași, Romania

Background. Copy number abnormalities of genes involved in lymphoid differentiation and cell cycle control have been shown to occur with a high frequency in B-cell precursor acute lymphoblastic leukemia. In order to reliably detect such alterations involving multiple targets in a single test, Multiplex Ligation-dependent Probe Amplification (MLPA) has been progressively exploited as a rapid technique for testing of large cohorts.

Materials and methods. Here we describe our experience using MLPA on 16 B-ALL patients (six adults and ten children) for the detection of risk-identifying aberrations. We used the SALSA MLPA probemix P335-B1 ALL-IKZF1 kit (MRC-Holland) containing 57 probes for tumor-associated genes, including IKZF1, PAX5, ETV6, RB1, BTG1 and the BTG1 downstream region, EBF1, CDKN2A-CDKN2B, PAR region, CRLF2, CSF2RA, IL3RA, P2RY8, ZFY, and JAK2 genes. As a control, 13 reference probes have been included targeting chromosomal regions that are relatively stable in ALL. The same patients were also investigated for the presence of four fusion genes: TEL-AML1, BCR-ABL (p190), E2A-PBX1 and MLL-AF4 and the immunophenotype of the malignant clone was assessed by multicolor flow cytometry.

Results. In six cases (three adults and three children) deletions/ duplications of one or more sequences in the above mentioned chromosomal regions were detected. In one pediatric case (girl, 10y), also carrying the p190 fusion gene, multiple gene deletions were identified (del 1/2 IKZF1 ex 4-7; del 1/2 CDKN2A; del 2/2 CDKN2B; del 1/2 PAX5 ex2 si 5; del BTG1 ex 1- 2).

Conclusions. Our data broaden previously reported evidence that MLPA may become a practical tool for the characterization of well-known lesions as well as the investigation of additional genomic changes in B-ALL.

P3. **Diffuse non-Hodgkin's lymphoma with large B cells and leukemic picture**

Adriana Coliță, Didona Vasilache, Adriana Dumitrescu, Anca Gheorghe, Camelia Dobrea, Iulia Ursuleac, Mihaela Mustață, Dan Coriu
Center of Hematology and Bone Marrow Transplantation, Fundeni Clinical Institute, Bucharest, Romania

We report the case of 70 years old, male without any medical history, which was admitted in Fundeni with fever (10 days before admission), jaundice, fatigue, easy bruising and splenomegaly. Hematologic findings: Hb 11g/dL, Ht 32%, Retic: 0,8%, WBC: 15.430/L, PLT: 50.000/L. On blood smear revealed polymorphic mononuclear cells (25%) with blastic appearance (big diameter, round or lobulated nucleus, fine chromatine, proeminent nucleoli, scanty basophilic cytoplasma) and left shift of differentials My: 1%, Mt: 1%, N 4%, S 54%, Ly 5%, Mo10%. Biological features: total bilirubin level 16 mg/dL; indirect bilirubin level 11 mg/dL; ALT: 76 U/L; AST: 212 U/L; TP: 4,6 g/dL, Alb: 2,10 g/dL, Glucose: 65 mg/dL; Urea: 65 mg/dL; Creatininemia: 0,8 mg/dL; LDH: 1359 nmol/L; 2m: 8,86 mg/L; Fbg: 421 mg/dL; INR: 1,18; APTT: 33 sec.; IgA: 421 mg/dL; IgG: 840 mg/dL; Coombs test: negative. Bone marrow aspirate: rich cellularity, 30-40% mononuclear cells, high diameter irregular cutting nuclei, proeminent nucleoli, basophilic cytoplasm and mitotic figures. Flow cytometry of the bone marrow aspirate revealed an infiltration with monoclonal B cells, CD45+, with moderate internal complexity (24% of total) which expresses CD38, CD19het., cCD79a+++, CD22het., CD5-/+ het., CD43+(low), CD79b+, CD11c+/-, FMC7+/-, CD20+, +, CD34-, TdT-, CD23-, Cd10-.
Conclusion: aspect compatible with the diagnosis of NHML with large B cells (DLBCL). Bone marrow biopsy: hypercellularity (75/25), malignant interstitial infiltration (35-40%) with large polymorphic cells; hyperlobulated nuclei with laxe chromatine and 2-4 nucleoli. At the immunohistochemistry, the tumoral cells are type B, CD20 positive; negative for BCL6, CD30, Cyclin D1 and CD3. CD34 negative. Conclusion: diffuse large B-cell lymphoma not otherwise specified (DLBCL- NOS) (CD10 negative, BCL6 negative) with bone marrow involvement.
Abdominal computer tomography: splenomegaly (15/8cm); polyadenopathies (perigastric, coeliac zone, retroperitoneal, inter aorto-caval). Pulmonary RX: normal. Treatment: antibiotics, antimycotic, supportive care, followed by Dexamethasone + Cyclophosphamide + Vincristine (as a prephase) and later Hyper-CVAD (2 cycles) with complete remission, but with a relapse in 3 months affecting the brain, meninges, the cervico-thoracal teritories. The treatment with HD-MTX plus i.t. MTX, VCR + Procarbazine (R-MVP) was not efficient. This case underline the importance of taking together the date of clinic, morphology, immunophenotyping, histopathology, immunohistochemistry for the correct establishment of the diagnosis in situations with an unusual morphologic picture. The presence of young cells, with blastic appearance was initially diagnosed as acute leukemia (possible monoblastic) but the flow cytometry demonstrated the diagnosis of a lymphoproliferative disorder with large B cells suggesting a NH malignant lymphoma. The immunochemistry of the bone marrow confirmed this diagnosis. This case revealved the importance of integrated diagnosis (clinical, hematological, pathological and immunophenotypical features); aproximatelly 1/3 of malignant non-Hodgkin's lymphomas with large B cells and bone marrow involvement present malignant cells in the peripheral blood.

P4. **Difficulties of flow-cytometry based diagnosis in T - or NK – cell malignant proliferations**

Daniela Constantinescu[1], Mihaela Zlei[2], Georgiana Grigore[1,2], Iuliu Cristian Ivanov[1,2], Angela Dăscalescu[1,2], Cătălin Dănăilă[1,2],

Cristina Burcoveanu[2], Eugen Carasevici[2]
1 University of Medicine and Pharmacy Grigore T. Popa of Iași, Romania
2 Regional Institute of Oncology, Laboratory of Molecular Biology, Iași, Romania

Whenever NK or T cell neoplasms include peripheral blood and/or bone marrow involvement, in order to decide upon the final diagnosis, according to WHO classification, results of flow cytometric characterization have to be corroborated with cytomorphology, histopathology and clinical findings. Here we present four cases of T and NK cell neoplasms diagnosed in our department by flow cytometry with discussions on the majors obstacles encountered for the final diagnosis. Bone marrow (BM, n=2) and peripheral blood (PB, n=2) samples from four patients with suspicion of chronic lympho-proliferative disorders were investigated by flow cytometry using a FACSCantoII (Becton Dickinson) cytometer and combinations of up to 6 colors. All patients were enrolled within the Hematology Department of Regional Institute of Oncology, Iasi, Romania. Case 1: BI, M, 39 y, BM: 15% NK cells (CD45+ CD56+ CD7+ CD2+ CD5- CD3s+ic- CD8-CD4- HLA/DR+ CD38+ CD161- CD94+ CD38+). Case 2: MV, F, 64 y, PB: (22 500 cells/ μL): 49,5% (5 700/ μL) NK cells (CD45+ CD56+ CD57+low CD26- CD7+ CD2+ CD5- CD3s+ic- CD8-/+ (6%) CD4- HLA/DR+). Case 3: VI, M, 72y, PB (46 000 cells/ l): - 82% T lymphocytes (CD45+ CD3+ CD8+CD45RA+TCRab+). Case 4: RS, M, 26y, BM: 3,3% T lymphocytes: CD45+low CD3s-CD3ic+CD5+ CD2+ CD7+ CD4- CD8- TCRab-TCRgd- CD1a- CD25- HLA/DR- CD56- TdT-. A major challenge for the diagnosis of T - or NK – cell malignant proliferations remains to be the establishment of the clonal nature of the expanded population.

P5. **Hepatosplenic T-cell γδ lymphoma. Presentation of case**

Doina Barbu, Oana Stanca, Anca Ciobanu,
Florentina Grădinaru, Anca Roxana Lupu
Coltea Hematology Department, Bucharest, Romania

Hepatosplenic T cell gamma/delta lymphoma is a rare clonal proliferation of T lymphocytes mature post-thymic, first described in 1990, clinical and paraclinical heterogeneous. In our presentation we discuss the case of a woman 40 year old, admitted to hospital, Coltea Hematology Department, corner marked asthenia, abdominal pain. Clinical examination reveald significant hepatosplenomegaly, generalized micropoliadenopaty and paraclinical lymphocytosis, anemia, thrombopenia. Flow cytometry reveald a predominent population CD3+ lymphocytes, CD2+, CD5-, CD7-, CD16-, TCR γδ+, CD20-, CD8-, CD4 -, CD56+. The peculiarity of the case: lymphocytosis, important splenomegely, important poliadenopatii generalized, superficial and deep. Differential diagnosis: reactive T proliferation was done, other lymphomas, acute lymphoblastic leukemia T. Conventional chemotherapy was established. The patient's progresion was favorable.

P6. **Blastic plasmocytoid dendritic cell neoplasm-case report**

Alexandra Gheaur[2] , Ana Manuela Crisan[1,2],
Sorina Bădeliță[2], Dana Elena Giza[2],
Camelia Dobrea[1,2], Anca Gheorghe, Daniel Coriu[1,2]
1 "Carol Davila" University of Medicine and Pharmacy, Bucharest,, Romania
2 Department of Hematology and Bone Marrow Transplantation, Fundeni Clinical Institute, Bucharest , Romania

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare hematopoietic malignancy, formerly known as blastic NK cell lymphoma or CD4+/CD56+ hemato-dermic neoplasm.
It is considered a clinically aggressive neoplasm derived from the precursors of plasmacytoid dendritic cells (also known as type 1 interferon producing cells or plasmacytoid monocytes).

We present the case of a young man with multiple bruise-like deep red plaques and left axillary adenopathy. The blood count showed Hb=8.9 g/dl, Ht=25,5%, MCV=90.1 fl, Wbc=58760/mmc Plt=18000/mmc (Metamyelocyte=1, Bands=1, Segmented= 4, Eosinophils= 1, Lymphocytes= 92). The blood film showed nuclear shadows, polymorphous lymphoid cells some blast-like and some monocytoid-like. The flow-cytometry exam of peripheral blood showed 92 % CD45 negative cells which express CD38+, CD4+, CD56+, CD123+, CD36+, CD33+/-, and do not express cyclina D3 si CD2. The bone marrow exam (aspirate and biopsy) showed hypercellular marrow by massive lymphoid infiltrate (80%) cu medium size cells with vesicular nucleus and rich cytoplasm (plasmacytoid-like) and reduced normal hematopoiesis. The whole body CT scan showed multiple supradyaphragmatic adenopathies. The immunohystochemical exam of skin biopsy showed CD56 + diffuse; CD4+ diffuse, L26/CD 20 –, CD 79a –, CD3 –, CD43 +, CD68 +, CLA +, TdT -, CD34-, CD8 –, CD30 –,Ki67 + in 30% tumour cells. The patient received 5 cycles of SMILE regimen and achieved complete remission. To consolidate the response to chemotherapy, the patient was eligible for autologous bone marrow transplant. Six months after autologous transplant, the patient relapsed and received salvage

chemotherapy as DeVic regimen. The chemotherapy regimens were delayed due to frequent bacterial and viral episode and achieved stable disease.

BPDCN needs to be considered as differential diagnosis of tumors involving the skin and flow-cytometry features of BPDCN have improved its recognition.

P7. **Flow cytometry – key investigation in autoimmune lymphoproliferative syndrome**

Mihaela Baica[1], Mihaela Bataneant[1], Mihaela Preja[1], Laszlo Marodi[2], Margit Şerban[1]
1„Louis Turcanu" Hospital, Timişoara
2Department of Infectious and Pediatric Immunology Debrecen, Hungary

Autoimmune Lymphoproliferative Syndrome (ALPS) is a genetically heterogenous syndrome that can be inherited in an autosomal-dominant or autosomal-recessive fashion or may arise due to somatic mutation in hematopoetic progenitors and consist in a chronic non-malignant lymphoproliferation caused by defective apoptosis due to mutations in genes involved in CD95 mediated apoptosis (CD95, CD95L, caspase 8 and 10).
The diagnosis of ALPS is based on clinical findings, laboratory abnormalities and identification of mutations in genes involved in Fas pathway of apoptosis. Increasing of CD3+/CD4-CD8-/TCR alfa/beta+ cells (so called TCR alfa/beta double negative T cells) in peripheral blood is the hallmark of the disease.

It will be presented the case of a three years old child who was investigated in January 2012 for splenomegaly and lateral cervical lymphadenopathy. Flow cytometry from pheripheral blood performed in our clinic revealed a TCRαβ DNT population of 12% from LTCD3+ (normal values typically <2,5% total T cells in healthy individuals). Genetic analysis performed in Debrecen revealed a c.814G>A heterozygous single base substitution in exon 9 of the FAS gene. All these findings along with lymph node biopsy and other clinical and laboratory findings established diagnosis of ALPS. Pacient is under observation in our clinic by repeated measurements of TCRαβ DNT population in peripheral blood.

P8. **Cell proliferation analysis in cervical cancer**

Mirela Dumitru, Maria Monica Vasilescu,
Marieta Elena Panait, Madalina Radu, Ileana Condrea, Raluca Nicolaescu, Iuliana Maria Gruia
Institute of Oncology "Prof.Dr.Alex.Trestioreanu", Bucharest, Romania

Cervical cancer is the first most common malignancy of the female genital tract in Romania. The molecular events involved in its proliferation and tumor growth are not well clarified.

The two epidermal growth factor receptors, the transmembrane proteins EGFR and HER-2 play an important role in the cell growth, proliferation, differentiation and migration. Overexpression of these receptors is associated with an aggressive behavior in cancer. Topoisomerase II-alpha (TOP2A), an essential enzyme for cell viability, is another proliferation-associated molecule. TOP2A levels are cell cycle-dependent and practically represents the percentage of dividing cells. Other indicators of cell proliferation rate are S-phase fraction and DNA ploidy. An increased S-phase fraction and DNA aneuploidy reflect a fast and uncontrolled tumor growth.

This study evaluated the expression of EGFR, HER-2, TOP2A and DNA content to characterize the proliferative potential of cervical malignant lesions.

Representative biopsies from 25 cases of cervical cancer, sampled from 2-7 different sites, were investigated immunohistochemical for EGFR, HER-2 and TOP2A expression and by flow cytometry for DNA content.

We observed co-expression of HER-2/EGFR in 3/25 cases and the co-expression of EGFR/TOP2A in 6/25 tumors. DNA aneuploidy was present in 9/25 carcinomas. The aneuploid cells were dominant in HER-2/EGFR positive cases and in HER-2/TOP2A positive tumors. A high S-phase fraction was detected in 7/9 HER-2 positive cases and in 6/10 TOP2A positive cases. The two carcinomas with EGFR, HER-2 and TOP2A co-expressed were DNA aneuploid and with a high S-phase fraction.

Our results suggest that cervical tumors HER-2 positive or with overexpression of TOP2A have a high proliferative activity. Thus, these data may be useful in evaluation of clinical outcome of patients with cervical cancer and have potential implications for the development of novel treatment strategies.

P9. **Analysis of in vitro cytotoxic effects of new synthesized drugs for DL50 establishment**

Sorina Dinescu[1], Bianca Gălățeanu[1], Diana Nuță[2], Marieta Costache[1]
1University of Bucharest, Department of Biochemistry and Molecular Biology, Bucharest, Romania
2University of Medicine and Pharmacy "Carol Davila"

Bucharest, Pharmaceutical Chemistry, Bucharest, Romania

Introduction: To reduce the number of animal experimental models for toxicity studies, a commonly used alternative is represented by the use of cell cultures. The in vitro systems allow successful assessment of cytotoxicity of chemical compounds with therapeutic potential both in terms of basic cell functions (those that occur in all cell types) and the particular functions (those that occur only in differentiated cell types).

The aim of this study was the synthesis of new N-(2-dialchilaminoetil) benzanilide hydrochloride or quaternary ammonium salts and the identification DL50 in standardized culture systems for prospective evaluation of their therapeutic potential. In this context, there have been synthesized a series of new N-(2-dialkylaminoethyl)benzanilides as hydrochlorides or quaternary ammonium derivatives and their chemical structures were confirmed by NMR and IR spectral analysis. Of these, five compounds were selected to determinate the therapeutic potential and to establish LD 50.

Materials and methods: N-(2-dialkylaminoethyl)-benzanilides (hydrochlorides) were obtained as a result of the alkylation reaction in anh. toluene of certain aromatic amines with N-(2-chloroethyl)-N,N-dialkylamine hydrochloride in the presence of sodium carbonate. The intermediary compounds are reacting with different aromatic acid chlorides using toluene and triethylamine as acid acceptor. The resulted benzanilides were turned into hydrochlorides by treating them with an cold etheric HCl solution or in quaternary ammonium derivatives by treating with methyl iodide in acetone. All the obtained compounds were purified by recrystallization.

The in vitro experimental model used was represented by VERO (ECACC) cell line. These cells were multiplied at 370C in MEM culture medium, supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere and 5% CO2. Cells were seeded in 96-well plates at an initial density of 2x104 cel/cm2. After 24 h of culture, the medium was refreshed and over the cells were added the tested compounds in concentrations ranging from 500 mM-10 nM.

At 24 h after treatment cell viability was assessed using MTT spectrophotometric test by incubating the cells for 4 hours in 1 mg / ml MTT solution. At this time the tetrazolium salt (MTT) was metabolized by viable cells and the purple formazan crystals appeared as a product of the process were solubilized in isopropanol. Formazan concentration, which is directly proportional to cell viability, was determined spectrophotometrically at 550 nm in isopropanol.

Live and the dead cells were detected simultaneously in the cultures treated with the tested compounds by fluorescence microscopy. Thus, at 24 h of culture, the medium was replaced with a 2 μM calceinAM and 4 μM ethidium bromide, which was maintained in contact with the cells for 15 minutes at room temperature at dark. CalceinAM is a non-fluorescent, cell permeable membrane that once entered into the cytoplasm is converted by the esterases into a fluorescent compound (Calcein). This compound, with a green fluorescence (ex / em: ~ 495 nm / ~ 515 nm) is well retained in living cells. Ethidium bromide is a compound that enters the cells only if the membrane is damaged and binds to nucleic acids, producing a strong red fluorescence (ex / em: ~ 495 nm / ~ 635 nm). After staining, the samples were inspected by fluorescence microscopy using the inverted fluorescence mycroscope Olympus IX71.

All samples were studied in triplicate and the results were compared with the control, which consisted in untreated cells. Quantitative data obtained by MTT assay were statistically processed using Prism software by Bonferoni test, ANOVA.

Results and discussions: Using the spectrophotometric MTT test, there has been determined the DL50 for all the five compound tested of in vitro cytotoxic potential. The LD50 varies with the structure. The quantitative results obtained from MTT test were confirmed by fluorescence microscopy images. From the results it is found that has the greatest influence substitution with halogen atoms, in particular on the remaining benzoyl chain, but also on the rest of the aniline chain. Double substitution on benzoyl radical is the most favorable biological activity, hydrochloride of N-(2-diethylaminoethyl)-N-(2,6-dimethylphenyl)-4-methoxybenzamide being the most active compound. Also, in the case of isosteric compounds 3 and 5 it was demonstrated the effectiveness of bioisosteric substitution with trifluoromethyl group, instead of chlorine. The absence of halogens in the structure of compound 1 led to a compound with lower activity, and to obtain quaternary ammonium salt (compound 4) did not improve the biological profile.

Conclusions and perspectives: There have been synthetized five new N-(2-dialkylaminoethyl) benzanilides as hydrochlorides or quaternary ammonium derivatives and their chemical structures were confirmed by NMR and IR spectral analysis. For all these compounds there has been identified the DL50 for VERO cell line and the results showed that the biological activity is closely dependent with the chemical structure. These promissing results allow the future developement of this study in order to evaluate the potential terapeutic efect of these compounds.

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P10. **Therapeutic efficacy evaluation of 177Lu - DOTA - NT and 177Lu – DOTA - SR48692 on RS-1 hepatoma**

Marieta Elena Panait[1], Diana Chiper[2],
Valentina Negoiță[1], Valeria Lungu[2],
Maria Iuliana Gruia[1]
1Institute of Oncology Prof. Dr. Al. Trestioreanu Bucharest
2Horia Hulubei National Institute for Research and Development in Physics and Nuclear Engineering

Introduction: This paper aims to bring a scientific contribution to elucidate the paradigm concerning the capture of neurotensin agonist (NT) or antagonist (SR48692) by tumor cell, concerning the competition between NT and SR48692 for receptors. For this reason, two radiolabelled compounds: 177Lu-DOTA-NT and 177Lu-DOTA-SR48692 were prepared and tested. Additionally, in order to evaluate the competition between NT and SR, either of these radiolabelled compounds was intermixed with its non-radiolabelled counterpart (177Lu-DOTA-NT/DOTA-SR48692 and 177Lu-DOTA-SR48692/DOTA-NT).
Materials and methods: Groups of bearing Wistar rats expressed in neurotensin receptors (RS-1 hepatoma) were injected intraperitoneally with 74-GBq (specific activity of 2Ci/mg) from each four compounds, as targeted therapy. Tumor control group included tumor bearing rats with no treatment. Animals of each group were sacrificed at 48h, 14 days and 60 days after administration, evaluating oxidative stress and cell proliferation parameters.
Results: All treated groups revealed some therapeutic benefits in contrast with control, consisting in tumor regression with statistically tumor volume reduction, decreasing in the number of cells in synthesis phase of DNA to normal range (S < 6,5%) and arresting (blocking) in G1, redox parameters proportionally increased with induced radiotherapy. The most obvious effects were recorded at 14 and 60 days after treatment.
Conclusions: The results demonstrated significant therapeutic effects of two radiolabelled compounds in RS-1 hepatoma, to be recommended in a complementary (alternative) treatment. In addition, the combination of radiolabelled compound with its non-radioactive counterpart may be a promising strategy to improve the therapeutic effects.

P11. **Assessment of the influence of phenyl lactic acid and its synergic activity with antibiotics on Pseudomonas aeruginosa resistant strains by flow cytometry**

Lia-Mara Dițu, Luminita Măruțescu,
Grigorie Mihaescu, Veronica Lazar,
Mariana-Carmen Chfiriuc
University of Bucharest, Faculty of Biology, Microbiology Immunology Department, Bucuresti, Romania

The phenyl lactic acid (PLA) is a soluble antimicrobial compound, known to be produced by Lactobacillus spp. probiotic strains, which is not acting by acid pH. In this study, we have investigated the influence of sub-inhibitory concentrations (sIC) of PLA, on the growth, viability and efflux pumps activity of MDR Pseudomonas aeruginosa strains, acting alone or in association with cefepime (FEP).
Materials and methods. Ten clinical P. aeruginosa strains were co-cultivated with sIC of PLA (1mg/ml) in Mueller Hinton broth medium (MHB) for 24 h and then the antimicrobial susceptibility pattern of the respective strains was performed using disk diffusion standard method. Two of the ten strains exhibited an increased susceptibility to FEP. These strains were further co-cultivated for another 24 h in MHB in the presence of sIC of PLA and different concentrations of FEP (MIC, 2xMIC, 3xMIC). The influence of PLA on efflux pumps activity and cell viability was investigated by the viable cell counts (VCCs) and flow cytometry methods. For flow cytometry analysis 1 ml aliquots was pelleted, washed twice with PBS and stained with 1 µg /ml propidium iodide (for viability) and ethidium bromide (for efflux pump activity). The fluorescence was evaluated using FL2 and FL3 channels.
Results. The fluorescence intensity on FL2 chanel (for propidium iodide) is depending on the MDR P. aeruginosa strain and growth conditions. For P. aeruginosa 289 strain co-cultivated with sIC of PLA in association with different concentrations of FEP (MIC, 2xMIC, 3xMIC), the fluorescence intensity was higher compared to the same strain co-cultivated with the antibiotics only. When the P. aeruginosa 304 strain was co-cultivated with sIC of PLA and 2xMIC or 3xMIC of FEP, the fluorescent signal decreased compared to the signal recorded for samples treated only with 2xMIC or 3xMIC of FEP. In contrast, for P. aeruginosa 304 strain co-cultivated only with sIC of PLA or sIC of PLA and MIC of FEP, recorded signal was more intense than the control strain or the strains grown only with MIC of FEP. For the two MDR P. aeruginosa strains, fow-cytometry analysis revealed that, in the simultaneously presence of the higher concentration of antibiotic and PLA, bacterial cells were protected by closing their efflux pumps.
Conclusion. The use of soluble factors secreted by probiotic bacteria in anti-infective strategy, may represent a new and ecological method, with a very important therapeutically value for treatment of

bacterial infections, particularly chronic infections caused by multidrug-resistant bacteria and with biofilm development potential.

P12. **Evaluation of the influence of lactic acid bacteria on Candida albicans B18 drug resistance by flow cytometry**

Luminita Măruțescu[2], Ionela Avram[1], Diana Pelinescu[1], Chifiriuc Carmen[2], Vassu Tatiana[1]
1University of Bucharest, Faculty of Biology, Genetic Department
2University of Bucharest, Faculty of Biology, Microbiology Immunology Department

Candida (C.) albicans is part of the commensal microbiota of the human gastrointestinal and urogenital tract. In healthy persons, strains of C. albicans generally cause superficial infections, while for immuno-compromised persons may determine serious invasive infections.

The frequency of systemic fungal infections has increased during the last decades. A higher risk of serious fungal infections is associated with clinical use of broad-spectrum antimicrobial compounds, immunosuppressant agents and various medical devices. Despite the effectiveness of antifungal compounds used to combat such infections, the emergence of resistance phenomena, their high toxicity and their low capacity to reach the target site, are still major problems in fight against systemic infections. Resistance of C. albicans strains to azoles class compounds (fluconazole, ketoconazole, etc.) is often correlated with increased activity of efflux pumps.

The main goal of our studies was to determine the efflux pumps activity of C. albicans B18 strain, in the presence of two lactic acid bacteria strains and fluconazole. Recent studies have shown that Nile red is a lipophilic fluorochrome that can be used as fluorescent substrate for evaluation of efflux pumps activity in yeast strains. Propidium iodide was used as fluorochrome in order to determine the viability of yeast cells. Two techniques (flow cytometry and fluorescence microscopy) were used.

Flow cytometry analysis using Nile Red showed an increased fluorescence of C. albicans B18 strain in the presence of two lactic acid bacteria strains and fluconazole. The increased fluorescence occurs due to binding of Nile red to lipids as a result of reduced activity of efflux pumps.

The results were confirmed by fluorescence microscopy. There was a noticeable decrease in fluconazole resistance of C. albicans B18 strain in the presence of lactic bacteria strains due to low activity of efflux pumps.

P13. **Detection of antimicrobial activity and cytotoxicity evaluation of some novel chemical compounds using flow cytometry**

Carmen Chifiriuc[1], Coralia Bleotu[2], Carmen Limban[3], Rodica Olar[4], Mihaela Badea[4], Veronica Lazar[1]
1University of Bucharest, Faculty of Biology, Bucharest, Romania
2 Virology Institute Ștefan S. Nicolau, Bucharest, Romania
3University Medicine and Pharmacy Carol Davila, Bucharest, Romania
4 University of Bucharest, Faculty of Chemistry, Bucharest, Romania

Classical microbiology techniques are relatively time - consuming in comparison to other analytical techniques, bing in many cases culture dependent. Flow cytometry (FCM) can be used for the quantitative assessment of the antimicrobial susceptibility and drug cytotoxicity in a rapid, accurate, and highly reproducible way. In this study FCM was applied to assess the antimicrobial activity of some newly synthesized compounds against different reference microbial strains, using staining with propidium iodide (PI) for membrane damage evaluation.

Material and methods. New organic compounds obtained by functionalization from chemical structures known for their pharmacological activity, as well as complex combinations of bivalent metals were previously characterized by elemental analysis, IR, 1H NMR and electronic spectroscopy. The compounds were solubilised in dimethylsulfoxide and screened for their in vitro antimicrobial activity against two reference strains, i.e. Pseudomonas aeruginosa ATCC 27853 and Staphylococcus aureus ATCC 25923. The effects of the newly synthesized compounds on the viability of the two reference strains were comparatively studied by means of classical assays and FCM.

Results. Our results showed that the new compounds exhibit antimicrobial activity, with MIC values ranging from 500 µg/mL to 62.5 µg/mL. The compounds exhibited a more intensive antimicrobial activity against P. aeruginosa ATCC 27853 strain. The absorbance of the microbial cultures grown in the presence of different substances and concentrations measured at 620 nm as well as the FCM assays gave comparative results concerning the MIC values. Variable results were observed between plate count results and flow cytometric data, which suggested the presence of a sublethally stressed subpopulation, not able to form colonies on agar plates. Following treatments, FCM assessment clearly discriminated three different subpopulations: viable, dead and injured cells. At the same time the cytotoxicity and influence on the

eukaryotic cell cycle by FCM allowed us to select the compounds with optimal antimicrobial activity and low cytotoxicity.

Conclusion. FCM enabled us to discriminate between bacteria with intact membrane and bacteria with damaged membrane after exposure to antimicrobial compounds by measurement at the direct target, the cytoplasmic membrane. This technique allows the monitoring of in vitro antimicrobial activity, the most outstanding contribution of FCM beeing the possibility of detecting the presence of heterogeneous populations with different responses to antimicrobial treatments.