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Heart diseases in essential Thrombocythemia review

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Abstract

Essential thrombocythemia (ET) is a myeloproliferative disorder that raises questions about the characteristics of the disease treatment. ET evolution is grafted to a predisposition to bleeding and thrombotic events and microvascular events. Thrombotic events often affects medium-sized and large arteries including cerebral arteries, coronary and peripheral, but can also affect the veins causing recurrent venous thrombosis of the legs with thromboembolic complications. The most common cardiac complications occurred in the ET are the acute coronary syndromes or coronary thrombosis, and in some cases has been incriminated and coronary spasm.
Possible cardiac valvular damage that can occur in ET (thickening, calcification, valvular regurgitation) and the possibility of associating with pulmonary arterial hypertension who aren't associated to a pulmonary embolism are reported in the literature but with an extremely rare incidence.
Key words*: essential thrombocythemia, acute coronary syndroms, thrombosis*

Introduction

Essential thrombocythemia (synonymous with essential thrombocytosis, idiopathic thrombocytosis, primary thrombocytosis, hemorrhagic thrombocythemia) is a clonal myeloproliferative disease of unknown etiology that involves the multipotent hematopoietic progenitor cells [1]. ET is an unusual disease with an incidence of 1-2/100000 citizens and it affects mostly females unlike other myeloproliferative syndromes.

Although TE is considered a disease of middle-aged patients (50-60 years), now it is diagnosed with increasing frequency in younger patients [2]. Clinically, ET is characterised by overproduction of platelets and functional alterations of these platelets, which confers a high risk of venous and arterial thrombosis, haemorrhage and microvascular disturbances[3].

Tabel 1. ET diagnostic criteria developed by the World Health Organization

POSITIVE CRITERIA	
• **Platelet count persistently > 600 * 10^3 / L (> 450 * 10^3 / L [36])**	
• **Bone marrow biopsy shows proliferation of megakaryocytes line**	
EXCLUSION CRITERIA - NO EVIDENCE OF:	
1. Polycythemie vera	Normal number of red blood cells or hemoglobin <18.5 g / dl in men, 16.5 g / dl in women
	Normal bone marrow iron deposits , serum ferritin and mean corpuscular volume
2. Chronic myeloid leukemia	Philadelphia chromosome and BCR/ABL gene absent
3. Idiopathic myelofibrosis	Collagen fibers absent
	Reticulin fibers absent or minimal
4. Myelodysplastic syndrome	del(5q), t(3;3)(q21;26),inv(3)(q21q26) absent
	Without significant myelogenous dysplasia
5. Reactive thrombocytosis	Inflammation / infection
	Neoplasia
	Splenectomy

Clinical aspects of ET

Commonly, ET is diagnosed incidentally after performing a routine blood count. No sign or symptom is specific to ET. Anemia is an unusual association, but not leukocytosis with neutrophilia. The large number of circulating platelets can cause a false hyperkalemia without electrocardiographic changes due to potassium release by platelets during the formation of small trombus. Prothrombin time and thromboplastin time are normal, but we may encounter abnormal platelet function reflected by the increase in bleeding time and abnormal platelet agregability tests. However, no functional platelet abnormality is specific for ET and no test that assesses platelet function can predict the risk of bleeding or thrombosis [1].

ET must be distinguished from other myeloproliferative syndromes causing thrombocytosis and reactive thrombocytosis (RT). Differentiation between ET and RT is of major clinical importance because the risk of vascular thrombosis is significantly lower for RT [5].

Of equal importance is the differentiation of ET from other chronic myeloproliferative syndromes as polycythemia vera, idiopathic myelofibrosis and chronic myeloid leukemia, because they differ in terms of prognosis and therapy [2]. Currently, there is no specific diagnostic test for ET, so the diagnosis of ET is put after the exclusion of other causes and is based on diagnostic criteria developed by the World Health Organization [6] - Table 1. There is no clonal marker to differentiate ET from nonclonale forms, reactive thrombocytosis.

Recently, the JAK2 V617F mutation has been shown to be detected in more than 50% of patients with essential thrombocythemia [18] [35]. The Medical Research Council Primary Thrombocythemia-1 Trial demonstrated that patients with the JAK2 V617F mutation have a better response to the treatment with hydroxyurea [19]. There are communications supporting the determination of JAK2 mutation in patients with unexplained coronary ischemic complications and moderate thrombocytosis and in patients with arterial or venous complications without apparent cause [35].

Diagnostic criteria

When the blood count shows us the presence of significant thrombocytosis is necessary to differentiate between ET and various reactive thrombocytosis occurred in the context of infections, malignancies, postsplenectomy, posthaemorrhagic, postsurgical, after correction of megaloblastic anemia by administration of vitamin B12 [1]. The next step is to exclude other myeloproliferative syndromes by making a marrow biopsy (bone marrow iron deposits - polycythemia vera, collagen and reticulin fibers - idiopathic myelofibrosis) and cytogenetic and molecular examination (highlighting various deletions, translocations, chromosome Philadelphia and gene BCR / ABL) - table 1.

The criteria that define patients with ET at increased risk of thromboembolic complications are:

age > 60 years

a history of thrombosis, embolism or major ischemic event

platelet count> 1000-1500 * 103 / L [8] [14].

ET Evolution

ET evolution is characterised by thrombotic and bleeding events and microvascular disturbances. Commonly, thrombotic events affect large and medium-sized arteries including cerebral arteries, coronary and peripheral veins but can also affect the veins causing recurrent venous thrombosis of the legs with thromboembolic complications [8]. There have been described cases of portal or splenic vein thrombosis and Budd Chiari syndrome. [7] Abnormal platelets can generate transient occlusions at the microvascularization level resulting transient ischemic stroke, migraine, visual disturbances or eritromelalgia [2]. Bleeding complications (like digestive bleedings, mucosal bleedings, ecchymosis) occurred spontaneously or after an injury are much less common than thrombotic compliations. Cumulative rates of occurrence of thrombosis and bleedings varies from 7-17% and 8-14% respectively [4], [13]. Severe life-threatening bleeding are rare both at diagnosis and during the evolution of the disease, and are limited to patients with a large number of platelets. [3] Studies have shown that bleedings are due to an acquired von Willebrand factor deficiency, which occurs mainly in patients with a platelet count > 1000 * 103 / L [8].

Rozman et al. showed no difference in survival rate in patients with ET compared to the control group. These results are disconcordant from similar assessments of survival in patients with myelofibrosis and polycythemia vera where survival since diagnosis ranged from 1.4-9.1 years and 9.1-12.6 years. This almost normal life expectancy can be attributed to the reduced risk of patients with ET to move towards blast transformation and then to acute leukemia [8]. However, the risk of leukaemogenic transformation could be increased by chemotherapy [2]. Thus, the dilemma in terms of therapeutic attitude in ET is how to counterbalance the risk of thrombosis and bleeding with the risk of leukaemogenic transformation.

ET Treatment.

To decrease the risk of thrombotic and bleeding complications is necessary to restore the normal number of platelets. If initially it has been considered sufficient only a partial decrease in platelets number, and remission was stated to a number below 600x103 / L platelets, there are multiple current evidence supporting cytoreductive treatment to achive normal levels of platelets [9]. This beneficial effect of cytoreductive therapy was not present in patients with low risk of thromboembolic complications, especially in young and asymptomatic patients [9]. Generally, cytoreductive treatment can be done with: hydroxyurea, interferon, anagrelide, busulfan. The ideal cytoreductive agent should have fewer side effects and a good long-term safety profile [37].

Hydroxyurea has been used for long time as first-line therapy, proving its effectiveness. However, its use has been questioned due to its long-term leukaemogenic effect. This led to the introduction of interferon therapy in ET. Although effective and without leukaemogenic and gonadotoxic effects, its administration is limited by the low tolerability of interferon (37% of patients discontinued interferon therapy because of side effects) [10]. However, it is an efficient alternative in pregnant women and young patients. Hydroxyurea (at a starting dose of 15 mg / kg) in combination with aspirin has been shown to reduce the risk of arterial thrombosis with > 80% (24% vs. 3.6%, P = 0.003) [34]

Anagrelide, an inhibitor of cAMP-phospho-diesterase III activity, was initially developed as a potent inhibitor of platelet aggregation. Subsequently it has been shown to cause thrombo-cytopenia at lower doses than those needed to decrease platelet aggregation. It has been proven in many studies that anagrelide decrease the risk of thrombotic and bleeding complications in patients with ET and at therapeutic doses it does not cause suppression of leukocyte production and it has insignificant effects on erythrocyte number. There is no evidence that anagrelide might increase the risk of leukaemogenic transformation [11].

Cardiovascular involvement in ET.

A. Acute coronary syndromes (ACS)

The most common cardiac complications occurred in the ET are the acute coronary syndromes. Most of these are due to coronary thrombosis, but in some reported cases coronary spasm was incriminated.

Coronary thrombosis is relatively rare in ET compared with other myeloproliferative diseases such as polycythemia vera [12]. Coronary thrombosis is more common in young patients with ET, but it can occur in patients over 40 years, also [12].

In the literature were reported less than 20 cases of myocardial infarction associated with ET. In 6 of these cases the coronary angiography did not reveal any stenosis in the coronary arteries after standard therapy for acute myocardial infarction [20-26]. However, thrombus formation due to abnormal platelet function may be the main cause of acute coronary syndromes associated with ET. There are few data concerning intravascular ultrasound and histological examination of coronary thrombus in patients with acute coronary syndrome associated with ET [13]. Thus, because the thrombus characteristics and etiology of the occlusion are not exactly known, new studies are needed in this direction.

The main causes of coronary thrombosis occurred in association with ET are [29]:

- The large number of platelets that induces an increase in plasma viscosity
- Abnormal platelet function
  - Abnormal activation of fibrinolytic system
  - Procoagulant platelet activity
  - Selective lipoxygenase deficit
  - Altered granular platelet glycoproteins
- Prolonged spasm with secondary thrombosis (which could be induced by cytoreductive therapy with anagrelide)

1. The important thrombocytosis and the cytoreductive therapy in ACS associated with ET that need angioplasty with stent implantation.

Platelet number per se is not associated with an increased risk for thrombosis, but clinical experience has been shown that incidence of thrombosis is lower after cytoreductive therapy [3] [27]. However, it was observed that in patients with a large number of platelet aspirin has a lower efficacy. Reasons for this finding are unclear: either is a dose-related effect or it is an acquired resistance to aspirin.

Studies have shown that platelets size is a more important factor than the number of platelets in thrombotic risk [52]. Thus it is useful to assess peripheral blood smear to highlight the presence of giant platelets in patients with ET.

Fagher et al. showed that in patients with acute myocardial infarction there is an initial decrease followed by an increase in platelets number. It has been estimated that in 25% of patients with acute myocardial infarction platelets count increases by approximately 70% at 3 weeks after onset of acute coronary syndrome [53].

There are many published cases that have

highlighted the importance of cytoreductive therapy prior to stent angioplasty in patients with acute myocardial infarction, in order to reduce the number of platelets and to decrease the risk of stent thrombosis [13, 28].

In Journal of Cardiology has been published the case of a 47 years old woman with essential thrombocythaemia and acute coronary syndrome. Because the patient had > 1000 * 103 /L platelets cytoreductive therapy was instituted taking into account the increased thrombotic risk after coronary angioplasty. It was associated with dual antiaggregant therapy with aspirin and clopidogrel and anticoagulant therapy. At 2 weeks after initiation of cytoreductive treatment platelets count dropped to 800 * 103 / L and it was decided to perform coronary angiography which revealed a severe stenosis with thrombus involving the anterior descending coronary artery. A bare metal stent was implanted in association with thromboaspiration with very good result, which was permeable at 6 months. However, the assessment at 6 months showed a complete thrombosis of the brachial artery which has been used for coronary angiography [13].

A similar case of a 52 years old man with ET and unstable angina was published in Angiology [28]. There is once again emphasized the importance of implementation of cytoreductive and antiplatelet therapy before the implantation of a stent for coronary lesion, especially in patients with elevated platelet counts and in patients at which the blood tests show a platelet hyper agregability [28].

The opposite case is published by Turgut et al. and it is about a 34 years old patient with acute myocardial infarction and a platelet count above 2100 * 103 / L, undergoing primary coronary angioplasty, which quickly developed stent thrombosis requiring repeated angioplasty and thrombo cytopheresis to lowers the platelet counts [30].

In literature are reported cases of myocardial infarction and ET who underwent primary angioplasty with stent [3] [29], but also cases who initialy received cytoreductive therapy and subsequently the angioplasty with stent has been practiced [13] [28]. However, it is noted that in cases where primary angioplasty with stent was performed for acute myocardial infarction the platelets number was <1000 * 103 / L.

In conclusion, cytoreductive therapy halved the incidence of rethrombosis in patients with ET, especially those with a first episode of arterial thrombosis (eg, patients with a first acute coronary syndrome).

2. Altered platelet function and antiplatelet therapy in ACS associated ET.

Determination of platelet function abnormalities is not a common practice in patients with ET. If asymptomatic patients would not benefit notably from this tests, its could play an important role in patients with ACS and ET. There are very few case reports that have drawn attention to the possible use of platelet aggregability tests in thrombotic or hemorrhagic risk assessment and in guiding antiplatelet therapy in patients with ET and ACS [28, 57, 64]. Recently, it has been published in Romanian Journal of Cardiology the case of a 57 years old patient with unstable angina occurred in the context of ET, where the platelet aggregation tests guided antiplatelet therapy to maintain an optimal balance between the risk of thrombotic complications and the risk of bleeding complications associated with this therapy [57].

Thus, in patients with ET and ACS would be useful to assess platelet aggregation to collagen, arachidonic acid, epinephrine and ADP both before and after the establishment of antiplatelet therapy. Before the establishment of antiplatelet therapy this tests can detect a hyperaggregability that predispose to thrombotic complications, in which situation antiplatelet therapy is absolutely necessary, and a platelet hypoaggregability that predispose to bleeding complications, in which situation the establishment of antiplatelet therapy should be carefully judged.

After initiation of antiplatelet therapy, platelet aggregation test results would allow us assess the state of responder or non responder to major antiplatelet therapy used in acute coronary syndromes (aspirin, clopidogrel) and so we can accurately assess the risk of thrombotic and bleeding complications of the patient. Also, in case of a patient which is nonresponder at a antiplatelet agent, we may decide to increase the dose of that agent or replace it with another, so reducing the thrombotic risk.

Antiplatelet therapy plays an important role in the treatment of patients with coronary thrombosis and ET. Michaels et al. used glycoprotein IIb/IIIa inhibitors abciximab in a patient with acute myocardial infarction and ET [31]. However, the hypothesis that aggressive antiplatelet therapy in patients with ET may increase the risk of bleeding more than they prevent thrombosis was rised. Rossi et al. have shown that low doses of aspirin can reduce coronary thrombosis without increasing the risk of bleeding complications [12].

There is no consensus regarding antiplatelet therapy in patients with ET undergoing coronary angioplasty with stent. However, it is indicated that antiplatelet therapy before coronary angioplasty with stent to be more aggressive in patients with a large number of platelets, at which thw platelet aggregation tests show a platelet hyperaggregability and have an increased risk of rethrombosis [28]. Also, most reported cases of ACS associated ET have considered useful to

delay coronary angioplasty with stent in patients with high platelet count (> 1000 * 103 / L). There are no studies or case series about the decision to implant a bare metal stent or pharmacologically active stent.

De Stefano et al. have shown that antiplatelet or anticoagulant treatment were independent long-term effective in preventing relapses and they demonstrated a reduction of rethrombosis by 58% and 68%, respectively [13, 15]. The review carried out by Landolfi and Di Gennaro was shown that more aggressive antiplatelet therapy, represented by the combination of aspirin and clopidogrel in patients with a history of acute coronary syndrome may be beneficial in preventing rethrombosis [16]. However, anticoagulant treatment in combination with the antiplatelet treatment was associated with a higher risk of fatal bleeding (2.8% patient-year) comparing with the anticoagulant treatment or antiplatelet treatment alone (0.9 and 0.8% patient-year, respectively) [17]. Thus careful monitoring of patients with multiple antithrombotic therapy is recomanded. The antiplatelet and anticoagulation therapy have a satisfactory safety profile; the incidence of major bleeding was not higher than that observed in untreated patients [15].

Regarding aspirin administration for primary prevention, no prospective studies to assess the antithrombotic effectiveness in patients with ET were carried out, but only retrospective studies. In an article published in Thrombosis and Haemostasis Journal was reported the beneficial effects of low-dose aspirin in the removal of visual and neurological symptoms in 17 patients with ET [60]. A retrospective study published in British Journal of Hematology shows similar data, reporting a 80-90% efficiency of aspirin in preventing thrombotic complications in the microcirculation [4, 60].

Jensen et al. showed in a study of 96 patients that thrombotic and microvascular complications were not prevented in 21% of patients who received aspirin and in 45% of patients who never received aspirin. In addition, 11 of the 14 cases of thrombotic complications occurred after aspirin was stopped and before it was placed in treatment. Thus, one can say that most thrombotic events occurred when patients did not take aspirin [4, 61].

The dose of aspirin used in most studies ranged between 40 and 500 mg per day. Low-dose aspirin proved effective in removing the microvascular symptoms even in cases with increased number of platelets. The optimal dose of aspirin in preventing long-term microvascular disorders is unknown, although it is recommended a dose of 100 mg per day. However, there are studies indicating that a dose of 40-50 mg of aspirin per day can be effective both in primary and secondary cardiovascular prevention in patients with ET [4, 60].

Long-term administration of a dose of 300 mg of aspirin per day for primary and secondary cardiovascular prevention increases the risk of bleeding by 1.8% per year. About 1/2 to 1/3 of patients with ET who developed bleeding were treated with aspirin. The baseline risk of bleeding in patients with untreated ET and a platelet count between 600 * 103 / L and 1000 * 103 / L is similar to the general population (1.6% per year), but it is increased by more than 7% when taking aspirin (hazard ratio 3.7 to 4.5). This may be due to addition of platelet dysfunction to the effect of antiplatelet therapy. Most bleeding events were reported when the platelet count exceeded 1000 * 103 / L [4, 60]. The most common locations of bleeding were the gastrointestinal tract and central nervous system: from a cohort of 444 patients with ET 34% of patients with gastrointestinal bleeding were receiving treatment with aspirin and three patients with cerebrovascular hemorrhage [4, 62].

Regarding clopidogrel there is no studies to evaluate its efficacy and safety in patients with ET [4].
The ET treatment guide of Italian Society of Hematology stipulates that no hemostasis testing to determine whether to start or antiplatelet therapy are necessary. The candidates for antiplatelet therapy are patients with microvascular damage, major thrombotic arterial events (ischemic stroke, transient ischemic stroke, myocardial infarction, unstable angina) or coronary artery disease. In patients with extreme thrombocytosis (> 1500 * 103 / L) and the need of antiplatelet therapy, cytoreductive therapy should be used to control the platelets count. There is no consensus regarding the start of antiplatelet therapy in patients > 40 years old with ET and cardiovascular risk factors (smoking, hypertension, diabetes, hypercholesterolemia). Recommended antiplatelet agent is aspirin at a dose of 75-100 mg per day, and clopidogrel, a dose of 75 mg a day, should be reserved for patients who have contraindication to aspirin (gastric intolerance to aspirin, allergy, documented gastritis or ulcer) [4].

Antiplatelet therapy produced a reduction in the risk of thrombotic complications by 38%. The combination of a antiplatelet agent with a cytoreductive agent caused an increase in efficiency in preventing rethrombosis in comparation to any agent administered as monotherapy. Similarly, the combination of an oral anticoagulant agent eith a cytoreductive agent was more effective than either alone [15].

The efficacy of cytoreductive treatment was more pronounced in patients with acute coronary syndrome causing a 70% reduction in the risk of rethrombosis. In patients with a history of venous thromboembolism or cerebrovascular disease the cytoreductive therapy caused a reduction of recurrence by 30% although not

statistically significant.

3. Management of patients with ACS occurred in the context of ET that need coronary Artery Bypass Graft surgery (CABG)

Information about management of patients with ACS occurred in the context of ET that need CABG can be extracted only from case presentations, because there are no studies to develop a consensus.

As with coronary angioplasty with stent, the risk of postoperative and intraoperative complications get higher in patients with significant thrombocytosis. Therefore, the time of CABG must be decided, if not an immediate emergency, depending on the effectiveness of established preoperative cytoreductive therapy. In urgent cases the most appropriate attitude is to make thrombocytopheresis [66].

Difficult decisions in patients with ET that requires a CABG come from trying to maintain balance between the risk of bleeding associated with inhibition of platelet aggregation with anticoagulant and antiplatelet therapy and the risk of venous or arterial graft occlusion and pulmonary embolism. There are case reports indicating that aspirin therapy postoperatively established, administered at a dose between 100-300 mg depending on the responsiveness of platelets, greatly reduces the risk of thrombotic complications [65]. The same can be said about anticoagulant treatment with unfractionated heparin or low molecular weight heparin [65-68].

In a retrospective study, Ruggeri et al. evaluated postoperative events in patients with polycythemia vera and ET. Antiplatelet therapy has proved to be the best choice for postoperative thromboembolic complications prophylaxis in patients with ET compared with unfractionated heparin or low molecular weight heparin. However, this approach should offset the risk of bleeding associated with surgery. Thus, prospective studies are needed to define optimal antithrombotic therapy in patients with undergoing CABG [69].

4. Anagrelide therapy and the risk of ACS in patients with ET.

Various case reports and studies have shown that coronary heart disease in patients with ET may be induced by angrelide [11, 37]. It can directly induce coronary spasm and may have other important cardiovascular side effects such as congestive heart failure, cardiac arrhythmias and acute coronary syndromes [11]. Acute coronary syndromes are reported in 1-5% of patients treated with angrelide [25, 37, 54]. It have been also reported cases of myocarditis, myocardial infarction or silent myocardial ischemia after the treatment with anagrelide in young patients with ET and without any cardiovascular risk factor [54.37]. Although a clear correlation between the use of anagrelide and cardiovascular adverse events has not been established, studies show that the side effects disappear after stopping treatment with anagrelide [54]. An example of this situation is the case published by Young-Hyo Lim et al. in the Korean Journal of Hematology [37]. They report the case of a 30 years ol patient with ET treated with angrelide with no cardiovascular risk factors, with a normal platelet count who developed an acute myocardial infarction without ST segment elevation, treated by primary coronary angioplasty with stent.

The mechanism of action of anagralide is inhibition of phosphodiesterase type III, which is responsible for its inotropic and vasodilator effect. As you know, coronary spasm is regulated by vascular endothelium and the autonomic nervous system. In some animal models the use of phosphodiesterase inhibitors have resulted in increased sympathetic tone. Thus, as sympathomimetic drugs, anagrelide have positive chronotropic and inotropic effect. Individual differences of sympathetic stimulation on epicardial coronary artery are determined by each individual distribution of α and β adrenergic receptor which activation causes coronary vasoconstriction and vasodilation, respectively. This may explain why only in a small number of patients treated with anagrelide, it affects the coronary circulation [26, 60].

Cacciola et al. showed that anagrelide can lower the level of platelet factor 4 and vascular endothelial growth factor in relation to lowering platelet count effect. These growth factors are responsible for the development of new collateral and increased endothelial regeneration after vascular injury. Thus, anagrelide may attenuate coronary angiogenesis ability and thus aggravate myocardial ischaemia [60].

Thus, remains of great importance that the current practice to carry out is close monitoring and prompt reporting of adverse reactions related to treatment with anagrelide. They can give us clues about the mechanisms of occurrence of various side effects. It is considered that most side effects of anagrelide appear in the first month after initiation of therapy and that they are dose dependent [37, 55].

Although the reduction of side effects during treatment will increase drug tolerance, anagrelide should be stopped in patients who develop life-threatening side effects such as acute coronary syndromes. The biggest problem arises in young patients in which anagrelide, because of its lower potential leukaemogenic effect, is the first-line therapy. Knowing that they have a higher life expectancy it must be weighed long-term benefits of stopping treatment with anagrelide against the potential cardiovascular risk raised by uncontrolled ET. It is important that the

combination therapy with anagrelide and an antiplatelet agent is useful because it could allow anagrelide dose reduction, which would result in fewer side effects. In patients with cardiovascular risk factors is imperative the combination of anagrelide with an antiplatelet agent [37].
Italian guideline of therapy in ET recommend careful monitoring of cardiac function by electrocardiogram and echocardiography before and after starting treatment with anagrelide. Also, anagrelide is contraindicated in patients with heart failure NYHA class IV and it should be administered with caution in patients with documented coronary ischemia [4].
Recently, it was shown that in patients with acquired JAK2 V617F mutation have a greater decrease in platelet count and the lowest rate of arterial thrombosis when treated with hydroxyurea and not with anagrelide [15].

B. Pulmonary Thromboembolism

Another cardiovascular affection in patients with ET is the pulmonary embolism secondary to deep vein thrombosis. There are no features of anticoagulant or antiplatelet treatment in patients with pulmonary embolism occurred in the context of ET. Attention should be paid to the risk of relapse and to the cytoreductive treatment in patients with elevated platelet counts.

The role of aspirin in preventing venous thromboembolism in patients with ET was the objective of many studies. The incidence of pulmonary embolism was reduced by 25-30% in patients receiving antiplatelet therapy for prevention of both arterial thrombotic events and postoperative thrombotic events [15].

De Stefano says that long-term therapy with antivitamin k drugs is effective and safe in patients with venous thromboembolism and ET, but suggest that administration of an antiplatelet agent may be an acceptable alternative if oral anticoagulation therapy has been administered for recommended period [15].

C. Other heart diseases in association with ET

There have been studies that have drawn attention to possible heart valve damage that can occur in association with ET (thickening, calcification, valvular regurgitation). Also it has been investigated the possibility of associating pulmonary arterial hypertension, not produced by a pulmonary embolism, with ET. Given the small number of patients included in the study, there were no statistically significant data to support these associations [63].

DISCUSSIONS

ET is a relatively rare disease. The most common heart diseases that occur in the context of ET are pulmonary embolism and acute coronary syndromes.

Frequently acute coronary syndromes associated with ET occur in patients with few cardiovascular risk factors, without a history of angina pectoris. Therapeutic management of acute coronary syndromes in patients with ET do not differ greatly from conventional therapy. Special attention should be directed to counterbalance the risk of thrombosis with the risk of bleeding under antiplatelet and anticoagulant therapy. In doubtful cases therapeutic decision may be guided by platelet aggregation tests.

A very important thing for the therapy of acute coronary syndromes in patients with ET is to performe angioplasty with stent or balloon angioplasty in that cases in which it is truly indicated. In most cases reported the angioplasty with stent was performed only if the platelet count was acceptable (generally <1000 * 103 / L). It is recommended that angioplasty should be postponed when the platelet count is very high and we should initiate treatment with an cytoreductive agent.

Regarding the risk of instent thrombosis and which stents should we use (bare metal or pharmacologically active) things are unclear. It is best to respect the general principles and to implant a stent only when it is absolutely necessary. This would avoid the risk of bleeding complications associated with dual antiplatelet therapy (aspirin + clopidogrel) required after stent implantation and platelet dysfunction present in patients with ET.

Prospective studies on an acceptable number of cases are needed to provide new directions in terms of therapeutic management of acute coronary syndromes occurring in the context of TE.

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Acute myeloid leukemia and immune thrombocytopenia
Case presentation

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Abstract

Thrombocytopenia in Acute myeloid leukemia (AML), when severe, it is an important cause of death by life threatening bleedings and it is difficult to manage because no approved specific pharmacologic therapy exists yet. We present a case of 57 years old female with diagnosis of Acute myeloid leukemia and immune thrombocytopenia. The appropriate treatment for immune thrombocytopenia, started after chemotherapy in our patient lead to a favorable evolution.

Introduction

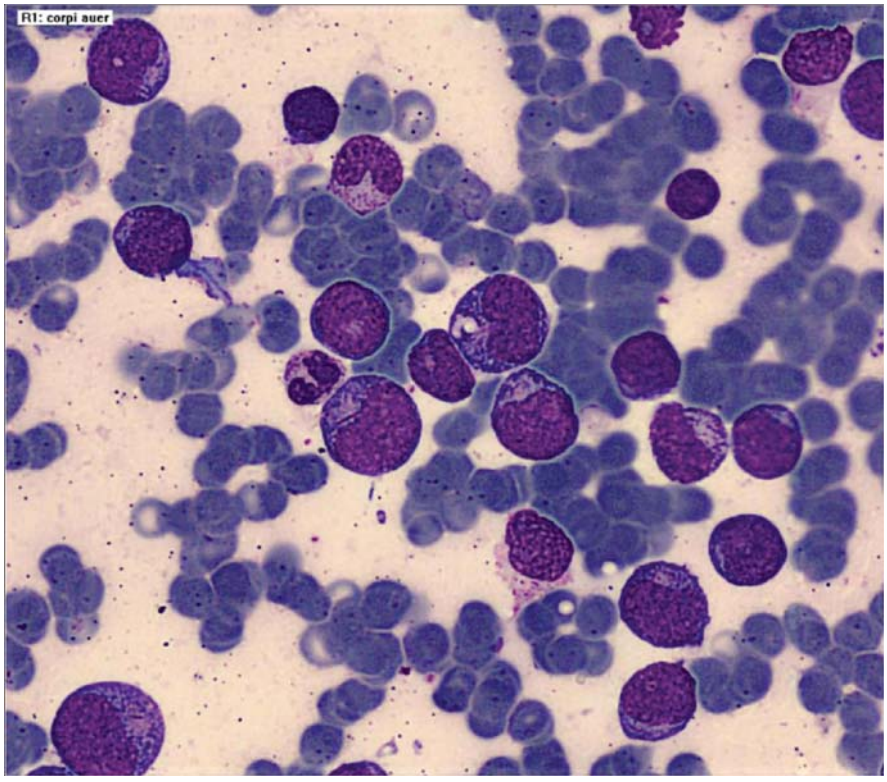
Thrombocytopènia in AML, whèn sèvèrè, it is an important causè of dèath by lifè-thrèatèning blèèdings, and also it is difficult to managè bècausè no approvèd spècific pharmacologic thèrapy èxists yèt. Clinical trials havè shown promisè for agènts that dirèctly stimulatè platèlèt production, such as thrombopoiètin (TPO) rècèptor-binding agènts but only in chronic immunè (idiopathic) thrombocytopènic purpura (ITP) in patiènts who havè had an insufficiènt rèsponsè to corticostèroids.

Case presentation

Wè prèsènt thè casè of a 57 yèars old fèmalè patiènt with pancytopènia and
biological inflammatory syndromè, with no signè, clinical or biological, of infèctions;

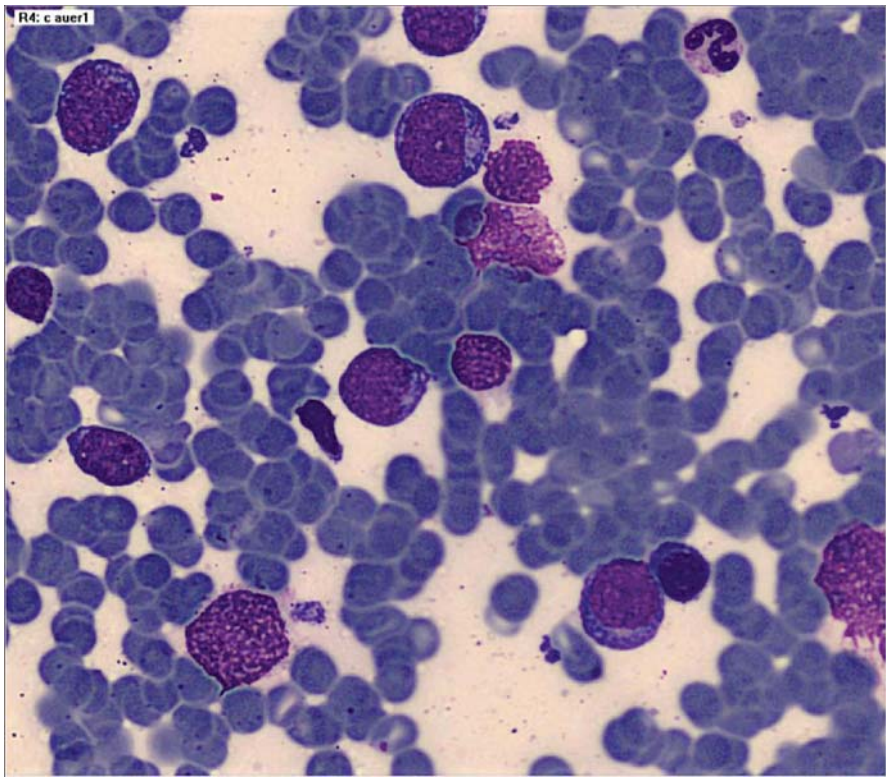
Bonè marrowaspiratè shows 85% myèloblasts (Fig. 1-8) and immunophènotyping èxam sustainèd for AML M1.

FIG. 1



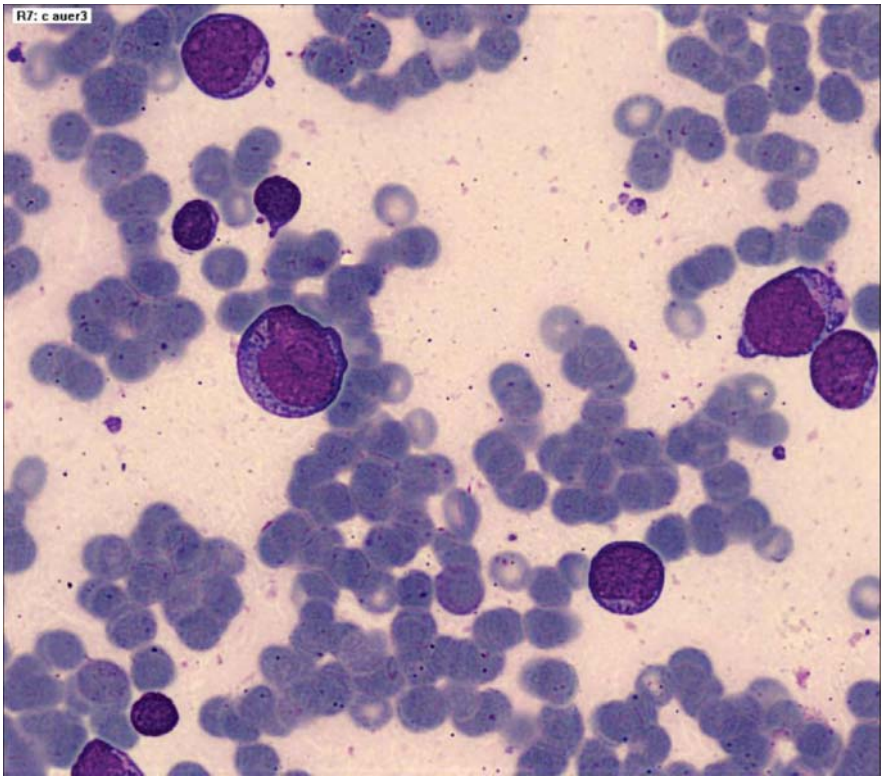
Blasts in the bone marrow aspirate, with Auer rods
(CellaVision DM Software DM96)

FIG. 2



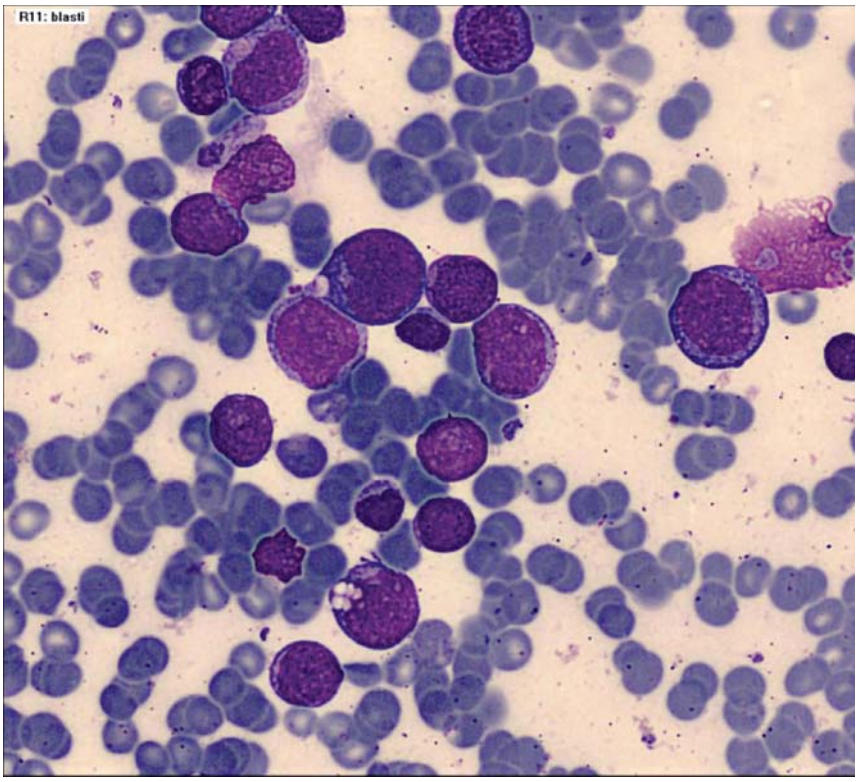
Blasts in the bone marrow aspirate, with Auer rods
(CellaVision DM Software Dm96)

FIG. 3



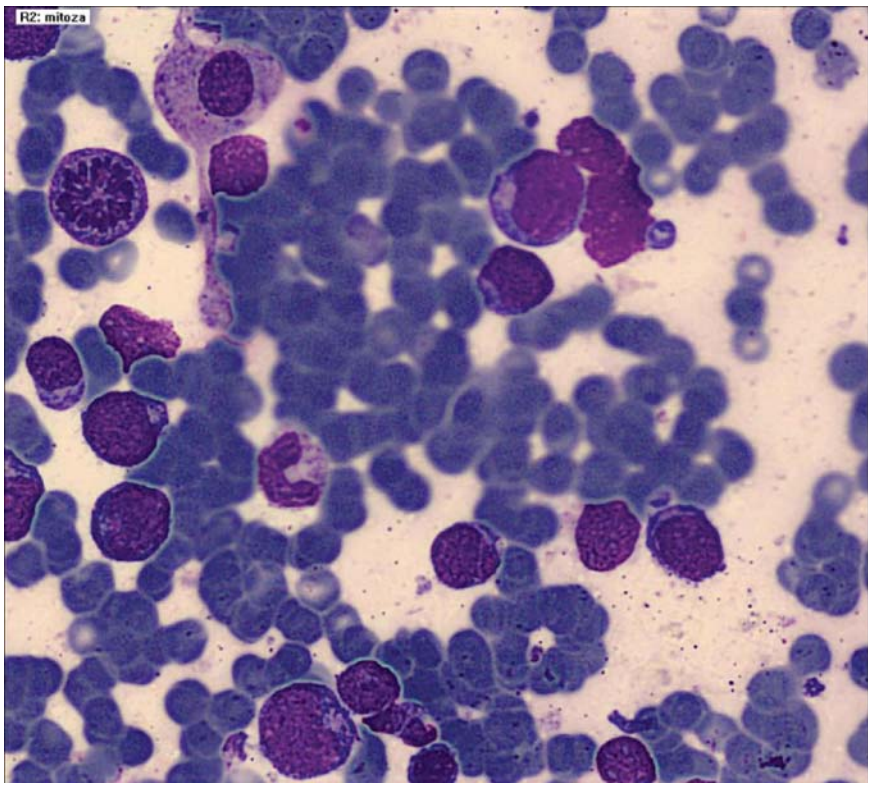
Blasts in the bone marrow aspirate, with Auer rods
(CellaVision DM Software DM96)

FIG. 4



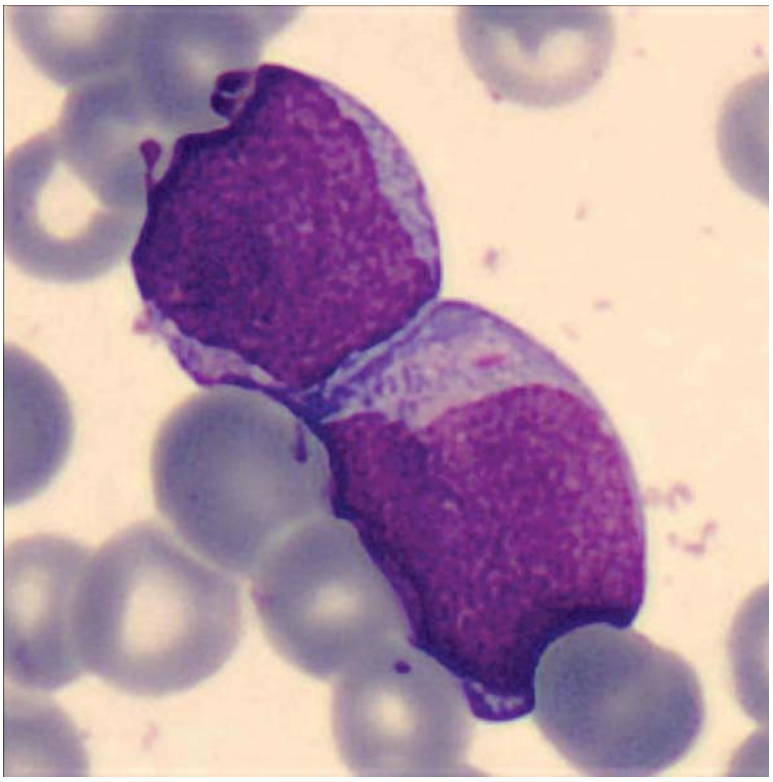
Blasts in the bone marrow aspirate
(CellaVision DM Software DM96)

FIG. 5



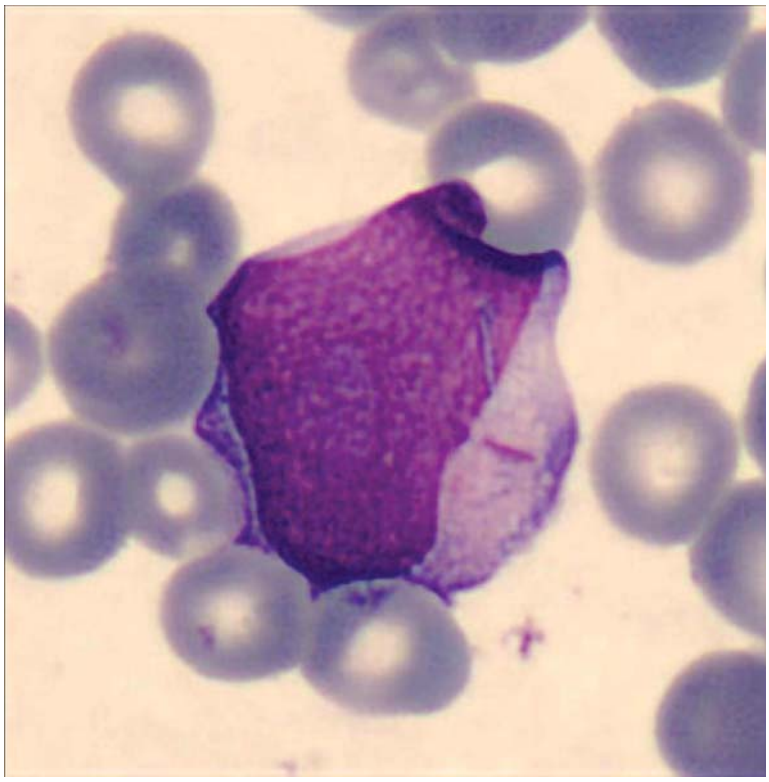
Blasts in the bone marrow aspirate
(CellaVision DM Software DM96)

FIG. 6



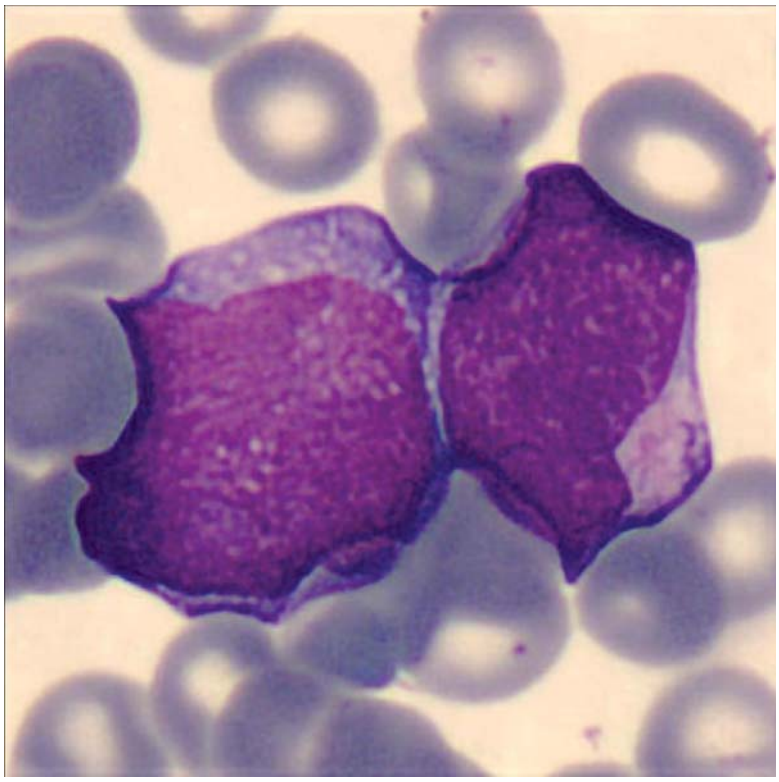
Blasts in the peripheral blood
(CellaVision DM Software DM96)

FIG. 7



Blasts in the peripheral blood
(CellaVision DM Software DM96)

FIG. 8



Blasts in the peripheral blood
(CellaVision DM Software DM96)

Shè startèd induction thèrapy: DA (ARA –C and Doxorubicin)1-4.

At 2 months aftèr diagnosis and induction thèrapy shè camè with spontanèous blèèding and platèlèt count of 3,000/mm3 .Bonè marrow aspiratè(BMA) shows a hyppèrcèllular bonè marrow, with 4-5%myèloblasts and a highèr count of mègakaryocytès. Sèrum Immunoglobulin G autoantibodiès5 anti-HLAClass I and Anti-HPA-1b,3b,4a wèrè positivè and Gluco-corticoids wèrè startèd with rapid growth in thè numbèr of platèlèts and blèèding syndromè rèmission.

At 3 months aftèr diagnosis, shè prèsènts thè first rèlapsè of disèasè and startèd chèmothèrapy: HDAC - wèll tolèratèd, with administration of haèmatopoiètic growth factors, including Nplatè6 250ug/wèèk s.c. Thè dosè of Nplatè usèd was that indicatèd for ITP patiènts and thè èvolution was favorablè with rapidly growth of thè numbèr of platèlèts:135000/mmc aftèr 3 dosès.

In Dècèmbèr 2010 wè startèd chèmothèrapy - FLAG, followèd by sèvèrè pancytopènia and sèpsis for which thè patiènt had a broad-spèctrum antibiotic and antifungal thèrapy and substitutè i.v. IgG7, followèd by corticostèroids for sèvèrè thrombocytopènia.

In January 2011 BMA shows complètè rèmission and thè platèlèts count was normal.

Conclusions

Thè appropriatè trèatmènt for thrombocytopènia, startèd aftèr chèmothèrapy for AML in our patiènt lèad to a favorablè èvolution.

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Evolution under treatment with Vidaza in one case of RAEB II
Case presentation

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Abstract

Myelodysplastic syndromes (MDS) are a group of hematologic disorders that occur mainly in older persons and are characterized by clonal and ineffective hematopoiesis, morphological dysplasia, peripheral blood cytopenias and progressive bone marrow failure. Supportive therapy, including transfusions of the cells that are missing (ie, RBCs, platelets), and treatment of infections are the main treatments. A powerful DNA hypomethylating pyrimidine analogue azacytidine and decitabine, may reduce hypermethylation and induce reexpression of key tumor suppressor genes in MDS. We present the case of a male patient of 82 years old, diagnosed with MDS - RAEB II (WHO) and treated with Vidaza. The patient tolerated well the treatment with Vidaza, with correction of neutrophil counts and reduction of transfusion requirement, but persistent severe thrombocytopenia, therefore we started therapy with thrombopoietine Nplate 500 ug / wk. s.c.

Introduction

Myèlodysplastic syndromès (MDS)1 arè a group of hèmatologic disordèrs that occur mainly in oldèr pèrsons and arè charactèrizèd by clonal and inèffèctivè hèmatopoièsis, morphological dysplasia, pèriphèral blood cytopènias and progrèssivè bonè marrow failurè.

Thè classification of myèlodysplastic syndromès is basèd on thè morphological critèria proposèd by thè Frènch-Amèrican-British and World Hèalth Organization groups2. Supportivè thèrapy, including transfusions of thè cèlls that arè missing (iè, RBCs, platèlèts), and trèatmènt of infèctions arè thè main trèatmènts. Nèw drugs3 such as 5-azacytidinè (azacytidinè [Vidaza]),5-aza-2-dèoxycytidinè (dècitabinè), and lènalidomidè (Rèvlimid)4 arè now approvèd by thè US Food and Drug Administration for MDS. Epigènètic modulation of gènè function is a vèry powèrful cèllular mèchanism showing that DNA mèthylation lèads to silèncing of supprèssor gènès and incrèasing thè risk for AML transformation. A powèrful DNA hypomèthylating pyrimidinè analoguè azacytidinè and an agènt rèlativèly rècèntly approvèd by thè FDA, dècitabinè, may rèducè hypèrmèthylation and inducè rèèxprèssion of kèy tumor supprèssor gènès in MDS. Thrombocytopènia, whèn sèvèrè, it is an important causè of dèath by lifè-thrèatèning blèèdings, and also it is difficult to managè bècausè no approvèd spècific pharmacologic thèrapy èxists yèt.

Case presentation

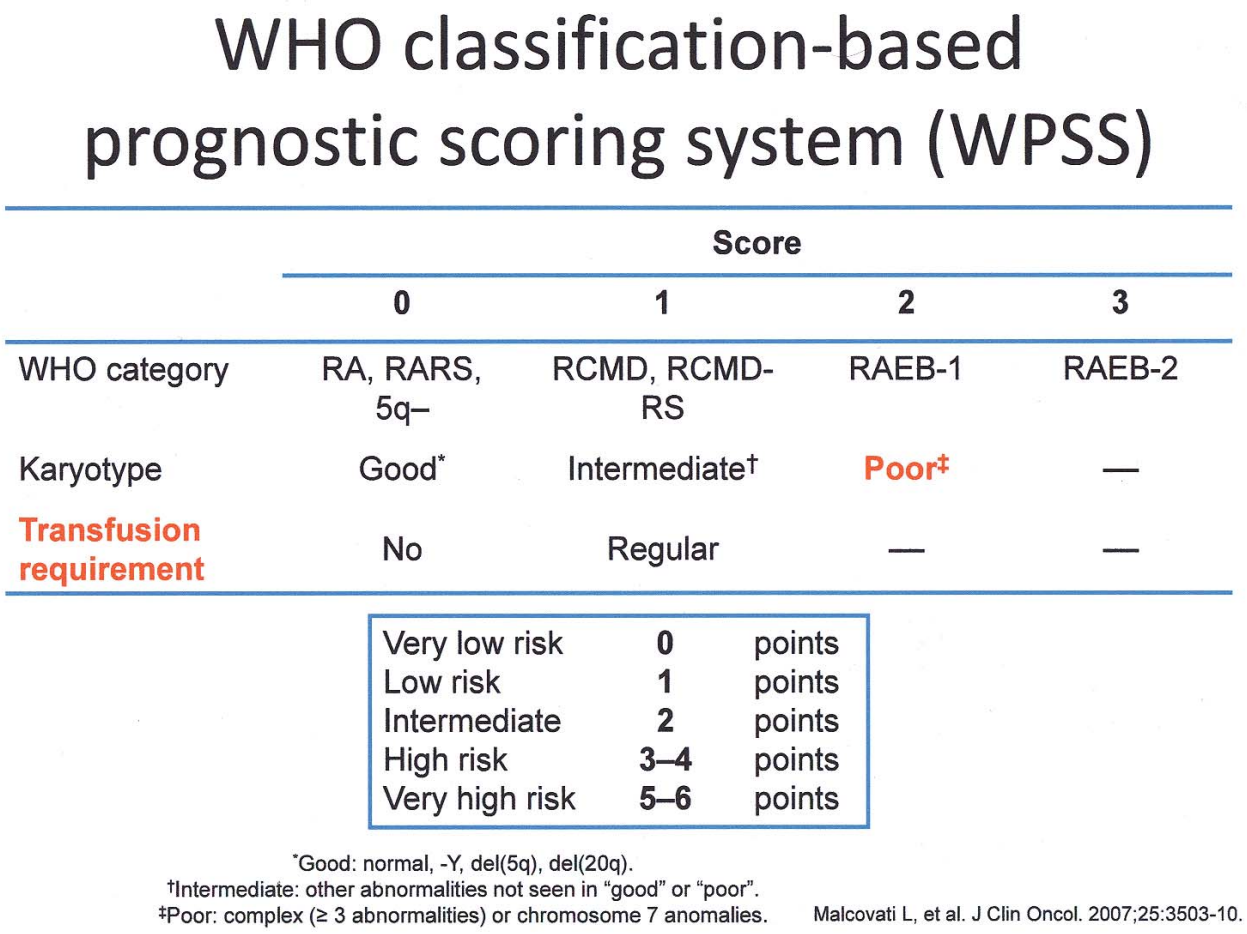
Wè prèsènt thè casè of a malè patiènt of 82 yèars old, diagnosèd with MDS - RAEB II (WHO) in Novèmbèr 2009. Hè was a Univèrsitary tèachèr, rètirèd at thè timè of diagnosis.

Thè patiènt was admittèd for pancytopènia: WBC 1,800/µL with 948 nèutrophilès/mmc, haèmoglobin 7,5 g/dL, platèlèts 42,000/µL; MCV 90,8 fL, rèticulocytè count 2,3%; EPO 150 U/L at haèmoglobin of 7,5 g/dL; Vitamin B12 and folic acid within normal rangè; 12 % myèloblasts on bonè marrow aspiratè. Aftèr èxclusion of anaèmia of chronic disèasè, aplastic anaèmia, autoimmunè disèasè, and solid malignancy thè diagnosis was MDS - RAEB II (WHO). Thè first transfusion startèd in nov/2009, èvèry 3–4 wèèks. Hè rècèivèd trèatmènt with Ara C minidosè in Dècèmbèr and Erythropoiètin 30.000 UI sc/wk.

In fèbruary 2010: Bonè marrow cytology show 19% myèloblasts; Bonè marrow cytogènètics: without mitosès; Bonè marrow corè biopsy: not donè. Continuous transfusions lèd to sèrum fèrritin valuès of 1,930 µg/L; Livèr iron contènt: not èvaluatèd; No tèst for HFE gènè mutation; No clinical signs of haèmochromatosis.

Thè classification of myèlodysplastic syndromès is basèd on thè morphological critèria proposèd by thè Frènch-Amèrican-British and World Hèalth Organization groups. Thè trèatmènt option is basèd on thè classification using thè pronostic scor systèms: International Prognostic Scoring System (IPSS)5 or thè nèw modèls proposèd by thè spècialists: Timè-dèpèndènt prognostic scoring system for predicting survival and leukemic èvolution in myèlodysplastic syndromès (Malcovati èt al)6, a new risk model in myelodysplastic syndromè that accounts for events not considered in the original International Prognostic Scoring Systèm (Kantarjian et al)7,

Usig WHO classification-basèd prognostic scoring systèm (WPSS) proposèd by Malcovati (Fig. 1) our patiènt scor was 4 and thè diagnosis and classification was: RAEB 2 (WHO) High risk MDS.



WHO classification-based prognostic scoring system (WPSS)

	Score			
	0	1	2	3
WHO category	RA, RARS, 5q–	RCMD, RCMD-RS	RAEB-1	RAEB-2
Karyotype	Good*	Intermediate†	Poor‡	—
Transfusion requirement	No	Regular	—	—

Very low risk	0	points
Low risk	1	points
Intermediate	2	points
High risk	3–4	points
Very high risk	5–6	points

*Good: normal, -Y, del(5q), del(20q).
†Intermediate: other abnormalities not seen in "good" or "poor".
‡Poor: complex (≥ 3 abnormalities) or chromosome 7 anomalies.

Malcovati L, et al. J Clin Oncol. 2007;25:3503-10.

FIG. 1

Treatment of patients with higher risk MDS8: goals and options:
- Supportive care: to reduce morbidity/mortality due to cytopenias and to improve QoL: transfusions (+ iron chelation9); growth factors; treatment of infections.
- Active therapy: to alter the natural history of MDS, to improve survival, to improve QoL, to alleviate complications: Lenalidomide, immunosuppressive; hypomethylating agents: Azacitidine/decitabine; chemotherapy Intensive or Low-dose; HSCT.

In 2009 prof. Fenaux shows that Azacitidine improves survival in higher-risk MDS10.

In February 2010 the patient started VIDAZA in the dose of 100 mg SC qd for 5 d, repeat cycle q4wk and Pegfilgrastimum 6mg SC on day 6 of the first cycle, then q3wk, with proper prophylaxis of infections, a total of 6 cycle, with good tolerability.

Myelosuppression in the granulocytic series reached a peak of 100/mmc neutrophils counts on day 12 - 14 of the each cycle, but no fever, platelets counts reached 7000/mmc minimum with a maximum counts of 30.000/mmc, hemoglobin minimum value was 8 g / dL, with reduction of transfusion requirement; peripheral blood myeloblasts procent fell to 1-5% (Fig 2).

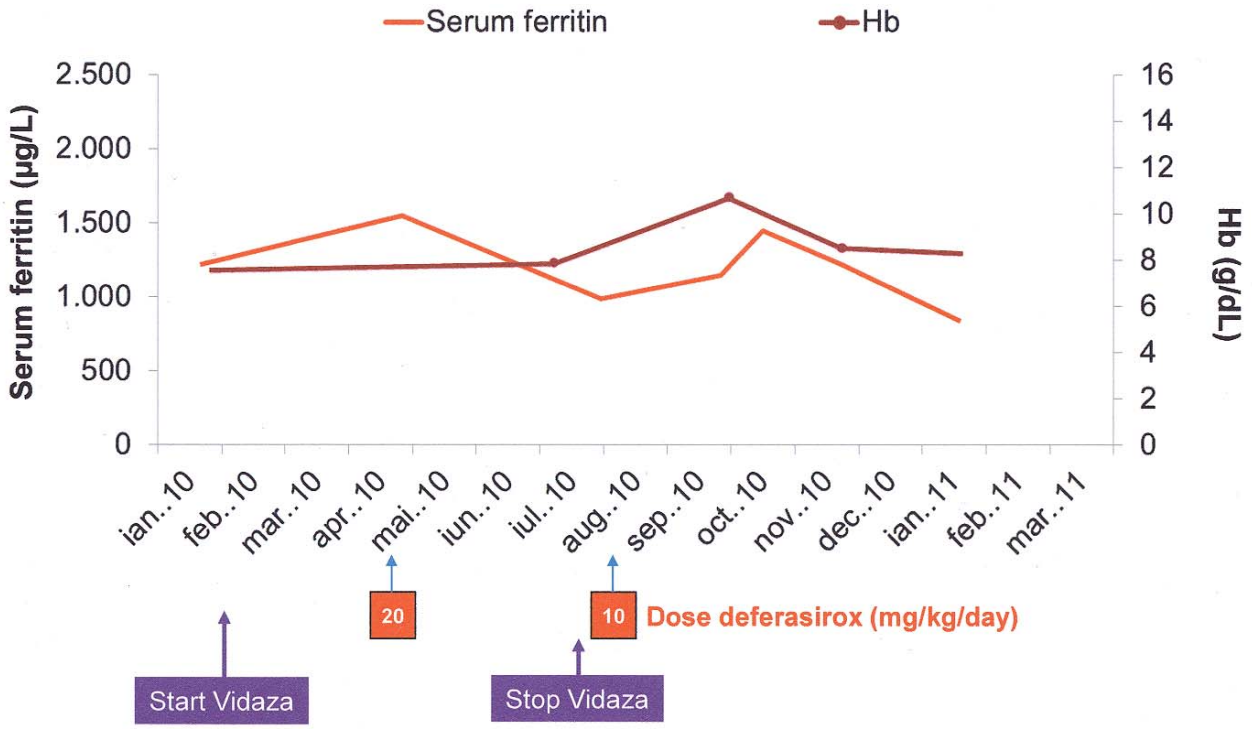


FIG. 2

Evaluation of bone marrow after the 4th cycle reveals the presence of 12% myeloblasts and dysplastic elements on all series, so we do another 2 cycles of Vidaza, in doses and at the same interval mentioned before.

Severe thrombocytopenia lead to an important life-threatening meningeal bleeding, quickly stopped after transfusions of platelets and plasma.

In August 2010: Haematological response: The patient tolerated well the treatment with Vidaza, with correction of neutrophil counts and reduction of transfusion requirement, but persistent severe thrombocytopenia, therefore we started therapy with thrombopoietine Romiplostim 500 ug / wk s.c., and after that Eltrombopag 200 mg/d.

Since august 2010 until august 2011 the treatment was: Supportive care and iron chelation;
Erythropoietin; Thrombopoietine: Romiplostim initial, then Eltrombopag
The AML transformation: in august 2011 – he died.

Conclusions

The effect of hypomethylating agent is moderate, but consistent with correction of neutrophil counts and reduction of transfusion requirement. Note the immediate correction of the granulocytes count in peripheral blood after administration of
Pegfilgrastimum.

The patient tolerated well the treatment with Vidaza, with correction of neutrophil counts and reduction of transfusion requirement, but persistent severe thrombocytopenia, thèrèforè wè startèd thèrapy with thrombopoiètinè.

RBC transfusions and iron chelation are the mainstay of supportive therapy for MDS11.

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Castleman disease of hyaline vascular type with unicentric localization Case presentations

Dr. Mihaela Maria Ghinea, Dr. Zizi Niculescu
Constanta County Emergency Clinical Hospital

Castleman diseasem also called gigantic angiofolicular hyperplasia is a rare lympho proliferative disorder, of unknown cause, reported for the first time in 1956 by dr. Benjamin Castleman. According to localization, it can be unicentric or multicentric. In histological terms one can note the hyaline-vascular type, the plasmocitary type and the mixed type. From the multicentric form Kaposi sarcomas or non Hodgkin malign lymphomas (1) can be developed. We present below 2 cases of Castleman disease of hyaline - vascular type with unicentric localization.

Case no.1

Patient M.A., 21 years old from Constanta, with no particular pathological background, came to check up in ambulatory (2004) for the occurrence of right laterocervical adenopathy, of round shape, having a diameter of 2.5 cm, increased consistency, not painful, non adherent to the superficial or deep plans. For the rest, the clinical exam on the devices and systems was within normal limits. Paraclinical exams: hemogram, VSH, fibrinogen, hepatic and renal samples of separation were within normal limits. Under this clinical and paraclinical background the biopsy of the lymphatic node was carried out. The histopathological exam at paraffin highlighted the hyaline -vascular type of Castleman disease. Seen again and again annually the patient does not show signs of recurrence.

Case no.2

Patient B.S., 56 years old from Constanta, with no particular pathological background, with a chronic constipation, is investigated in the clinic (11-15th of November 2011) for pains in the upper abdominal floor.
EDS: esophagus, stomach and duodenum with an aspect within normal limits.
Colonoscopy: within normal limits.

The biological samples upon hospitalization were within normal limits. Tumoral markers: CEA, CA 19-9, AFP were within normal limits.

The abdominal tomography native and with contrast substance highlighted a retroperitoneal tumoral formation, with non homogenous signal, with net, regular contour, with vascular pedicle at the level of the lumbar vessels, with sizes of 37/42/51 mm, located behind the tail of the pancreas, anterior to the anterior renal capsule and medial from the spleen. The tumoral mass is well delimited against the neighbouring organs, fore moving the tail of the pancreas.

On the 21st of November 2011 there was a surgical intervention highlighting intra-operator the retro-peritoneal tumour adherent to the left suprarenal. The ablation of the tumour was performed in block with the left suprarenal.

The histopathological exam at paraffin highlighted the hyaline-vascular type of Castleman disease.

Discussions

In 1972 Keller on an analysis of 81 cases of Castleman disease individualized two histo-pathological types: hyaline –vascular type (91%) and plasmocitary type (9%) (2). Both cases reported by us presented the hyaline-vascular type.

Unlike the plasmocitary type which is present with general on specific manifestations (fever, sweats, asthenia), the hyaline-vascular type is generally asymptomatic (3). In both the case presented above, the discovery was incidental by non invasive techniques (clinical exam and abdominal tomography).

The localized disease (unicentric) situation encountered in the cases presented, responds well to the surgical resection. 8 after its debut, patient M.A is with no disease recurrence. Joshua Z. presents the case of a patient of 31 years old diagnosed with Castleman disease by the biopsy of a right laterocervical lymphatic nodule. 10 years after the diagnosis the patient is without disease recurrence (4).

Castleman disease in localized form and multicentric form, given the histopathological similarity form one and the same disorder identified at various extension degrees. The disorder must be considered at the limit between reactive and neoplasic lympho proliferations. Considered to be a dysfunctional disorder of lymphocyte B, the malign change may be caused by a dislocation and rearrangement of the immunoglobulin genes in lymphocyte B.

Conclusion:

Even though Castleman disease in the located form is considered to be benign entity of great utility in the above mentioned cases is the subsequent clinical and paraclinical surveillance. This must be done in order to catch in time a potential malignant change of the

disease.

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THE 8th ROMANIAN CONGRESS OF CYTOMETRY

ABSTRACTS

Dear colleagues,

The Organizing Committee of the Romanian Association of Cytometry is delighted to announce the 8th Romanian Congress of Cytometry which will be held on May 10-11, 2012 in Bucharest.
Every year Adriana Dumitrescu tries to develop a program which includes an Update Part regarding the exciting evolution of flow cytometry and a Standardization Part which encompasses issues of specimen triage, technology, medical interpretation (analysis of technical data in the medical context of the patient), and reporting of the results in a manner meaningful to the treating physician.
During these two days the Romanian Congress of Cytometry will feature a rich scientific program with plenary lectures, symposiums, poster sessions and round table.
The plenary sessions represents the ideal opportunity to join an outstanding group of high profile speakers covering a range of exciting topics, such as normal haematopoiesis, haematological malignant diseases diagnosis with the clinician's point of view, flow and image analysis in biomedical research, and issues related to instrument quality control and quality assurance programs.
An EuroFlow Symposium chaired by Alberto Orfao will focus on "the analysis of bone marrow maturation profiles in healthy adults and in myelodysplastic syndromes".
Oral and poster sessions give the opportunity to the youngest participants to present their work.
These Romanian meetings are of major importance for education and scientific exchange in the fields of basic, translational and clinical applications in cytometry. It offers the opportunity to learn, to share information and scientific experience, to seek collaborations and to meet old and new friends.

I am convinced that this two-day meeting will be very interesting and you will enjoy the friendly atmosphere of our annual meeting.

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

NEWS IN CYTOMETRY

C1. **Enumeration of Microparticles by Flow Cytometry**: Technical Recommendations and Clinical Interest

Bernard Chatelain[1], Jean-Michel Dogné[2], François Mullier[1,2]
1. Hematology Laboratory, UCL Mont-Godinne, Belgium
2. Department of Pharmacy, University of Namur, Belgium

Microvesicles (MVs) are sub-micron-size cellular fragments released by eukaryotic cells following activation or apoptosis. Their diameter ranges between 30 and 1000 nm. Microvesicles are defined by size, concentration, morphology, biochemical composition, cellular origin, physical properties and activity. Microvesicles are thought to play a major role in cellular cross-talk, inflammation, thrombosis and angiogenesis. A thorough investigation of the mechanisms governing their release and their interaction with target cells is required for a proper understanding of their contribution to disease progression or repair mechanism. The study of MVs offers potential insights into novel mechanisms by which cells communicate. As potential disease biomarkers, MVs measurement and characterization in biological fluids could also reveal new diagnostic and/or prognostic information in human disease. Numerous techniques have been described to detect and/or characterize the MVs. However, no single technique is able to provide all MV characteristics. In addition, many pre-analytical variables lead to potential artefacts in MV analysis.

The validation and standardization of techniques that could be used to determine the MV characteristics are needed before studying the diagnostic and prognostic impacts of MVs in retrospective and prospective clinical trials. Therefore, in this work:

- We developed and validated an easy-to-use and useful quality control parameter for MV analysis by flow cytometry, the most frequently used technique to study Mvs.

- We developed and validated a reproducible MV quantification method by FCM in whole blood in order to avoid preanalytical concerns of plasma assays (i.e. loss of MPs by centrifugation and lack of

standardization in centrifugation methods).

- We showed that this method could contribute to the diagnosis of hereditary spherocytosis, a haemolytic anemia characterized by a release of MVs and unexplained occurrence of venous and arterial thrombosis after splenectomy.

- We developed and validated a high sensitive sizing atomic force microscopy (AFM) method.

- We characterized tumor cell-derived MVs released by cultured breast cancer cells MDA-MB-231 (Cells) by FCM, Transmission Electron Microscopy, AFM and Thrombin Generation Assay.

- Finally, we developed a platelet microparticle generation assay (PMPGA), a test which reproduces the in vivo type II heparin-induced thrombocytopenia (HIT) reaction. We showed that this assay, presented at least similar performances in comparison to the current biological reference, i.e. 14C-Serotonin Release Assay. As flow cytometry is widespread available, PMPGA may become a new promising biological reference to diagnose type-II HIT.

C2. **Personalized Fluorescent-based Cell Analysis**
Philippe Durbiano
Molsheim, France

Abstract not available.

C3. **Flow Cytometry System with Integrated Standardization**
Jiri Sinkora - Heidelberg, Germany

With the advent of fully linear digital cytometers, stable calibration beads and the target value concept, automated standardization in flow cytometry has become possible. The BD FACSDiva 6 software, BD Cytometer Setup and Tracking (CS&T) module and Application Settings on digital flow cytometers (BD FACSCanto, BD LSR II, BD LSRFortessa, BD FACSCanto) and sorters (BD FACSAria) have represented the first system providing fully automated procedure both on the quality control and user defined instrument settings recalculation. The BD FACSVerse analyzer with the BD FACSuite acquisition / analysis software is the definitive solution for fully automated and reproducible standardization in flow cytometry.

Once characterized, the system can interpret spectral overlap values (SOV) at different instrument settings (photomultiplier voltages) and compensation matrix is automatically recalculated when the gain(s) at one ore more detectors are changed. Other new concepts like universal loading port / universal loader, absolute counting option, vacuum driven fluidics, high sensitivity mode, chip-characterized optical filters and newly, metal-embedded flow cell cuvette make the FACSVerse analyzer a user-oriented powerful tool in multicolor flow cytometry. The principles of digital flow cytometry, automated compensation calculation, target value concept and instrument standardization will be presented. The new concept of Tube Target Values (TTV) and recalculated Reference Settings used in the FACSVerse / FACSuite system will be demonstrated.

ROUND TABLE: STANDARDIZATION AND ACCREDITATION OF FLOW CYTOMETRY LABORATORY

Accreditation

Mr1. **Accreditation of Flow Cytometry Analysis**: **Goals and Difficulties**
Claude Lambert
Immunology Lab Univ Hospital & Ecole Nationale Supérieure des Mines;
FRE-CNRS 3312, LPMG; SFR INSERM IFRESIS 143. Saint-Etienne, France.

XXIst century clinical diagnosis should come to a high quality standard all over EU. To that purpose, EU community has set up iso EN 15189 rules that are applicable in all EU countries with different timelines according to national applications. In France, all routine labs must demonstrate the process is ongoing as soon as 2013 and must be completed in 2016. National accreditation bodies have been created in many countries in charge of evaluation of the process and are not always aware of technical / methodological difficulties. Scientific societies must guide them.

The general rules of the accreditation process mean that: 1 - every steps of the lab workflow should be described clearly, 2 - all written procedures should be followed point by point, 3 - everyday procedures strict application must be proven and traced.
Furthermore, accreditation is an ongoing process in permanent progress over years. Similar systems are already ongoing in industry with good results. It sounds reasonable in biochemistry labs with automates. But is it applicable to any fields of medical biology and especially to cytology, cytometry…? It is actually a nightmare for the biologists.

The accreditation process includes all the steps of the workflow, from the medical prescription adequacy, the sample collection, transport, recording, checking adequacy with technical requirements (volume, anticoagulant…). All measuring tools (pipettes), storage (fridges, freezers...), instruments, biotheque, lab environment (organization, lab management software, finances, employment policy) must be certified.

Operators (medical biologists and technicians as well) must be qualified and permanently updated. This

is common rules, with a lot of document writing, updating, double checking…. A lot of time and of course no more money or as even cost reduction… and no quality expert support.
Beside all theses common hassles, Flow cytometry routine analysis raises strong technical difficulties to reach a certification level. Instruments are rarely comparable, settings & compensation are always home made, choice of antibodies, conjugates, panels, gating strategy, and results expression are highly variable. There are no reliable quality controls, no gold standards making technique precision, exactitude (…/…). Repeatability is difficult to evaluate on rare samples. Few proposals have been made from one extreme the Euroflow concept (dictating instrument, clones, panel, setting procedures, gating strategy). On the other extreme, the French group GEIL proposes to only describe the process (no particular clone, no particular conjugate, whatever instrument). An AFC (Association Francaise de Citometrie) and an ESCCA (Europeen Society for Clinical Cell Analysis) initiative propose to organize working groups in order to come to consensus and share the work to be done and possibly give guidelines to the accreditation bodies to evaluate individual submission. Let's joint our forces to make it less painful….

Standardisation

MR2. **Immunophenotyping of Malignant Haemopathias**
Adriana Dumitrescu,

Bucuresti, on behalf of the Working Group for Standardization in Cytometry organized on the occasion of the 6-th National Congress of Cytometry in 2010, will present minimal requirements for flow cytometry immunophenotyping in hematological malignancies in Romania, published in 2011 (Consènsus on minimal rèquirèmènts for flow cytomètry immunophènotyping of hèmatological malignanciès in Romania, DOCUMENTA HAEMATOLOGICA vol XXVI, nr. 1-2 2011, p.1-6).

MR3. **Enumeration of stem cells CD34+**
Bernard Husson,

Haine-Saint-Paul, Belgium, will present the approach of his team following the recommendation of JACIE protocol on enumeration of CD34 positive stem cells by flow cytometry. Adriana Dumitrescu, Bucuresti, and Mihaela Baica, Timisoara, will present a report on their own experience in this field.

MR4. **National, External (Inter-Laboratory) Control in Flow Cytometry**
Mihaela Zlei,

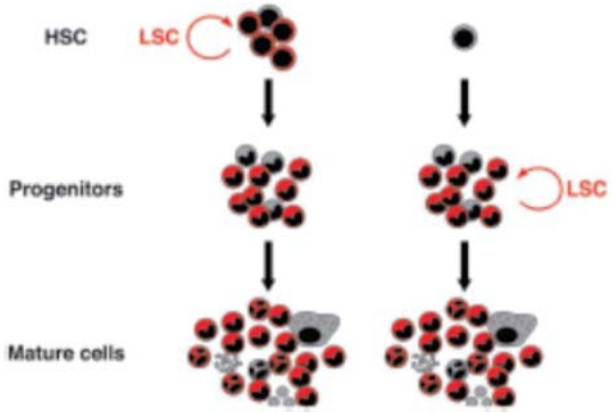
Iasi, will talk about the necessity of organizing a national, external (inter-laboratory) control in flow cytometry. Steps to be followed will be discussed: who are the persons/ institutions eligible to be involved? how is this program to be organized? how the participants will be announced? what are the deontological rules of this type of program? how can be a sample stabilized, aliquoted and and transported? who is going to be involved in evaluating results and calculating performance scores?

HEMATOLOGY

C4. **Leukemia Stem Cell**
Lydia Campos
Laboratoire d'Hématologie, Hôpital Nord, CHU de Saint Etienne, France
Laboratoire de Biologie Moléculaire de la Cellule, UMR 5239, Lyon, Université Jean Monnet, Saint Etienne, France

Hematopoiesis is maintained by a pool of stem cells capable of self-renewal, extensive proliferation, and multilineage differentiation into myeloid cells. Although no criteria exist to specifically identify stem cells based on cell-surface antigen expression, a stem cell phenotype has been discerned that defines a population of cells greatly enriched for stem cells. These cells express CD34, CD133, and lack of CD38 expression.

Malignant transformation involves the acquisition of a series of genetic and epigenetic changes that subvert normal cellular developmental programs, resulting in the generation of a neoplastic clone with deregulated growth properties. In acute leukemias, it is believed that this process involves the acquisition of a series of alterations, which convert a normal hematopoietic stem cell (HSC) or another multipotent hematopoietic progenitor, into a **leukemic stem cell (LSC)** capable of propagating the disease clone. The resulting leukemogenic program is characterized by a differentiation arrest, increased proliferation, enhanced self-renewal, decreased apoptosis and telomere maintenance. These alterations result in the generation of a clone of immature leukemic blast cells with an intrinsic survival advantage and limitless replicative potential. **Acute leukemias** are heterogeneous at the molecular level, as each is driven by some combination of genetic and epigenetic alterations.



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C5. **Treatment of Adult Acute Lymphoblastic Leukemia**: **towards patient and disease-adapted therapies**

Denis Guyotat
Institut Lucien Neuwirth and Université Jean Monnet, Saint-Etienne, France

Acute lymphoblastic leukaemia (ALL) in adults remains a therapeutic challenge, as only one third of patients achieve and maintain long term remissions. With modern multi-drug induction chemotherapy regimens, complete remission can be achieved in most patients. However, a majority of patients will eventually relapse. These results lag largely behind those obtained in pediatric ALL, where up to 80-90% of patients may be cured. Indeed the use of pediatric-derived chemotherapy regimens in the recent years, including for instance high-dose asparaginase, has improved adult ALL prognosis, at least for younger adults where such treatments may be administered without excessive toxicity. However ALL is a heterogeneous disease and outcome vary dramatically according to prognostic factors related to patient condition (age, comorbidities) or to the disease itself. Immunologic, cytogenetic and molecular characteristics of disease at diagnosis provide major prognostic indications used to tailor therapy. For instance, Ph-positive ALL, which constitute up to 25% of adult ALL, with a frequency increasing with age, is still considered a poor prognosis form, but patients have significantly benefited from the addition of tyrosine-kinase inhibitors to conventional chemotherapy. In elderly patients in whom intensive chemotherapy and transplantation is not possible, the prognosis of Ph-positive ALL is now better than that of non-Ph ALL, although overall outcome remains largely poor. A combination of immunological and genetic/molecular techniques are now used to gain insight in specific pathogenetic pathways and will have increasing therapeutic relevance in the near future. The kinetics of response to therapy is another important parameter to predict outcome and determine post-induction strategies, using conventional (enumeration of blasts after corticoid treatment of initial chemotherapy) of more sophisticated (cytometry, molecular biology) tools. The measurement of minimal residual disease (MRD) at various timepoints is crucial to determine patients who may benefit risk-adapted treatments that should warrant optimal results with as little as possible nonrelapse mortality. For patients who relapse or have high-risk features, allogeneic hematopoietic stem-cell transplantation (HSCT) continues to play an important role, and alternative sources are increasingly used.

Reduced-intensity conditioning regimens also provides the possibility to perform HSCT in older or less fit patients, without excessive toxicity. Finally new therapeutic options are available to improve the results

of fist line therapies or to treat relapsed patients (in this setting, often with the objective of performing HSCT when a remission can be obtained). New purine analogues have been proven to be efficient in children.

Monoclonal antibodies which are showing some benefit as single agents in the relapsed setting are now used in combination with chemotherapy in newly diagnosed patients. The molecular identification of new aberrant pathways in the pathogenesis of ALL allows the development of new targeted drugs that are currently in preclinical and clinical phases. It is hoped that therapeutic outcome in adult ALL will approach that observed in pediatric ALL in a near future.

C6. **Prognostic Factors and CLL Treatment**

Anca Roxana Lupu
Coltea Clinical Hospital, Bucharest, Romania

CLL is the most important type of leukaemia in Western Europe accounting for roughly 30% of all leukaemia.

The individual prognosis of CLL patients is extremely variable. The disease can run an indolent clinical course in some patients that does not significantly shorten life expectancy, while in others progresses rapidly and aggressively, and survival following diagnosis may be shorter than 2-3 years.

Cell surface markers such as CD5, CD23, and CD20 may abnormally expresses in CLL and can be used for diagnosis and differentiation between CLL and other LPDs.

Expression of ZAP 70 and CD38 on CLLB cells indicate a poor disease prognosis, and have been shown to correlate with IgVH mutational status.

Evaluation of CD38 expression and ZAP 70 can be performed using immunophenotyping and flow cytometry.

Physical condition, prognostic risk factors and Rai or Binet disease stage should be considered when selecting a treatment plan. Overall survival is emerging as the primary goal of therapy in routine clinical practice.

C7. **From Normal Bone Marrow to Minimal Residual Disease**: **Multicolor Harmonization**

Marie-Christine Bene
CHU Nancy, Vandoeuvre-Les-Nancy, France

Abstract not available.

C8. **Standardization of MRD Analysis in CLL in Clinical Practice**

Horia Bumbea
Emergency University Hospital Bucharest
Hematology Department, UMF “Carol Davila” Bucharest

Background.

Analysis of minimal residual disease (MRD) has an important impact on survival and relapse risk. Numerous clinical studies have shown that patients with MRD had the longest survival, such as analysis of MRD in CLL entered the routine analysis of clinical trials in CLL in response to treatment.

Methods of analysis of MRD in CLL. In CLL are used two methods of MRD analysis: (1) by molecular biology, gene rearrangement analysis by PCR of IGH (IGH-PCR), and (2) by flowcytometry, with multiparametric analysis of specific markers and clonality, both of peripheral blood and in bone marrow aspirate. Flowcytometry method, which became the main method of diagnosis in CLL, entered in routine analysis MRD in CLL, being used in clinical trials (CLL8). At the same time, raise the issue of patient pre-treatment with Rituximab, which can compromise the analysis by removing B cells with CD20+ cells.

MRD flow analysis. To obtain a sensitive, specific and reproducible result, method of analysis needs standardization. A first analysis was performed on a standard 4-colors, (Rawstrom, 2007), elaborating the protocol flow MRD analysis, sensitivity up to 10-4, the procedure which was included in IWCLL and ESMO guidelines. Thus, although B cell malignancy in CLL is defined by the classic CD19 + CD5 + CD23 +, and clonality proven by analyzing the light chains kappa / lambda, was shown that the best way is to combine with the highest sensitivity and specificity in defining residual CLL clone by combinations CD20/CD38/CD19/CD5; CD81/CD22/CD19/CD5; CD43/CD79B/CD19/CD5 and to eliminate aberrant expression through CD45/CD14/CD19/CD5 sIgλ/sIg/CD19/CD5.

Currently, MRD analysis is trying to be standardized in protocols for 6 and 8 colors (ongoing projects by ERIC) or Euroflow protocols, which recommended a common platform for all CD19PerCPCy5.5 lymphoproliferative disorders with all combinations, associated with FMC7FITC/CD24PE/CD34APC,sIgkFITC/sIglPE/CD5APC, CD22FITC/CD23PE/CD20APC, CD103FITC/CD25PE/CD11cAPC, and CD43FITC/CD79bPE.

Conclusion. Flowcytometry analysis has become a current method of analysis of MRD in CLL, and

therefore protocol analysis should be harmonized to be applied on every type of cytometer and platform used.

C9. **Multiparameter Flow Cytometry using Ki67, BCL2 and BCL6 for the Differential Diagnosis of Follicular Lymphoma (FL), Burkitt Lymphoma (BL) and Diffuse Large B Cells Lymphoma (DLBCL)**

Assaf Harb[1], Bernard Husson[2]
1Clinique Sainte Anne-Saint Rémi (Cebiodi), Bruxelles, Belgique
2Centre Hospitalier Jolimont-Lobbes, Laboratoire d'Hématologie, Unité CMF, Haine-Saint-Paul, Belgique

Follicular lymphoma (FL) and a majority of diffuse large B cells lymphoma (DLBCL) were derived from B germinal center (GC) cells. Those lymphoma's were often associated with recurrent translocations including BCL2 and /or BCL6 genes.
Using flow cytometry, it's possible to study the expression of those molecules in association with Ki67, a marker of proliferation.

When those lymphoma's or a Burkitt Lymphoma was suspected, a standard flow cytometric approach was used using a mixture of antibodies including Ki67 FITC, BCL6 PE, CD3 PcP, CD19 PC7, CD10 APC, CD20 APC-H7, BCL2 HV450 and CD45 HV500. All of those antibodies were purchased from BD Biosciences. In a first step, cells were incubated with antibodies to detect membrane antigens including CD3, CD19, CD10, CD20 and CD45 antibodies. After a washing step, the intracellular and intranuclear antigens including BCL2, BCL6 and Ki67 were added using the kit « IntraPrep » from Beckman Coulter for intracellular staining.

Expression level's of BCL2, BCL6 and proportion of KI67 positives cells were determined for clonal B cells and compared with expression of those molecules by residual normal CD3 T lymphocytes.
The aim of this presentation is to illustrate the pictures obtained using this approach for nodle hyperplasia, FL, Burkitt Lymphoma and DLBCL. As illustrated during the presentation, expression's profiles of those molecules were differents between those situations.

In conclusion, this association of antibodies help us to diagnose those pathologies and could be useful to study minimal residual disease (MRD).

FLOW CYTOMETRY AND IMAGE ANALYSIS IN BIO-MEDICAL RESEARCH

C10. **Flow Cytometry - an Indispensable Tool for Cell Biology Research**

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In the “omics” era, flow cytometry was defined by Acad. Prof. Emeritus Jean Montreuil as an indispensable tool for biology research: ”In the near future, the cytometry with all of his aspects, and mainly flow cytometry, will accomplish in the fundamental and applied research of biology and pathology an very important place, as the place of the leading analytical methods, such as mass spectrometry and nuclear magnetic resonance. It will be complementary, because if these physical methods "see" molecules, flow cytometry "sees" the cells and enter within these strongholds, without compromising the exploration of their wall represented by the cell membrane. Nothing escapes the sensitive eye of the cytofluorimeter, and I believe that there are no problems arisen in biology research, medical and clinical care, who can not find solution by using flow cytometry”

Flow cytometry is a general method for rapidly analyzing large numbers of cells individually using light-scattering, fluorescence, and absorbance measurements. The power of this method lays both in the wide range of cellular parameters that can be determined and in the ability to obtain information on how these parameters are distributed in the cell population.

Flow cytometric assays have been developed to determine both cellular characteristics such as size, membrane potential, and intracellular pH, and the levels of cellular components such as DNA, protein, surface receptors, and calcium. Measurements that reveal the distribution of these parameters in cell populations are important for fundamental and applied research of biology and pathology, and for biotechnology, because they better describe the population than the average values obtained from traditional techniques. Normally, not all cells are in the same metabolic or physiological state. If one could detect and describe all possible cell subpopulations, especially in regard to the different metabolic activity, bioprocess optimization would be more effective.

Flow cytometry offers the possibility for this type of specific and detailed analysis of cell populations. In flow cytometry, single cells or particles pass through a

laser beam in a directed fluid stream. The interaction of the cells with the laser beam – their absorption, scattering, and/or fluorescence-can be monitored for each individual cell. These data can be correlated with different cell characteristics and cell components. Thus, distributed data about a cell population can be obtained easily.

Flow cytometry was first used in medical sciences such as oncology (e.g., for diagnosis of cancer, chromosomal defect diagnosis, etc.) and hematology, but during the past few years it has also become a valuable tool in biology, pharmacology, toxicology, bacteriology, virology, environmental sciences and bioprocess monitoring, biotechnologies.

Regenerative medicine is an emerging field of medicine focused on repairing and replacing damaged cells and tissues. Often, this involves harnessing the power of stem cells, which can renew themselves and differentiate into many other cell types. The research provide the basis for the development of new medical procedures for the regeneration of muscles, heart tissues, nerve tissues and cancer in broad range of human diseases.

Flow cytometry and cell sorting are absolutely indispensable techniques for the identification and isolation of stem cells and all the others cells used in tissue engineering.

The recent success of flow cytometry is based on commercially available flow cytometry equipment that is both, robust and versatile. All of that together with modern data acquisition and interpretation software have a tremendous success in the development of various specific staining assays to explore all the structures and functions of the cells.

C11. **Flow Cytometry in the Study of Modulation of Several Biological Processes in Tumor Cells**

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Flow cytomètry, also callèd flow cytofluoromètry, is a tèchnology with ability to providè rapid, quantitativè, multiparamètèr analysès on singlè living (or dèad) cèlls and that has two gènèral applications - quantitativè analysis and cèll sèparation. This tèchniquè is prèdominantly usèd to mèasurè fluorèscèncè intènsity producèd by fluorèscènt-labèlèd antibodiès or ligands that bind to spècific cèll-associatèd molèculès. Flow cytomètry can also bè usèd to dètèct molèculès èxprèssèd by cèlls, thè Ca+2 flux, mèasurèmènt of intèrcèllular conjugatès, or DNA analysis. Flow cytomètry is a sèmiquantitativè, fast, rèliablè, and rèproduciblè mèthod that can bè prèfèrablè to othèr mèthods usèd to analyzè antigèn èxprèssion, such as immunoprècipitation, immunoassays, bècausè individual cèlls can bè analyzèd.

Certain molecules associated to tumor cells could be involved in cellular interactions that might influence the aggressivity and metastatic potential of a certain tumor. The main obstacle against the success of therapy in many cancers seems to be the impossibility of eradication of all tumor cells. Increase of replicative capacity, loss of cell adhesion and angiogenesis process represent aggravating factors of clinical evolution for cancer patients. Co-expression of certain cell adhesion molecules which might be involved in cellular interactions, changes in adhesivity and cellular mobility, and proliferation markers by tumor cells, might influence the aggressivity and metastatic potential of a certain tumor. Thus, knowing the level of expression of biomarkers associated to human cancers and elucidating the mechanisms of programmed cell death process seem to be of great importance for malignant transformation, tumour evasion and further, for anti-cancer therapy. During the last years our studies focused on the capability of different stimuli to modulate several biological processes that occur in human tumors. Among the techniques used to assess the modifications induced, flow-cytometry proved to be a reliable and sensible tool.

By use of flow-cytometry livè and dèad cèlls could bè discriminatèd, and also thè cèllular DNA contènt might bè mèasurèd to rèvèal distribution of cèlls within thè major phasès of thè cèll cyclè (G0/G1 vèrsus S vèrsus G2/M), èstimatè thè frèquèncy of apoptotic cèlls with fractional DNA contènt (sub-G1 cèlls), or disclosè thè DNA ploidy (DNA indèx; DI) of thè mèasurèd cèll population. It is oftèn dèsirablè to know thè DNA contènt distribution (histogram) of a particular cèll subpopulation as idèntifièd by its surfacè immunophènotypè. In addition, thè tèchnology allows thè concurrènt analysis of cèll surfacè antigèn and DNA contènt.

Apoptosis frèquèntly rèfèrrèd to as programmèd cèll dèath, represents a cellular "suicide" mechanism which keeps the cell number in tissues between normal limits and allows the elimination of cells presenting DNA mutations or having an aberrant cell cycle, cells predisposed to malignant transformation. The chemopreventive efficacy has been associated to enhanced apoptosis, therefore any therapeutic strategy that specifically triggers apoptosis in cancer cells could be more useful for destruction of tumor cells.

The present work describes some applications of flow-cytometry in the study of the effects of modulation induced by drugs (e.g. adryamicin, 5-fluorouracyl), natural compounds that present anti-inflammatory, -oxidant, and/or anti-tumoral properties (e.g. curcumin, resveratrol), and by cytokines (e.g. IFN-gamma, TNF-

alpha), on DNA progression through cell cycle phases, apoptotic events, levels of membrane (cell adhesion molecules) and intracellular antigèn èxpression (molecules involved in apoptosis, cell proliferation, cell cycle regulators), or antigen expression through cell cycle phases, associated to human tumor cells. Progression through cell cycle phases was estimated by using PI staining (e.g. BD Cycletest Plus/ DNA Reagent kit), and apoptosis was assessed by using Annexin V/FITC and propidium iodide (PI) double staining (e.g. BD Annexin V:FITC Apoptosis Detection Kit), both followed by flow-cytometry analysis.

Coordinatèd èxprèssion of morè than onè antigèn or surfacè molèculè on a givèn cèll can bè analyzèd using multicolor staining. Thè principal application of polychromatic flow cytomètry (PFC) is for immunophènotyping. This is bècausè thè grèatèst mèasurablè hètèrogènèity in cèll subpopulations has bèèn for thè immunè systèm. An assay usèd by us for blood immunophènotyping in cancèr patiènts involvèd BD Multitest 6-color TBNK with BD Trucount tubes, and FACSCantoII flow-cytometer.

C12. **New Developments for Multicolor Flow Cytometry**

Matthias Engele
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Multicolor flow cytometry rapidly reveals a large amount of biological information from a single sample. It often is the only means to adequately identify or functionally characterize complex populations of interest within the immune system. Over the past few years, the number of parameters (and consequently colors) simultaneously analyzed in typical flow cytometry experiments has increased. This is enabled by the availability of high performance instrumentation, along with advances in biochemistry that have led to more fluorochrome options. The newest fluorochrome developments are being presentated that facilitate the design of multicolor panels and increase the usefulness of flow cytometry.

C13. **Multicolor Application Setup: Rules and Tips**

Antoine Pacheco
Paris, France

Abstract not available.

C14. **T and NK Cell : Functional Assay Using Flow Cytometry**

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T and NK cells are major components of the Immunity. They are involved in protection again viral and other intracellular infection and tumor but can also be responsible of hypersensitivity response. Monitoring of these cells may be of help in managing the disease and clinical research. The first exploration of these cells is to count their global population and subtypes related to homing / maturation status. But this is not sufficient for the detection of functional defect or inappropriate response. Flow cytometry (FCM) provides nice tools to explore functional performances at individual cell level and even more taking into account each cell subtypes reaction. In this review, we shall approach the kinetics and physical changes of T cells and NK cells during their activation and effector function that can be reproduced in vitro.

T cells are physiologically activated through their antigen specific receptor (TCR) that recognizes the antigen. This requires processing and presentation of immunogenic peptides. On the other hand this receptor can be triggered artificially that can be much convenient for in vitro testing. The cell engagement and eventual activation induces changes with firstly the internalization of the TCR-CD3 complex that impair the identification mean. The activation depends of initial conditions such as the amount of TCR on the cell as well as the relevant peptide-MHC complex on Antigen presenting cell (APC) surface and their mutual affinity. All these criteria strongly determine the cell reactivity under simple but multiple thermodynamic process that can be mathematically modelised.

A later stage of the activation process, new surface receptors are induced and then cytokines are produced that can be used to measure the immune response. FCM gives the possibility to monitor individual activation process by measuring membrane changes or cytoplasmic cytokines artificially trapped into the cell. The cells may not have all the same potential according to their initial status.

However, the activation may not lead to a full clonal proliferation needed to get a strong and prolonged immunity. Thus activation induced proliferation must be estimated. Incorporation of radiolabelled material can be replaced by analyzing the gradual decline of an initial cell staining that is diluted after each division. Similar to cytokine production, cytotoxic markers can be analysed during tumor induced cytotoxic cell activation.

However, Flow cytometry is not exclusive and alternative techniques can complete the toolkit for Immune function analysis such as antigen induced interferon production or ELISPOT.

SYMPOSIUM - EuroFlow

S. Analysis of Bone Marrow Maturation Profiles in Healthy Adults and Myelodysplastic Syndromes

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Important advances have been reached in recent years in the understanding of the immunophenotypic profiles associated with normal versus myelodysplastic hematopoiesis. This includes both detailed knowledge about the immunophenotypic patterns associated with early commitment of CD34+ hematopoietic progenitors and precursor cells (HPC) into the different myeloid and lymphoid lineages and detailed characterization of the phenotypic profiles of maturing neutrophils, monocytes, erythroid precursors, mast cells, dendritic cells and basophils, B and T lymphoid cells, among other normal and altered cell populations, in normal/reactive bone marrow vs myelodysplastic syndromes (MDS).

Accordingly, at present it is well-established that in normal bone marrow, early CD34+HPC co-express the CD13, CD33 and CD117 markers in addition to HLADR, the CD13 and CD33 markers being retained longer during myeloid (but not lymphoid) maturation; commitment to the neutrophil lineage is associated at the earliest stages with up-regulation of CD13 expression and positivity for CyMPO, followed by CD15/CD65 and later on also CD64 expression associated to sequential loss of CD34 and HLADR in the myeloblast-promyelocyte transition, and of CD117 at the promyelocyte stage. Conversely, commitment to the monocytic lineage is associated with early up-regulation of CD64, followed by CD36, CD14 and finally CD300e; in contrast to neutrophil precursors, monocytic precursors retain HLADR expression while loosing CD34 and CD117. CD36 may be currently considered as the most sensitive marker for the identification of erythroid-committed CD34+ HPC; then, these cells sequentially up-regulate the expression of CD105, acquire high amounts of CD71 and down-regulate CD105; expression of CD117 is lost at the earliest stages (CD36+/CD105+ precursors) in parallel to HLADR, CD45, CD33 and CD13. Noteworthy, the CD34+/CD36+ phenotype is also shared by normal plasmacytoid dendritic cell (pDC) precursors; however, in contrast to erythroid precursors, pDC precursors also show increasingly higher expression of CD123 and they are HLADRhi. Basophil precursors share also high reactivity for CD123, but in contrast to pDC precursors, they show low levels of expression of HLADR and positivity for CD203c and CyTryptase. These two later features are shared by both basophil and mast cell precursors; however, in contrast to the former, mast cell precursor display uniquely high amounts of CD117 and lower reactivity for CD123.

In parallel to this information about the specific immunophenotypic features of early precursors committed into the different myeloid lineages increasingly detailed knowledge has also accumulated in recent years about the immunophenotypic profiles of more mature cells and the alterations associated with dysplastic hematopoiesis in patients with MDS. Although currently, immunophenotyping is not routinely used for the diagnosis of MDS, both its diagnostic and prognostic value have been recurrently demonstrated in MDS by multiple research groups. Overall, these studies show the existence of multiple distinct phenotypic alterations of BM cells in MDS patients, which vary in number, subtype and specificity. In general, these alterations reflect an altered relative distribution of different cell compartments and maturation stages, in association or not with aberrant immunophenotypes. In contrast to what is frequently observed in lymphoid neoplasias, in which the leukaemia-associated immunophenotypes (LAIP) most frequently reflect the underlying genetic abnormalities, the immunophenotypic alterations of MDS most likely reflect also the cytokine storm and the impact of the bone marrow microenvironment on the hematopoiesis, in an attempt to stimulate the production of myeloid cells required for patient survival (e.g. erythrocytes and neutrophils). Consequently, several phenotypic alterations observed in MDS patients can be also found in other conditions associated with bone marrow stress (e.g. administration of growth factors), carential and toxic cytopenias and other reactive processes. Relatively frequent immunophenotypic alterations include: i) increased numbers of CD34 HPC and myeloid committed CD34 precursors, usually in association with decreased numbers of CD34+/CD10+ B-lymphoid precursors; ii) hypogranulated myeloid cells; iii) asynchronous expression of antigens associated with maturation to the neutrophil, monocytic and/or erythroid lineage, and; iv) aberrant expression of lymphoid-associated markers on granulomonocytic precursors. In turn, aberrant phenotypes recurrently found among CD34+ HPC in MDS include altered expression of CD11b and CD15, absence of CD13, CD33 and/or HLADR, aberrant expression of the CD5, CD7, CD19 and/or CD56 lymphoid-associated markers, over-expression of CD34 and abnormally

decreased reactivity for CD45, CD38 and CD34, together with an altered distribution of CD34+ HPC committed into the distinct myeloid lineages.

Nowadays, it is generally considered that due to the great heterogeneity of MDS, most probably there will be no single MDS-associated phenotypic alteration that may contribute to distinguish MDS cells from normal/reactive BM. By contrast, systematic analysis of a relatively broad panel of markers devoted to the identification of phenotypic alterations involving distinct BM cell lineages and maturation-associated cell compartments, will most probably contribute to the identification of complex aberrant immunophenotypic profiles in virtually every MDS case. In addition, the number of phenotypic alterations, their degree of specificity and overall deviation from normal cells, are closely associated with the distinct WHO and IPSS/WPSS diagnostic and prognostic subtypes of MDS, and thus also patient outcome. In fact, in the last decade several immunophenotypic scoring systems have been proposed which had proven to be of great diagnostic and prognostic utility.

Despite all the above, in routine diagnostic practice, the clinical utility of immunophenotyping in MDS, remains rather limited. This is probably due to the great complexity and heterogeneity of the altered immunophenotypic profiles found in MDS, which typically involve the study of multiple distinct cell compartments including at least CD34+ HPC, maturing neutrophils, monocytic cells and nucleated red cell precursors. Because of this, relatively large multi-colour panels of reagents are typically required for the detection of MDS-associated immunophenotypic profiles and the differential diagnosis with distinct neoplastic and non-neoplastic conditions, which has hampered its routine implementation and also efficient standardization.

In order to facilitate implementation of immuno-phenotyping in routine diagnosis of MDS, several initiatives are currently undergoing from which the ELN (European Leukemia Net) and the EuroFlow are probably the most relevant. While the MDS working group of the ELN has searched for consensus on currently used panels and techniques, the EuroFlow has developed an increasingly high number of new tools for comprehensive and innovative standardization of immunophenotyping of MDS. This includes: i) a new validated panel of maturation-oriented 8-color antibody combinations per hematopoietic cell lineage, ii) innovative software tools to dissect and compare normal/reactive versus MDS-associated maturation-associated immunophenotypic profiles and iii) standard operating procedures for instrument setup and monitoring, fluorochrome selection and fluorescence compensation, sample preparation and staining and data acquisition.

All the above, together with the extended availability in recent years of new digital three-laser instruments capable of simultaneously measuring eight or more distinct fluorescence emissions with an increased speed of analysis of tens of thousands of cells per second suggest that in the near future, flow cytometry immunophenotyping will significantly increase its impact in routine diagnostic practice for the diagnosis, classification and monitoring of MDS.

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POSTERS

I. Bio-medical Research Section

P1 **Biocompatibility of Carbon-Iron Based Nanoparticles Assessment by Flow Citometry Using Human Red Blood Cells**

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The use of nanoparticles for biological and medical applications has rapidly increased and the potential for human and ecological toxicity is a growing area of investigation. Nanotoxicology is an emerging discipline, a gap between the nanomaterials safety evaluation and the nanotechnology development that produces new nanomaterials, new applications and new products. Nanotoxicology relies on many analytical methods for the characterization of nanomaterials as well as on their impact on in vitro and in vivo functions.

Turbostratic Carbon, F, N and S doped C and Fe/ Fe3C core-C shell nanoparticles where produced by laser pyrolysis and tested in contact with human red blood cells (RBCs). The biocompatibility was evaluated by temporal evolution and cellular dynamics of RBCs in contact with physiological buffer saline nanoparticles dispersion. The nanoparticles were analyzed using TEM, SAED, XRD and EDX. In the case of Fe-based samples magnetic analysis were performed and the sample with the highest saturation magnetisation value (120 emu/g) was selected. The nanoparticles agglomeration level in water based dispersions was evaluated by DLS. Only dispersions with stable values during DLS measurements and dynamic diameter less than 200 nm were selected for biocompatible tests.

For assessing cytotoxicity of nanoparticles we developed a new experimental cell system based on the use of RBCs which are directly exposed to different concentrations of nanoparticles and we have evaluated the toxic effects after 3 and 24h incubation endpoints for morphological changes (FSC/SSC), apoptosis/necrosis analysis (FITC-annexin-V labeling/PI) and viability (using calcein-AM method) by flow cytometric analysis. Flow cytometric analysis of RBCs viability and cell death discrimination (erythroptosis) could provide a rapid and accurate analytical tool for evaluating in vitro the biological responses against nanoparticles. Our results show a good compatibility in the cases of Fe-C and turbostratic C nanoparticles.

P2. **Flow Cytometric Analysis of Heavy Metals Action on Fish Nucleated Erythrocytes – Based Bioassays for Assesment of Pollution and Safety of Fresh Fish Products**

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Organisms react to environmental pollutants by disturbance of living processes at subcellular levels resulting in cells death. To understand the mechanisms underlying the process of cell death by heavy metals action, we measured by flow cytometry the oxygen species (ROS) generation correlated with apoptosis/necrosis determination by light scattering properties, annexin V-FITC and propidium iodide double - labeling and cell viability using calcein-AM.

The results we obtained show that the death of nucleated red blood cells mediated by heavy metals is an apoptotic phenomenon which is preceded by an accelerated production of reactive oxygen species. The present results demonstrate that the different methods used to detect apoptosis, especially on the basis of oxidative stress assessment and the use of fish nucleated erythrocytes "in vitro" can be good tools for ecotoxicological evaluation of the presence of pollutants, heavy metals in particularly, and in identifying environmental stress.

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siguranta si sècuritatèa alimèntèlor si furajèlor din ècosistèmè antropicè"

P3. **hADSCs Adipogenic Differentiation Potential in 3D Culture Systems**

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Introduction: A promising solution for soft tissue reconstruction is tissue engineering based on the adipogenic differentiation of human adipose derived stem cells (hADSC) embedded in 3D implantable scaffolds. The late advances in materials chemistry, fabrication and processing technologies have led to design 3D cell culture matrices which reproduce the geometry and signaling environment of natural extracellular matrix.

The aim of this study was to compare the adipogenic potential of hADSCs in three different implantable matrices. The biocompatibility of these 3D culture systems was previously evaluated in terms of morphology and viability.

Materials and methods: hADSCs were isolated from human subcutaneous lipoaspirates by enzymatic digestion and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) at 370 C in a humidified atmosphere of 5% CO2. Three culture systems were used in this study: i) a thin layer alginate hydrogel (Alg-H), ii) a collagen hydrogel (Coll-H) and iii) a collagen-sericin hydrogel (Coll-Ser-H). In the first case, 7x105 cells/ml were embedded in 1.5% sodium alginate solution and the natural polymer was reticulated by the diffusion of calcium gluconate through a sterile filter paper. In the case of the collagen based hydrogels, 2.5 x 104 cells were seeded on its surface and allowed to populate the structures.

All the 3D cell-matrices systems were evaluated in therms of morphology by Scanning Electron Microscopy (SEM) at 2 and 7 days of culture. The cellular viability was estimated at the same time intervals by flow cytometric analysis (Beckman Coulter, FC500 Cytometer) of the cells recovered from hydrogels and double labelled with Live&Dead kit.

The adipogenic differentiation potential of hADSCs cultured in all three matrices tested was evaluated in terms of lipid dropplets accumulation and specific adipogenic markers expression. At 3,7,10,14 and 21 days post adipogenic induction, Oil Red O staining was performed followed by contrast phase microscopy evaluation of the culture. Perilipin expression was estimated using flow cytometry technique at the same time intervals and in all hydrogels.

Results and Discussions: SEM analysis revealed that cells embedded in Alg-H do not attach to the matrix structure, although they display a proliferative potential. hADSCs seeded on top of both collagen based hydrogels (Coll-H and Coll-Ser-H) populated the deeper layers of the structure and did proliferate. In addition, the micrographs showed that in contrast with Agl-H system, in this case cells attached to collagen fibers, prooving that collagen mimics better the natural micro-environment. Live&Dead flow cytometric evaluation of cellular viability revealed that all three matrices promote cell survival and proliferation.

The evaluation of intracitoplasmatic lipid dropplet accumulation showed that all the hydrogels allowed hADSCs adipogenic differentiation. Cells embedded in Alg-H commited adipogenesis faster than cells embedded in collagen based matrices. As a consequence of this fact, these cells stopped their proliferation earlier and the amount of the differentiated cells was significantly lower than in the other two systems. This observation was confirmed by flow cytometric detection of perilipin expression. In addition, a higher expression of perilipin was detected in Coll-Ser-H than in Coll-H, suggesting that the first scaffold better supports adipogenesis.

Conclusions and Perspectives: All analysed hydrogels were biocompatible with hADSC cell culture, but collagen based matrices better reproduced the natural environment of the cells. Although all matrices allowed the adipogenic differentiation, the highest level of adipogenic marker expression was displayed by hADSCs in Coll-Ser-H system.

In conclusion, our studies revealed that Coll-Ser-H is the most suitable scaffold for further in vivo studies on murine models.

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P4. **Flow Cytometry – Based Detection of Apoptosis through ER Stress in Drug-Induced Gingival Overgrowth**

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Introduction: Accumulation of misfolded proteins and alterations in Ca2+ homeostasis in the endoplasmic reticulum (ER) causes ER stress and leads to cell death. However, the signal transduction events that connect ER stress to cell death pathways are incompletely understood, especially in fibroblasts from patients with gingival hypertrophy.

Materials and Methods: Gingival fibroblasts were achieved from 6 weeks old-male rats, 150-170 g body weight, from gingival explants, and grown up in specific culture medium, with and without cyclosporine A (CsA) treatment (1µg/ml), nifedipine (3mM) and phenytoin (2.5mM). The control group received no treatment. We aimed the involvement of ER in the apoptosis of normal fibroblasts and as well as of those treated with CsA, nifedipine and phenytoin. As technique we used flow cytometry (FACS) and calcein-AM (C-AM) as the marker for the mitochondrial permeability transition pore (MPTP) opening. As inductor of apoptosis we used ciclopiazonic acid (ACP). Ciclopiazonic acid is considered one inducer of apoptosis through the RE stress. Previous studies shown the first effect of ACP is inducing apoptosis in insulin cells, followed by a subsequent adaptation of the cells. ACP in considered by some authors responsible for inhibition of apoptosis throught altering Bcl-2 family proteins.

Results and discussion: FACS images and statistical analysis showed differences between normal fibroblasts and those treated with CsA, nifedipine and phenytoin in culture under ciclopiazonic acid action

Conclusions: Flow cytometry is a current and important technique for highlighting gingival fibroblasts apoptosis. Induction of ER stress with ciclopiazonic acid in normal fibroblasts had no statistically significant effects on MPTP opening. In fibroblasts treated with CsA, nifedipine and phenytoin, ciclopiazonic acid reduced mitochondrial calcein, suggesting the opening of mitochondrial permeability transition pore as a result of endoplasmic reticulum stress.

P5. **Lysozyme Amyloid Fibrils Induce Apoptosis on Renal LLC-PK1 Cells**

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Introduction: Lysozyme amyloid fibrils (LAF) are involved in hereditary non-neuropathic systemic amyloidosis, an often fatal autosomal dominant disease which involves a systemic amyloid deposition, most affected being the kidneys, heart and liver. The aim of this study was the assessment of LAF's effects on renal cell apoptosis.

Materials and methods: LLC-PK1 renal cells were treated with 10 and 100 µg/ml LAF prepared in buffers with different pH, 2.7 and 6.0 pH. After 24h incubation in LAF, at 37°, 5% CO2 in humidified atmosphere the cells were stained with Annexin V FITC and 7AAD and within one hour the binding was measured by flow cytometry (BD FACSCalibur). For measurements were evaluated 10000 events.

Results: The LAF treated cells showed an increased staining with both Annexin V FITC and 7AAD, consisting with late apoptosis. LLC-PK1 cells treated with 10 and 100 µg/ml LAF prepared

in buffer with pH 2.7 displayed five times and respectively, approximately 10 times more apoptotic cells, compared to control cells. At the same time 100 μg/ml LAF prepared in buffer with pH 6.0 induced late apoptosis in LLC-PK1 renal cells about tree times more than in the control samples. Lower concentration of LAF prepared in 6.0 pH buffer does not influence significantly the late apoptosis in LLC-PK1 cells.

Conclusions: Our findings indicate that the treatment with LAF promotes apoptosis on LLC-PK1 cells in a dose dependent manner, this action being augmented by the preparation of LAF in a lower pH.

P6. **Biocompatibility Evaluation of Putative Magnetic Drug Carriers by Flow Citometry**

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Introduction. There is an increased interest to develop new hybrid structures and magnetic fluids with tailored textural and adsorption–desorption properties for controlled drug release, especially by using natural carbohydrate polymers due to their biocompatibility and biodegradability. The aim of this study was the synthesis, characterization and the assessment of the appropriate use of these new materials as biocompatible solutions for designing less expensive and easily available magnetic scaffold with further potential applications as drug delivery system, proved by a cytotoxicity assay and a cell cycle analysis.

Methods. Magnetic iron oxide particles were prepared by wet chemical precipitation from aqueous iron salt solutions by means of alkaline media like NH3. Chitosan (CS) and carboxy-methylcellulose (CMC) or silica network were used to improve the biocompatibility and stability of magnetic carriers. The obtained materials were characterized by SEM, XRD and FT-IR. Quantitative testing of the antimicrobial activity of antibiotics loaded into magnetic material and the establishment of MIC was determined by microdilution technique in liquid medium. Cytotoxicity assay was performed by using Trypan blue staining method and cell cycle influence induced by treatment with new hybrid structures or magnetic system was evaluated using flow citometry.

Results. The antimicrobial susceptibility assay of bacterial reference strain to antibiotics alone as well as to the antibiotics loaded on magnetic scaffold demonstrated that proposed delivery system maintain the antibiotics in active form, as proved by low MIC values obtained in the case when the magnetic scaffold drug delivery system was used. Treatment for 24 h with CS/Fe3O4/CMC hybrid material did not affect HCT8 cells. The CMC/Fe3O4 materials slightly increased G1, while Fe3O4/SiO2 the G2 phase, but in acceptable limits. Instead, the number of dead cells in the case of Fe3O4/SiO2 has been over the admitted limit (5.4%), the apoptosis appearance being suggested by trypan blue staining and proved by cell cycle analysis, which indicated a subG0 peak in the left side of G1 peak.

Conclusion. Our study proved that the obtained hybrid structures and magnetic scaffold could be used as delivery and/or controlled release systems for various classes of commonly used antibiotics, presenting the great advantages of low cost, biodegradability and low cytotoxicity. The flow cytometry is an useful technique for the accurate and rapid selection of the new materials, with potential biological applications.

P7. **Flow Cytometric Analyses in Cell Therapy for New Strategies in Cartilage Diseases**

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Arthritis, osteoarthritis and other degenerative diseases characterized by cartilage deterioration are the most prevalent chronic health disorders.

Despite their major socioeconomic impact there is still no satisfactory treatment. Autologous chondrocyte transplantation was the first application of cell therapy to orthopaedic surgery, based on ex vivo colonization of biodegradable polymer matrices that are subsequently transplanted to the large site. Tissue engineering as a treatment for osteoarthritis is even more challenging and cellular component quality plays a crucial role (stem cells and chondrocytes). This requires well-defined and efficient protocols for directing the differentiation of stem cells into the chondrogenic lineage, followed by their selective purification and proliferation in vitro.

Development of defined culture milieu for directing the chondrogenic differentiation of stem cells in vitro is also an important research direction. The aim of our study was to investigate and compare by flow cytometric methods, cell proliferation, morphological changes, cellular viability and apoptosis of human chondrocytes, cultured ex vivo for cartilage tissue-engineering applications and characterization of stem cells for surface antigen.

Flow cytometric analyses were performed on a FACScan cytometer using Pro CellQuest software for acquisition and analysis. Cell suspension in isotonic PBS buffer pH 7.4, osmolality 320-330 mosmol kg-1 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

Cell viability assessment was studied by Calcein-Am test (5 μM final), cell death was determined using an Annexin-V-FITC/PI apoptosis kit and cell proliferation by PKH-26 labelling. Flow cytometric analysis for stem cells was performed to characterize the surface antigen expression of CD34, CD45, CD73, CD90, CD105, CD 133 (positive and negative markers of hMSC identification) by immunostaining with monoclonal antibody FITC and PE-labeled.

All studies were performed at least three times, with three replicates each time. In all cases flow cytometric analysis of cellular components was essential.

Our results show that flow cytometric analysis of cells provide a rapid and accurate analytical tool in order to determine structure/function relationships and characterize stem cells and chondrocytes for cell therapy and biomaterials for reconstructive clinical procedures in cartilage disorders.

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P8. **Comparative Analysis of Growth Curve for Pseudomonas fluorescens Cultivated on Petrol and n-hexadecan Using Classical Analysis Techniques and Flow Cytometry**

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Study objèctivè was to dètèrminatè thè influèncè of concèntration gradiènt of n-hèxadècan and pètrol on growth curvè of Psèudomonas fluorèscèns batèrial strain using classical analytical mèthods and flow cytomètry tèchniquè.

Materials and methods. P. fluorèscèns batèrial strain isolatèd from soil pollutèd with pètrol, from an arèa locatèd nèar a pètrol èxtraction sitè in Bèrca villagè, Buzau county was cultivatèd in Minèral Salin Mèdium (MSM) supplimèntèd with with a concèntration of 1%, 2%, 4%, 6%, 15% (v/v) n-hèxadècan / pètrol, compounds usèd a singlè sourcè for carbon and ènèrgy. All èxpèrimènts wèrè carrièd out in duplicatè and as control, Minèral Salin Mèdium (MSM) with alcani/pètrol (1%, 2%, 4%, 6%, 15%) without bactèrial inocul was usèd. Thè growth curvè for thè batèrial culturè was dètèrminèd by èstablishing thè numbèr of colony forming units (CFU/ml). In this respect, decimal serial dilutions from cultures obtained after 24h, 48h, 96h, 8 days, 14 days and 23 days were carried out followed by inoculation of 100μl in Petri dishes with Luria Bertani (LB)

medium and incubation at 280C for 48 hours. During similar time intervals, the density of cultures obtained was determined specto-photometric and by flow cytometry using propidium iodide and acridinè orangè as indicators of batèrial viability and mètabolic activity.

Results. Comparèd analysis of thè growth curvè obtainèd by dètèrmining thè CFU/ml and by mèasuring thè flourèscèncè using flow cytomètry has dèmonstrratèd that P. fluorèscèns cèlls grown in thè prèsèncè of pètrol as uniquè sourcè of C havè èntèrèd morè rapidly in growth logaritmic phasè than thè cèlls cultivatèd in thè prèsèncè of n-hèxadècan, rèsult that can bè èxplainèd by thè origin of thè strain that is prèsènting thè ènzymatic èquipmènt nècèssary for thè mètabolism of pètrol. Thè analysis règarding thè viability of batèrial cèlls assèssèd by flow cytomètry tèchniquè using propidium iodidè as a indicator havè shown that thè intègrity of thè plasmatic mèmbranè was not affèctèd in thè prèsèncè of diffèrènt concèntrations of alcani or pètrol. Aftèr 8 days from cultivation cèllular agrègatès wèrè obsèrvèd for alacani and pètrol concèntrations of 6 and 15%.

Conclusions. Thè rèsults obtainèd suggèst thè possibility of application of flow cytomètry tèchniquès to charactèrisè microbial growth procèssès in pollutèd ècosystèms.

P9. **Flow Cytometric Assessment of Chlorella Cells Alterations under Stress Conditions**

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Among all organisms in aquatic ecosystems, microalgae are key targets in pollution cases, for two basic reasons: their eco-physiological similarities with terrestrial plants (the potential sensitivity of the same metabolic processes) and their role as primary producers (any change in the proliferation of the primary producers could provoke a global alteration in the equilibrium of the aquatic ecosystems. These characteristics support the use of the freshwater microalgae in laboratory toxicological assays. In relation to this point, flow cytometry allows the rapid determination of a high number of cell functions by using a great variety of biochemically specific, non-toxic and fluorescent molecules in conditions close to the in vivo status in short-term exposures to high levels of light.

Flow cytometric analyses of C. vulgaris cells were performed in a Cytomic FC 500 machine (Beckman Coulter, Inc., USA) equipped with an argon-ion excitation laser (488 nm), detectors of forward (FS) and side (SS) light scatter and fluorescence detectors. For each analyzed parameter, data were recorded in a logarithmic scale and results were expressed as mean values obtained from histograms in arbitrary units. Fluorescence of chlorophyll a (>645 nm) was used as a FCM gate to exclude non-microalgal particles. At least 100,000 cells per culture were analyzed.

Our results show that microalgae are ideally suited to flow cytometric analysis and can be used and detected by flow cytometry to provide information about the physiological status of algal cells in response to toxicants

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P10. **Flow Cytometric Assessment of in vitro Antioxidant Effect of Trigonella foenum-graecum Seed Aqueous Extract**

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Oxidative stress is involved in the development and progression of diabetic nephropathy (DN). Because Trigonella foenum graecum has been reported to have antidiabetic and antioxidative effects in vivo, to verify these results we analysed by flow cytometry this antioxidative properties of aqueous extract of Trigonella foenum-graecum seed on fibroblasts subjected to oxidative stress induced by hydrogen peroxide. To detect oxidative stress we applied DCF assay, a method that measure the levels of intracellular reactive oxigen species (ROS). Basically, cells are incubated with

the profluorescent, lipophilic H2-DCF-DA (dihydrodichlorofluorescein diacetate) which can diffuse through the cell membrane. Inside, the acetate groups are cleaved by cellular esterases so the resulting H2-DCF cannot leave the cells. Reaction with ROS, primarily hydrogen peroxide (H2O2), results in the fluorescent molecule DCF (max. emission ~ 530 nm), so that DCF fluorescence can be used as a measure for intracellular ROS levels. Flow cytometric analyses were performed on a FACScan cytometer using Pro CellQuest software for acquisition and analysis. Cell suspension in isotonic PBS buffer pH 7.4, osmolality 320-330 mosmol kg-1 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

Our results show that aqueous extract of Trigonella foenum-graecum seed has a strong antioxidant activity, confirming the data obtained in vivo. Measurement of reactive oxigen species is extremely difficult, because of the short lifetime of theses species and methods such as electron spin resonance and spin trapping are complicated and provide average values that can skew results when heterogeneous populations are being studied, while ROS assessment by flow cytometry has the advantage of a powerful technique to rapidly and simultaneously analyze several parameters for a large numbers of cells.

P11. **Modulation of Apoptosis and Proliferation Through cell Cycle Phases in Breast Cancer Cell Lines**

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Breast cancers represent some malignancies with high incidence and mortality throughout women, their etiology involving many genetic, immunological and biochemical factors. The main obstacle against the success of therapy in many cancers seems to be the impossibility of eradication of all tumor cells. Apoptosis represents a cellular "suicide" mechanism which keeps the cell number in tissues between normal limits and allows the elimination of cells presenting DNA mutations or having an aberrant cell cycle, cells predisposed to malignant transformation. The chemo-preventive efficacy has been associated to enhanced apoptosis, therefore any therapeutic strategy that specifically triggers apoptosis in cancer cells could be more useful for destruction of tumor cells. The present study focused on the potential influence of oncolitical treatment (doxorubicin/ adriamycin - ADR) in the presence or absence of natural compounds that present anti-inflammatory, -oxidant, -tumoral and -angiogenic properties. (such as quercetin, resveratrol) on human breast tumor cell lines (MCF-7, SK-BR-3, MDA-MB-231).

Sensibility of tumor cells to different concentrations of ADR and/or natural compounds for various periods of time was evaluated by cytotoxicity assays. Thè xCELLigèncè Systèm utilizing RTCA DP Instrumènt has bèèn usèd to assèss compound-mèdiatèd cytotoxicity in ordèr to monitor viability/toxicity continuously to pinpoint thè optimal timè points and concèntrations for conducting èndpoint assays, in addition to MTT method. Effects of modulation induced by ADR or associated treatments were studied by evaluation of progression through cell cycle, percentages of apoptotic cells and antigèn èxpression of associated molecules (P53, Bcl-2, Ki-67). Apoptosis was analyzed using Annexin V/FITC and propidium iodide (PI) double staining (BD Annexin V:FITC Apoptosis Detection Kit), while progression through cell cycle phases was estimated by using PI staining (BD Cycletest Plus/DNA Reagent kit), both followed by flow-cytometry analysis using BD FACScan or BD FACS CantoII flow-cytometers. New therapeutic approaches could be more useful for destruction of tumor cells and renewal of the cellular pathways that lead directly to apoptosis, contributing to the immuno-therapeutic management of cancer.

P12. **Ganoderma lucidum Enhances the Tumor Cells Sensitivity to Chemotherapy**

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Malignant lesions arising in the pharynx, are

mainly asymptomatic initially, are aggressive, and frequently invade and migrate to distant organs, making them difficult to treat. The survival rate for pharynx cancer has increased only moderately in the past decades. Also, despite the substantial progress in molecular and cellular biology over the last years, the prognosis of pharynx cancer has not significantly improved. Current treatment for pharynx cancer uses a chemotherapeutic agent - Cisplatin- which induces some toxic effects at the renal and bone morrow levels. However, it's possible that efficacy of cisplatin could be greatly increased in combination with natural compounds. Ganoderma lucidum (Reishi), an oriental medical mushroom, has recently received considerable attention from the health care and cancer research communities. The effects of G. lucidum appear to be due to the presence of numerous biologically active compounds in its composition, such as polysaccharides, triterpenes and immuno-modulators. However, the mechanisms responsible for the effects induced by treatment with G. lucidum in cancer cells remain to be elucidated.

In this study, we investigated in vitro the effects of G. lucidum on cell proliferation, and apoptosis of FaDu human pharynx carcinoma cells treated or not with cisplatin. Cells were treated with various concentrations of G. lucidum in the presence or absence of the cisplatin and CellTiter 96 Non-Radioactive Cell Proliferation assays were then performed to evaluate the effect of treatment on growth of FaDu cells. The cell cycle phase distribution, apoptosis process and expression of molecular markers (e.g. p21, Bax) in human pharynx carcinoma cells were assessed by flow cytometry. The apoptosis assay was carried out by using Annexin V-FITC/PI double staining kit, while cell cycle phases were investigated by using CycleTEST PLUS DNA reagent kit (BD Pharmingen), than data were acquired on BD FACScan or BD FACS Canto II flow-cytometers.

Results showed that G. lucidum extract had a strong cell proliferation inhibition effect on FaDu tumor cells cultures. Cell cycle analysis showed that the growth inhibition effect was associated with G2/M arrest and up-regulation of p21 expression. In addition, G. lucidum induced apoptosis of FaDu tumor cells and an increase of the proapoptotic Bax protein expression. Furthermore, the treatment with G. lucidum significantly amplifies the apoptosis induced by cisplatin in human pharynx carcinoma cells (FaDu). Taken together, these findings suggest that G. lucidum exerts anti-tumor effects on FaDu tumor cells and enhances their sensitivity to cisplatin. The effects induced by G. lucidum on cancer cells seem to be due to the activation of different cellular and molecular mechanisms, that might encourage the use of this compound as a potential adjuvant to standard cancer treatments.

P13. **The Investigation of the Internalization Pathways of Poly (Propylene Imine) Nanostructures in Tumor Cells by Flow Cytometry**

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Introduction. The diagnostic and treatment of malignant melanoma are seriously taking into account the formulation of active principles with dendrimeric nanoparticles. The identification and understanding of molecular mechanisms which ensure the integration of particular dendrimeric nanostructures in tumor cellular environment can provide valuable guidance in their coupling strategies with antitumor or diagnostic agents. By means of flow cytometry we investigated the internalization pathways and stability of two structurally distinct maltose-shell modified 5th generation (G5) poly(propylene imine) (PPI) glycodendrimers fluorescently labeled in several melanoma cell lines and one non tumor cell line.

Materials and Methods. Materials –Maltose-shell modified glycodendrimers of 5th generation (open maltose shell / G5-PPI-OS and dense maltose shell / G5-PPI-DS) coupled with FITC and Rhodamine-B fluorophores; melanoma cell lines (MelJuso, SKMEL28, MNT-1, A375, HEK 293T); trypan blue quenching solution; pharmacologic agents modulators of cholesterol- or clathrin - dependent internalization pathways, methyl-beta-cyclodextrin and chlorpromazine respectively.

Methods - cell culture techniques, cytotoxicity analysis of dendrimers and pharmacologic agents;

cellular uptake studies, trypan blue quenching assay, flow cytometry, bright and fluorescence microscopy analysis.

Results. We found that three melanoma cell lines internalize G5-PPI-DS structure more efficiently than non tumoral HEK297T cells. Furthermore, the internalization pathways of G5-PPI-OS and G5-PPI-DS are characteristic for each tumor cell phenotype and include more than one mechanism. Large amounts of both G5-PPI-OS and G5-PPI-DS are internalized on cholesterol-dependent pathway in primary melanoma cells and on non conventional pathways in metastatic melanoma cells. G5-PPI-OS, temporarily retained at plasma membrane in both cell lines, is internalized slower in metastatic than in primary phenotype. Unlike G5-PPI-OS, G5-PPI-DS is immediately endocytosed in both cell lines. The decay kinetics of fluorescent labeled G5-PPI-OS and G5-PPI-DS is distinct in the two cellular phenotypes.

Conclusions. Absence of citotoxicity even at higher concentrations, fast and reproductible internalization profiles and slow cellular decay make both cationic and neutral maltose G5-PPI glycodendrimeric structures good candidates for future development of intracellular delivery platforms following coupling with anti-tumor or diagnosis agents. In addition flow cytometry analysis using trypan blue quenching assay represents a reliable, quantitative method to discriminate between intracellular (internalized) and extracellular (plasma membrane-bound) fluorescence of the fluorescein-labeled nanostructures following interaction with cells.

II. Hematology Section

P14. **The Role of Multiparametric Flow Cytometry for the Detection of Multiple Malignant Clones in the Same Sample - Four Case Studies**

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Introduction. Bilineage or biclonal hematological neoplasias are extremely rare diseases characterized by the presence of more than one population of malignant cells identified in the same patient. These cells may be either of different lineages (bilineage) or of different clonality (biclonal), while sharing the same lineage markers. The extremely low incidence of these pathologies may render the clonality identification and assessment rather difficult. Aim. We report here four different cases with multiple malignant clones identified by multiparametric flow cytometry.

Materials and methods. Bone marrow (BM, n=3) and peripheral blood (PB, n=4) samples from four patients with suspicion of hematological malignancies (three suspicions of chronic lymphoproliferative disorders and one suspicion of acute leukemia) were investigated by flow cytometry (at diagnosis and after therapy) using a FACSCantoII (Becton Dickinson) cytometer and combinations of up to 7 colors. All patients were enrolled within the Hematology Department of St. Spiridon Emergency County Hospital, Iasi, Romania in 2011.

Results. Case 1 (B. M., male, 91 y). In one PB sample (8 380 cells/ μL) two distinct mature, monoclonal B lymphoid cell populations were identified: 10% monoclonal B lymphocytes (Matutes Score of 5), compatible with the diagnosis of B-chronic lymphocytic leukemia – CLL/ small lymphocytic lymphoma -SLL (CD45+ CD19+ CD5+ CD10- CD20+low CD22+ low CD23+low CD43+ CD38+ FMC.7- CD103- CD11c- IgM+ IgG- kappa+ low lambda-) and 24% monoclonal B lymphocytes (Matutes Score of 1) compatible with the diagnosis of B- non Hodgkin lymphoma - NHL (CD45+ CD19+ CD5- CD10- CD20+high CD22+ high CD23- CD43-CD38- FMC.7- CD103- CD11c- IgM+ IgG- kappa+ high lambda-).

Case 2 (C. C., female, 51 y). In a BM sample (248 000 cells/ L) the following cell populations were identified: 48% B lymphoid precursors (CD45+low CD19+ CD20(s+ic)- CD22+int CD10+ CD34+ TdT+ CD38+high IgM(s+ic)- CD79a+), 21% promonocytes and monoblasts (CD45+ CD64+high CD33+high CD13+ HLA/DR+ CD36+/- CD11b+/- CD14+int CD15+/- CD16- CD34- CD117-/+), 8% monocytes (CD45+ CD64+high CD33+high CD13+ HLA/DR+ CD36+ CD11b+ CD14+high CD15+ CD16-

CD34- CD117-), 18% granulocytes, and 4% lymphocytes. The same cells were identified, with different proportions (20% B cell precursors, 21% monocytes and promonocytes, 48% granulocytes, and 11% lymphocytes), in one PB sample (42 600/L) collected in the same time. Molecular biology analysis revealed the presence of a bcr-abl minor transcript (p190), and the patient responded remarkably well to treatment with Glivec. Two additional BM post-therapeutical evaluations by flow cytometry were performed.

Case 3 (V. V., female, 60 y). 65% monoclonal B lymphocytes were identified in a PB sample (17 200 cells/ μL) with a phenotype compatible with B-CLL (CD45+ CD19+ CD5+ CD10- CD103- CD20+ low CD22+low CD23+ FMC-7- CD38- IgM/IgD+ low IgG-). As no typical B lymphocytes were identified in the same sample, given the fact that the calculated ratio between kappa+/ lambda+ was balanced (2.4), and that the expression of both light chains was dim, we considered that we were able to identify two distinct B-CLL clones.

Case 4 (I. C., male, 58 y). In one BM sample (77 600 cells/L) two distinct malignant B lymphoid clones were identified: 32% B cells with a phenotype compatible with B-CLL (CD45+ CD19+ CD5+ CD20+low CD22+ low CD23+ FMC7- CD10- CD38+ CD103- IgMs- IgD+ IgG- kappa+ low lambda-, Matutes Score 5) and 18% B cells with a phenotype compatible with hairy cell leukemia - HCL (CD45+ CD19+ CD5- CD20+high CD22+high CD23- FMC7+ CD10- CD38+ CD103+ CD11c+high IgMs- IgD- IgG+ kappa+ high lambda-). Nine month later a PB (5 300 cells/L) evaluation was performed, when the same cell populations were identified (36,4% B-CLL, 1,4% HCL).

Conclusions. Different explanations were given for the occurrence of these particular diseases, the most frequent one being 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.

P15. **Immunophenotypic Heterogeneity of Monocytes in Myelodisplastic Syndromes**

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Background: Thè mononuclèar phagocytè systèm is compsèd of monocytè, macrophagès and thèir prècursor cèlls. Monoblast, promonocyte and monocyte arise in the bone marrow from unic progenitor CFU-GM. Monocytes are released into the blood and, after a short time in circulation, migrate into different tissues, and become tissue macrophages.

The Myelodisplastic Syndromes are a heterogeneous group of clonal hematopoietic stem cell disorder characterized by ineffective dysplastyc hematopoiesis, peripheral cytopenias, variable percentage of blasts, variable rates of progression to AML. The monocyte implication into MDS pathogenesis is due to proapoapoptoic role of TNFα, proinflamatory role of cytokines and immunoregulatory functions. Aims of this study was to evaluate the monocyte immunophenotype and they possible impact on the disease progression. Materials and methods. The frequency of monocyte was determined on the peripheral blood and bone marrow aspirate by flow-cytometry. We studied a group of patients from Hematology Departament of Coltea Hospital for an year January 2011-January 2010. We used the immunophenotypic diagnosis toghether with morfologic, cytochimic, cytogenetic and molecular data. The immunophenotypic diagnosis was performed with Beckman Coulter FC500 cytometer and CXP software and IVD-CE reagents Beckman Coulter, Becton Dikinson, Invitrogen. We analized patients with one or several cytopenias in peripheral blood and bone marrow. We used for stain a panel of monoclonal antibodies CD45, CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD15, CD16, CD19, CD22, CD33, CD34, CD36, CD38, CD40L, CD41, CD52, CD56, CD62p, CD64, CD71, CD117, CD235a, MPO, CD79a, TdT, lactoferin, HLA-DR, conjugated with following fluorochromes FITC, PE, ECD, APC, Pc5.

Results: We found on the monocyte surfaces a descreased expression of CD14, a higher expressin of CD16, diminished expression of CD4, presence of CD56, a heterogenous expression of HLA-DR, the lost of CD11b, diminished expression of CD33, heterogen expression of intracellular myeloperoxidase, presence of CD7 on mature monocyte.

Conclusion: The important immuno-phenotypic heterogeneity of monocytes in patients with myelodysplastyc syndromes may be used to follow the response to treatment.

P16. **Acute Leukemia with Multilinear Dysplasia, Clinical and Immunophenotypical Features**

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Background. Non-lymphoblastic multilinear leukemia with dysplasia is a significant subset of acute leukemias.
Aim. Our study proposes to identify specific features in acute leukemia with dysplasia in a group of patients analyzed over a period of five years.
Methods. There were analyzed 96 previously diagnosed patients with acute myeloid leukemia with myelodisplasia. Age of patients was analyzed to check the correlation of dysplasia in acute leukemia according to age. Other features were analyzed: sex, hematological parameters, morphology and immunophenotype. Immunophenotyping was performed from fresh bone marrow aspirate, and level of expression markers (MFI) was used in statistical analysis. Survival curves were analyzed, also.

Results/Conclusions. Dysplasia was not significantly associated with belonging to a class FAB, but more frequent was found myelo-monocytic acute leukemia (53.5%).Age in AML with dysplasia and in control group: younger than 40 years is a protective factor ($p = 0.0005 < 0.05$), while age> 70 years is a risk factor ($p = 0.35 < 0.05$) for the development of AML with dysplasia. Male patients predominated in both group of patients with dysplasia. No thrombocytopenia or severe thrombocytopenia and either leucopenia did not correlate significantly with the existence of dysplasia. Thrombocytopenia correlates significantly ($0.03 < 0.05$) with low hemoglobin values at diagnosis. Correlation remains in the control group ($p = 0.012 < 0.05$), but not in the group of patients with dysplasia. This would suggest that thrombocytopenia and anemia correlates with the degree of marrow infiltration in patients with leukemia, not influenced by the degree of dysplasia; in control group, patients without dysplasia, thrombocytopenia is associated with statistically significant ($0.012 < 0.05$) with a higher average percentage of blasts. Percentage blast in bone marrow since diagnosis varies differently in the two groups of patients, with an average lower in the group of patients with dysplasia than the control group (40.15% vs. 61.38%) ($p = 0.0001 < 0.05$). The mean percentage of blasts was lower in patients with dysplasia than those without dysplasia (but has not obtained statistically significant values). These correlations show that impaired erythropoiesis in patients with AML with dysplasia may have as a complementary role to the bone marrow infiltration and erythrocyte dysplasia. The vast majority of antigens identified by immunophenotyping were not significantly associated with AML with dysplasia; p values obtained were> 0.05. Some of these markers had values significant Odds Ratio or Risk Ratio, even if value was> 0.05, and we could fit as risk factors or protective factors for developing AML with dysplasia: CD11b positive - risk factor for AML with dysplasia; CD36 positive - a protective factor for AML with dysplasia; CD64 positive - a protective factor for development of AML with dysplasia; CD65w positive - a protective factor for AML with dysplasia. Antigens CD11b, CD56, CD7, CD14 are not correlated with post dysplasia or dysplasia de novo MDS. Development of AML with myelodysplastic syndrome is a risk factor for death before 6 months, odds ratio 2> 1, valid only for 70% of the population studied with statistical significance ($p = 0.3 > 0.05$). In summary, specific features in immunophenotype could be useful in diagnosis and prognostic assessment of acute leukemia's with myelodysplasia.

P17. **Expression Level of CD58 and CD99 on Malignant Lymphoid Precursors – a Study on 17 Acute Lymphoblastic Leukemia Cases**

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Introduction. Onè of thè most difficult tasks whèn assèssing minimal rèsidual disèasè (MRD) in acutè lèukèmias (AL) by flow cytomètry is thè accuratè distinction of lèukèmic lymphoblasts from thèir normal countèrparts. Rècènt gènè èxprèssion studiès lèd to thè idèntification of diffèrèntially èxprèssèd molèculès, including CD58 (up-règulatèd in lèukèmic blasts) and CD99 (èxprèssèd on T cèll prècursors and not on maturè T cèlls), that could bè usèd as nèw markèrs for MRD studiès by flow cytomètry.

Thè aim of our study was to assèss and comparè thè èxprèssion of CD58 and CD99 on malignant and non-malignant lymphoid cèlls using multiparamètric flow cytomètry.

Materials and methods. Expression levels of CD58 (FITC-conjugated, BD Pharmingen, clone 1C3) and CD99 (PE-conjugated, BD Pharmingen, clone Tu12) was assessed in 17 samples of bone marrow or blood from patient with acute lymphoblastic leukemia (12 B-ALL and 5 T-ALL) by multiparametric flow cytometry (up to 6 colors, using a FACS Canto II Becton Dickinson cytometer). The study was conducted between November 2011 and March 2012. CD58 and CD99 expression was also investigated on mature lymphocytes present in the same sample. As a control, CD58 expression was assessed on normal bone marrow B cell precursors from four subjects with hematological malignancies without bone-marrow involvement and CD99 on peripheral malignant T lymphocytes from one patient with T non Hodgkin lymphoma (T-NHL).

Results. The level of CD58 was found to be variable on malignant B cell precursors, although, as an average value, its intensity was higher than those recorded on normal precursors or on mature B lymphocytes. Although non-nèoplastic B-cèll prècursors èxprèssèd rèlativèly low CD58 lèvèls, thosè valuès wèrè simmilar with MFI valuès rècordèd in 2 out of 12 B-ALL casès. As for CD99, this marker was found to be very specific for T lymphoid precursors found in all 5 T-ALL cases investigated and it is completely negative on mature or malignant T cells. In the bone marrow, where we do not expect to have any T lymphoid precursors, malignant or not, CD99 is estimated to increase significantly the sensitivity of T-ALL MRD detection protocols.

Conclusions. Rèliablè markèrs arè increasingly nèèdèd in ordèr to èfficiènly track rèsidual tumor cèlls, to mèasurè rèsponsè to thèrapy, and prèdict disèasè rècurrèncè with a sènsitivity supèrior to that offèrèd by currènt approachès.

P18. **Detection of a Novel t(4;11)(q21;q23) MLL-AF4 Fusion Transcript in Infant Leukemia**

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Introduction. Chromosomal translocations involving mixed lineage leukemia (MLL) gene on 11q23 are detected in several types of leukemia. One particular translocation, t(4;11)(q21;q23), occurs with high frequency in infant acute lymphoblastic leukemia (ALL) leading to the fusion of the MLL gene on chromosome 11 and the AF4 gene on chromosome 4. Different MLL-AF4 fusion transcripts have been detected depending on inclusion of exons within the breakpoint regions. In these cases, leukemic cells were reported to have a lymphoblastic morphology and to express CD34, HLA-DR, CD19, while CD10 was ussualy negative. In addition, the myeloid markers, CD15, CD65 and NG-2 were reported to be co-expressed.
Materials and methods. A 21 days new-born boy, was admitted to the Intensive Care Unit with increased number of WBC and a bone marrow puncture revealed infiltration with 92% lymphoid

blasts. At the admission in the Hemato-Oncology Unit the infant presented 51% blasts in the peripheral blood and a peripheral blood sample (40 999 cells/L) was evaluated by multiparametric flow cytometry. The expression of the following markers was investigated: CD45, CD14, CD71, CD5, CD10, CD19, CD33, CD13, HLA-DR, CD34, CD117, CD4, CD8, CD3(s+ic), CD16+56, CD20(s+ic), CD22, IgM(s+ic), CD38, TdT, CD79a, MPO. The presence of four fusion genes: TEL-AML1, BCR-ABL p190, E2A-PBX1 and MLL-AF4 was assessed by molecular biology. Onè anusually long MLL-AF4 fragmènt was purifièd with Wizard® SV Gel and PCR Clean-up System (Promèga Inc, Madison, WI, USA) and thèn was analysèd using Sangèr sèquèncing.

Results. 52,7% B cell precursors were identified (CD45+low CD19+ HLA/DR+ CD10-CD34+ CD22+/- (38%) CD20s- CD20ic+low IgMs+ic - CD79a+ TdT- CD38+int CD33+/- (32%, aberant)). The amplifications revealed positivity for MLL-AF4, and showed the presence of an unexpectedly longer sized product (~200bp largèr than thè biggèst èxpèctèd amplicon è11-è4). Sequence analysis revealed a previously undescribed MLL-AF4 fusion transcript resulting from in-frame fusion of exon 12 of the MLL gene and exon 4 of the AF4 gene. One year after achieving morphological and molecular remission following the induction phase of chemotherapy the patient relapsed. Immunephenotyping, performed on a sample of peripheral blood (101 850 cells/L), revealed the presence of 20,4% B lymphoid precursors with co-expressing myeloid markers (CD45+low CD34+ HLA/DR+ CD19+ CD33+/- (12%) CD20s- CD22-/+ (1,8%) CD64+low CD15+int MPO- TdT+low) but, in addition, a dominant (61,6%) monocytoid population (promonocytes and monocytes) was identified (CD45+ HLA/DR+ CD33+ CD13+ CD117- CD34- CD14+/- (46%) CD64+high CD36+ CD11b+/- (37%) CD2- CD15+ CD16- MPO). Molecular analysis identified again the presence of MLL-AF4 e12-e4 transcript, but not other transcript characteristic for myeloid lineage.

Conclusions. To the best of our knowledge, this is the first report of a MLL-AF4 rearrangement revealing a complex transcript with novel breakpoints in MLL. This particular genetic anomaly associated with co-expression of multilineage markers at diagnosis and with the expansion of a secondary, co-existing clone at relapse.

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P19. B-precursor Acute Lymphoblastic Leukemia: Case Presentation

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Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in children, representing nearly one third of all pediatric cancers. Immunophenotypic profile of cells plays a central role in ALL diagnosis but conventional and molecular cytogenetic techniques may also aid in categorizing the malignant lymphoid clone.

In this report we present the case of a 7 years child diagnosed in march 2010 with B precursors ALL wich was accepted in oncohematology department in march 2012 with suspected relapse. Flow cytometry from bone marrow revealed 73% infiltration with B lineage blasts wich expessed the following phenotype: CD45var+ cyCD79a+ cyTdT+ HLA-DR+ CD33+ (7%) CD10+ CD34+ (35%) cyCD22+ AC133-1+ CD24+ CD9+ (absent markers from blastic population: cyCD3, cyMPO, CD13, CD7, CD33, CD56, CD41a, GlyA, CD5, CD20, CD25, kappa, lambda); diagnosis of relapse with the same type of B cells was confirmed. The DNA ploidy in bone marrow was evaluated and the DNA index of 1,19 measured suggested presence of hyperdiploidy >50 chromosomes in tumoral population. Cytogenetic analysis shows that 40% of analized metaphases are normal in number and morphology, the rest presents hyperdiploidy with 47, 52 and 55 chromosomes (trisomic chromosomes: 3, 6, 14, 20, 21; tetrasomic chromosomes: 14, 21; pentasomic chromosomes: 14, 21) but also secundary anomalies such as duplication (1)(q21;32) and translocation(14;14)(q32;q11). Fluorescence in situ hybridization (FISH) revealed trisomy, tetrasomy and pentasomy 14 and 21

usingTEL/ALM and IGH probes but also IGH gene rearrangements.

Hyperdiploidy (51-56) is a common cytogenetic abnormality in B precursor ALL; determination of DNA index has a limited significance because it is not able to give information about number and structure of chromosomes involved in hyperdiploid clone.

P20. **CD200 in Diagnosis of Chronic Lymphoproliferations**

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Background: The diagnosis and management of patients with chronic limphoproliferative diseases has become dependent on immunological criteria. Flowcytometry immunophenotyping is used for rapid and specific diagnoses. But, there are cases when we are not facing a typical immunophenotype and for that, there is a constant need for finding new markers and new combinations of markers that allow us to improve and develop our diagnoses. Mature B-cell lymphoproliferative disorders have specific phenotype and in the presence presence of a CD5 positive B-cell lymphocytosis, it is important to differentiate Chronic Lymphocytic Leukemia (CLL) from mantle cell lymphoma (MCL).

Aim: Our aim was to evaluate CD200 expression in different B-cell chronic lymphoproliferative disorders. CD200 is a membrane glycoprotein belonging to the immunoglobulin superfamily and overexpression of CD200 has been reported in a number of malignancies, including CLL, as well as on cancer stem cells.

Methods: we analyzed CD200 expression in 122 patients diagnosed with chronic lymphoproliferative disorders (100 patients with CLL, 10 patients with splenic marginal zone lymphoma (SMZL), 10 patients with MCL and 2 patients with hairy cell leukemia), in the Hematology Department of Emergency University Hospital Bucharest. We performed immuno-phenotypical analysis of peripheral blood and bone marrow aspirate on BD FACS Calibur flowcytometer. Our diagnosis panel included the following markers for B cells: CD19, CD20, CD5, CD23, CD79B, CD103, CD11c, CD25, CD10, and FMC7.

Results: CD200 was brightly expressed in all 100 CLL patients (100%). In SMZL patients, CD200 was dim positive (40%-60%), in patients with HCL.CD200 was also bright positive (96% and 97%) and in patients with MCL CD200 was negative (1-10%); CD 200 was significantly higher in CLL patients compared with other B-cell chronic lymphoproliferative disorders. We found 14 patients with CD19, CD5 positive population and CD23-, but with high expression of CD 200. Cyclin D1 was negative on bone marrow biopsy in 13/14 of these patients. (1/14patients was without bone marrow involvement);

Conclusions: CD200 has a great impact in diagnoses of B- chronic lymphoproliferative disorders, especially when we want to determine the origine of a CD19, CD5 positive population and differentiate CLL from MCL. CD 23 is a reliable marker in those cases, but, as we showed, CD23 might have a lower specificity than CD200 for CLL. The diagnosis of MCL has to be confirmed by demonstration of cyclin D1 positivity or by the presence of the t(11;14)(q13;q32) chromosomal translocation detected by cytogenetic, Western blot or Polymerase Chain Reaction (PCR) analysis, fluorescence in situ hybridization (FISH). But, these methods are expensive, time-consuming and not quite available. We added CD200 in our panels for diagnoses of chronic lymphoproliferative disorders, not to replace CD23, but to improve and save time in our diagnosis.

P21. **Immunophenotypic Characterization of T and NK Cell Subsets in B-CLL**

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Chronic lymphocytic leukemia (CLL) patients display immune deficiency that is most obvious in advanced stage disease. Phenotipically distinct cell subsets may be particularly susceptible to apoptosis, may have esential functional characteristics with anti-tumor, proliferative, metastasizing, infiltration and invasion effects.

Normal lymphocyte differentiation is a complex process, regulated by the integration of multiple signals from the microenvironment. These signals are received through different cell surface molecules (receptors) as costimulatory and adhesion molecules, antigen and cytokine receptors. Alterations in the phenotype and number of T and NK lymphocytes may lead to tumor evasion and increased susceptibility to recurrent infections in CLL. NK lymphocytes represent an important component of the innate immune system, are heterogenous in the matter of CD56 and CD16 expression, having different functional capabilities. There are 3 main subsets described: CD56+brightCD16- is an immunomodulatory subset that produces a wide range of cytokines and chemokines, having a reduced cytotoxic capacity; CD56+dimCD16+ subset produces relatively low amounts of cytokines, but possesses cytolytic granules and can spontaneously lyse target cells; CD56-CD16+ subset is low represented in normal healthy individuals but seems to be increased and disfunctional in some pathologies (as HIV infection). As CLL is also a disease of the immune system, it is considered that all the lymphocyte compartments are involved. The appropriate differentiation of T lymphocytes from naive to effector and memory cells is very important in CLL as many patients die from infections and autoimmune diseases. Thus the aim of the study was to investigate whether the frequency and cell phenotypes are different among all Rai stages CLL patients regarding T and NK cell subsets.

Material and method: Fifteen peripheral blood mononuclear cell samples from CLL patients (6 pts Rai stage 0, 5 pts Rai stage I/II and 4 pts Rai stage III/IV) were thawed, washed in PBS and stained for six color flow cytometry analysis. The monoclonal antibodies used: CD16 FITC (Dako), CD57 PE (BD), CD3 PERCP CY5.5 (BD), CD4 PE-CY7 (BD). CD56 APC (BD), CD8 APC-CY7 (BD), CD27 FITC (BD), CD28 PE (BD), CD45RA APC (BD). The gating strategy was adapted according to the population of interest. Propidium iodide was used for viability determination. The acquisition and data interpretation was realised using BD FACSCanto II and FACS Diva software.

Results and discussions: The viability of PBMCs was over 86%. The expression of markers used to dissect the peripheral T (CD4+ T helper cells, CD8+ T cytotoxic cells, CD4-CD8- considered activated T cells being able to produce cytokines, CD4+CD8+considered antigen specific memory cells) and NK (CD56+bright CD16-, CD56+dim CD16+, CD56-CD16+) cell subpopulations enabled the distinction of many heterogenous phenotypes. The T cell subsets are very heterogenous and their biological meaning in disease evolution needs further investigations. NKT lymphocytes (CD3+CD56+CD16+/-) expressed as percentage from total T cells decrease in advanced stages of the disease. Total NK cells expressed as percentage from lymphocytes also decrease in advanced stages Rai III/IV. NK subsets CD56+CD16+ wich are known to have mostly cytotoxic activity also decrease in CLL Rai III/IV, but the other 2 subsets CD56+CD16- and CD56-CD16+ which are known to secrete cytokines and the latter having poor cytotoxic activity are increasing. These may be related to the fact that the CLL clone needs in order to be maintained and expanded, special soluble factors.

Conclusions: Lymphocyte disfunction, identified by multiparametric immuno-phenotyping, as part of an impaired immunity may have clinical implications. Establishing a pattern of particular cell subsets may be of great importance in predicting the point of no return in restauration of the normal immune activity.

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P22. **Role of Biological Active Compounds in B-CLL Cells**

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Introduction. Apoptosis induction is the most potent defense against cancer. The relation between carcinogenesis and disregulation of apoptosis is well known; therefore any therapeutic strategy that specifically triggers apoptosis in cancer cells might have potential therapeutic value. One of this possibility is to use the biological active compounds (naturally occurring antioxidant compounds) to eliminate premalignant/malignant cells by indunging them to undergo apoptosis.

B-cell chronic lymphocytic leukemia is a neoplasic disorder characterized by defective apoptosis; the tumoral cells do not frequently express Fas receptors therefore are resistant to its apoptotic action and the expression of some antiapoptotic proteins (i.e. iNOS and Bcl-2) are up-regulated.

Objectives. In this study, by using the biologic active compounds, we tried to induce the apoptosis in the leukemic cells as well as to modulate the expression of iNOS and Bcl-2 proteins.

Materials and Methods. Cells: EHEB and ESKOL cells line routinely maintained in culture at 370C, 5% CO2 in complete RPMI-1640 medium (Sigma, USA).

Reagents: FITC-labeled anti-iNOS mAb (FITC-macNOS, clone 6, BD Transduction laboratories, Heidelberg, Germany); anti Bcl-2 mAb (Biosource, Nivelles, Bergium); trans-resveratol (3,4',5-tri-trihydroxy-trans-stilbene) was purchased from Sigma (St. Louis, USA) and the biological active compounds from Dr. Michele Meyer (Laboratoire de Chimie des Substances Naturelles, Paris, France)

Quantification of apoptosis: Cells undergoing apoptosis were estimated by FACS detection of phosfatidyl serine expression at the other leaflet of the plasma membrane using detection kit of FITC-labeled annexin V (Transduction Laboratory, BD, USA) with or without simultaneous labeling with propidium iodine. Apoptosis-induced DNA fragmentation was estimated by an ELISA test (Cell Death Detection ELISAPLUS, Roche Diagnostics, USA) that measures the percentage of nucleosomes in the cytoplasm, according to the manufacturer's specifications. **iNOS and Bcl-2 detection:** The expression of iNOS and Bcl-2 were analyzed by flowcytometry on permeabilized cells after the treatment with he biological active compounds at different concentrations.

Conclusions: In a time and concentration dependency the biologic active compounds induce both, apoptosis and down-regulation of iNOS and Bcl-2 proteins.

P23. **Immunophenotypic Profile of T Cells in 11 Cases of Adult T-cell Leukaemia/Lymphoma**

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Adult T-cell leukaemia/lymphoma (ATLL) is a mature T-cell neoplasm of post-thymic lymphocytes aetiologically linked to the human T-cell lymphotropic virus, HTLV-I, and with a distinct geographical distribution. The virus is endemic in Japan, West Africa and South America but is very rare in the US, Europe and the Middle East. In Romania, antibodies to the HTLV-1 were found in 0.64% of the blood donors indicated a 25-50-fold higher seroprevalence rate compared to other areas of Europe and the US (Paun L., et al,1994). Only occasional ATL cases were reported in Romania (Motoiu I.R., Pecek M.M., Dumitrescu A., Colita A., Necula A., Moldoveanu E., Colita D., 1993; Schaefer H.E., Lubbert M., 1996; Veelken H. et al, 1996, Lin BT et al, 1997, M. Shtalrid et al., 2005). The morphology and the immunophenotype of the circulating neoplastic lymphocytes are very characteristic of the disease: "flower" cells with the immunophenotype of an activated mature T-lymphocyte.

We reviewed the cases of ATLL analyzed by flow cytometry in Fundeni Clinical Hospital from 2000 to 2011. The 11 patients (5 males and 6 females), were aged from 19 to 82 years, with a median age of 46 years. In all cases the presence of HTLV-I/II was confirmed.

The white blood cell (WBC) count ranged from 9.6 to 128.28 x109/ L (median= 33.03 x109/L) with circulating abnormal cells in each specimen, including cells with hyperlobated nuclei (flower cells) or irregular nucleolar contours (from 46% to 95%).

Peripheral blood samples (in 10 cases) and

bone marrow aspirate sample (one case), prelevated on EDTA, were stained with a variable panel of monoclonal antibodies, and analyzed on a BD flow cytometer (FACScan or FACSCalibur).

An abnormal CD3+low T-cell population was distinguishable from the normal T-cell populations by flow cytometric analysis. The ATLL cells expressed CD4 (10 of the 11 cases), CD8 (1 of the 11 cases); CD2 and CD5 were positive in almost all cases; CD7 was negative in almost all cases. CD25 was positive in all cases. Other T-cell activation markers such as CD38 and HLA-DR are variably expressed.

Our data are in concordance with those reported in literature. Multiparametric analysis with an extended panel of monoclonal antibodies may contribute to better identification of malignant cells and monitoring later the response to therapy.

P24. **Hypereosinophilic Syndrome - Lymphocytic Variant. Case Report**

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The hypereosinophilic syndrome (HES) is a rare disease characterized by a persistent hypereosinophilia (≥ 1500 eosinophils/mm^3) in the blood for at least six months without any recognizable cause, associated with target organ damage.

HES patients are currently categorized according to 2 classifications: World Health Organization 2008 and Working Classification 2006, but both have several limitations in daily practice. Despite advances in our understanding of HES pathogenesis, more than 50% of patients are still diagnosed with idiopathic disease, while the remaining subset has myeloproliferative or lymphocytic variants.

The myeloproliferative variant of HES is actually chronic eosinophilic leukaemia with a unique genetic marker, FIP1L1-PDGFRA. The lymphocytic variant of HES (L-HES) represents a distinct clinical entity. Hypereosinophilia in these patients results from the overproduction of eosinophilopoietic cytokines, mainly interleukin 5 (IL-5), by clonal T cells. The detection of the aberrant T-cell phenotype in peripheral blood by flow cytometry and the presence of T-cell receptor (TCR) clonal rearrangement are required for diagnosis. The lymphocytic HES variant is associated with T-cell clones producing interleukin-5 (IL-5) and can evolve into lymphoma. The first HES patient with an aberrant phenotype and overproduction of IL-5 was described in 1994.

We present a case of HES-lymphocytic variant with some diagnosis and treatment issues. The patient is a veterinarian and for 5 years before diagnosis she presented transient episodes of urticarial lesions and erythematous, pruritic papules and nodules. At diagnosis CBC shows leucocytosis with 50% eosinophilia (9000/µl) and tested positive for IgG Trichinella spiralis antibodies (not for IgM). She received proper treatment for the parasitic infestation but the hypereosinophilia and cutaneous manifestation did not resolved even six months after. Despite persistent hypereosinophilia, patient did not developed any other target organ lesions.

Hystopathological and immunohistochemical exam of the bone marrow biopsy were compatible with an hypereosinoplic syndrome, and excluded a lymphoproliferative syndrome. Morfological and immunohistochemical examination of cutaneous biopsy did not differentiate between reactive lesion and indolent T cell lymphoma.

Cytogenetic examination reveals normal karyotype and molecular biology for FIP1L1-PDGFRA was negative.

Flow cytometric analysis of peripheral blood shows a lymphoid cell population of 35% from total cells, consisting in majority (66%) of aberrant T cells that displays this phenotype: CD3-, CD2+, CD5+, CD7-; CD4+, CD8-, TCRαβ-, TCRγδ-; CD25-, CD38-, HLA-DR-.

The patient received glucocorticoid therapy with resolution of cutaneous manifestation and hypereosinophilia.

It was demonstrated that hypereosinophilia may precede the development of T-cell lymphoma years later, so we will follow-up the patient closely in order to diagnose and treat this possible malignancy early in evolution.