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REVISTA SOCIETĂȚII ROMÂNE DE HEMATOLOGIE
ȘI A SOCIETĂȚII NAȚIONALE DE TRANSFUZIE SANGUINĂ
DIN ROMÂNIA

ROMANIAN SOCIETY OF HAEMATOLOGY

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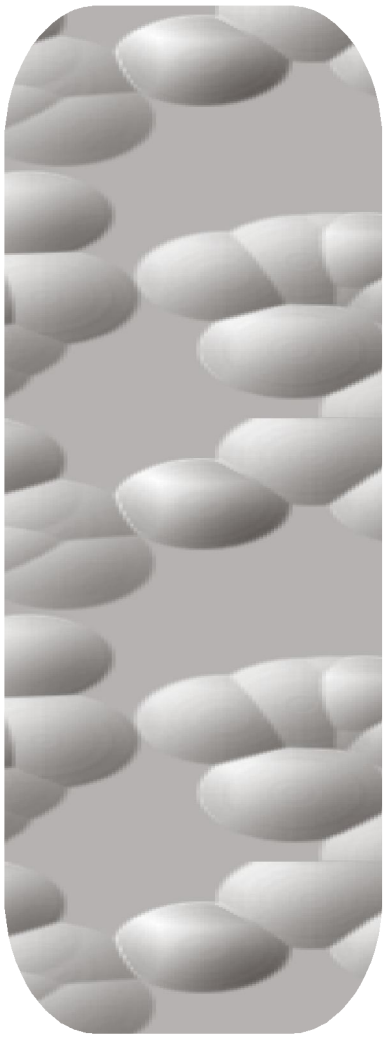

NATIONAL SOCIETY OF BLOOD TRANSFUSION FROM ROMANIA

DIAGNOSIS AND TREATMENT PROTOCOL FOR HEMOPHILIA AND VON WILLEBRAND DISEASE

References:

1. European "Guidelines for the management of hemophilia", Srivastava A et al, Haemophilia 2012
2. WFH Guidelines for the de management of hemophilia, Second print edition
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I. DISORDER DEFINITION

Hemophilia is a congenital hemorrhagic disorder transmitted hereditary X-linkat, characterized as reduced quantitative or altered qualitative synthesis of coagulation factor VIII (Hemophilia A) or of factor IX (Hemophilia B).
Hemophilia A is more frequent than hemophilia B, representing 80-85% of the total cases of hemophilia. The incidence of hemophilia A is of 1 case in 5000 – 10000 male newborns. 75% of the hemophilia cases are hereditary, and the remaining 25% (and in the opinion of certain authors even up to 50%) represent de novo hemophilia.

Taking into account the serum level of the coagulation factor, there are 3 forms of Hemophilia:
- Light form, quantity of coagulation factor >5%
- Moderate form, quantity of coagulation factor 1–5%
- Severe form, quantity of coagulation factor <1%

Hemorrhagic events

- The characteristic phenotype of hemophilia represents the tendency to bleed.
- The severity of the hemophilia hemorrhagic episodes is generally correlated to the coagulation factor (Table 1).

Hemophilia Severity	Coagulation factor level	Hemorrhagic episodes
Severe	<1 UI/dl or <1% of the normal value	Spontaneous hemorrhages at the level of the joints and muscles, in general without an identifiable cause.
Moderate	1-5 UI/dl or 1-5% of the normal value	Occasional spontaneous hemorrhages; prolonged hemorrhages following minor traumatisms or surgeries.
Light	5-40 UI/dl or 5-40% of the normal value	Severe hemorrhages following major traumatisms or surgeries; spontaneous hemorrhages are rare.

Table 1. : Correlation between the severity of the hemorrhagic episodes and the coagulation factor level

- Very often the hemorrhagic episodes are internal, at the level of the joints and muscles (Table 2).
- Some hemorrhagic events (cerebral, gastro-intestinal, neck, larynx) can endanger life and require emergency treatment

Hemorrhage location	Frequency (%)
Haemarthrosis	70-80
Muscular hemorrhages	10-20
Other major hemorrhages	5-10
SNC hemorrhages	<5

Table 2. Frequency of hemorrhagic episodes depending of their location

II. INITIAL HEMOPHILIA DIAGNOSIS PROTOCOL

II.1. Postnatal diagnosis

Diagnosis grounds
- history (characteristic hemorrhagic events, family history – family tree)
- active diagnosis for boys coming from families with hemophilia (family tree)
- approximately 50% of our newly diagnosed cases do not have family history (rare forms)

Confirming the diagnosis and determining the type of hemophilia
- activated partial time of de thromboplastin (TPTA)
- prothrombin consumption time
- global coagulation time, Howell duration have different frequently normal values in the non-severe cases and they are presented as screening tests
- correction of the assimilation duration of prothrombin or TPTA with fresh plasma, old serum and plasma absorbed on barium sulfate

Possible diagnosis	TP	TPTA	Bleeding time	No. of thrombocytes
Normal	Normal	Normal	Normal	Normal
Hemophilia A or B	Normal	*Prolonged*	Normal	Normal
Von Willebrand disease	Normal	Normal *or prolonged*	Normal *or prolonged*	Normal *or reduced*
Thrombocytes defect	Normal	Normal	Normal *or prolonged*	Normal *or reduced*

Table 3. Interpretation of the screening test

Establishing the level of severity of hemophilia
- establishing the plasma concentration of factor VIII/IX through a metric coagulation / chromogenic method (<1% severe forms; 1 - <5% moderate forms; 5-40% light forms)
- establishing the anti-FVIII or anti-FIX inhibitors, recovery test and establishing the period for administering half of the quantity of FVIII and FIX

II.2. Prenatal diagnosis
- analysis of the family tree
- diagnosis of the gender of the fetus
- bio-molecular tests in order to determine the disease's genetic substrate

• III. HEMOPHILIA TREATMENT PRINCIPLES
- The main purpose of the treatment is preventing and treating the hemorrhagic episodes.
- Prophylaxis in children suffering from severe hemophilia is recognized as standard treatment. Prophylaxis in adults can also be initiated when it is necessary, depending on the doctor's decision.
- The treatment for patients suffering from hemophilia is best managed in Comprehensive Centers for treating hemophilia.
- The acute hemorrhagic episodes must be treated as soon as possible, preferably within the 2 hours as of the moment of commencement. If there are any diagnosis doubts regarding the hemorrhagic episode, the recommendation is to administer treatment.
- The diagnosis and rapid initiation of the treatment limits the destruction of articular tissues.
- In the event of an acute hemorrhagic event, an evaluation must be performed in order to establish the stage of the hemorrhage (if it is not

obvious from the clinical perspective) and treatment with deficient coagulation factor must be initiated.

- In the event of severe, life threatening hemorrhages, especially those located at the level of the brain, neck, larynx, digestive tube, treatment with deficient coagulation factor must be initiated immediately, even before having finalized the diagnosis tests.
- In order to facilitate the correct management of emergency situations, all patients must carry with them an identification card that shall contain the following information regarding the disease: diagnosis and severity of the disease, inhibitors status, product and doses utilized for treating the disease and contact details of the attending doctor.
- If the hemorrhagic episode is not solved despite having administered the adequate dose of coagulation factor, the plasma level of the coagulation factor must be measured; if this level is significantly low, then the presence and level of the inhibitors must be measured.
- Patients with inhibitors who do not react or who are not eligible for immune tolerance induction therapy should be administered a prophylactic treatment with bypass agents.
- The decision on selecting concentrates of coagulation factors for treating patients suffering from hemophilia should not be taken only based on the cost criteria but also based on the quality criteria of the said product; the patients suffering from hemophilia must be provided with access to safe and efficient concentrates of coagulation factors in the optimum dosage; in the case of prophylaxis in children that were not previously treated (PUP), recombined concentrates of coagulation factors.
- Preserving vein access is of vital importance in patients suffering from hemophilia.
- Avoiding products that lead to platelet dysfunction, especially those that contain aspirin. The anti-inflammatory medication without steroids must be administered with caution. For example it is recommended to administer acetaminophen with or without codeine in order to control pain; when administering combined medication you must take into account the interaction between the medications.
- At home treatment administered with coagulation factor can be initiated in children of young ages with adequate vein access, after having adequately trained the family members; home treatment reduces the costs and complications associated with hemophilia and it should be a standard for patients suffering from hemophilia.

IV. HEMOPHILIA TREATMENT PROTOCOL

IV.1. Prophylaxis of hemorrhagic accidents

IV.1.1.Definitions:

Continuous primary prophylaxis: continuous and regular treatment before the articular affection documented clinically and/or by means of imagery, before the occurrence of the second haemarthrosis at the level of major joints* and before the age of 3.
Continuous secondary prophylaxis: continuous and regular treatment, initiated after the occurrence of two or several haemarthrosis at the level of major joints* and before the joints are affected and documented clinically and/or by means of imagery.
Tertiary prophylaxis: continuous and regular treatment, initiated after the joint has been affected and has been clinically documented by means of imagery.
Intermittent prophylaxis (periodical): treatment administered in order to prevent bleeding for a period of time that does not exceed 45 weeks during one year.
*Major joints: ankle, knee, hip, elbow and shoulder
Continuous treatment is defined as the treatment intent for a period of 52 weeks per year and with a minimum number of dosages defined in advance with at least 45 weeks (85%) per year.

IV.1.2.Objectives: preventing hemorrhagic accidents, relieving chronic articular disease, improving the quality of life of the patients suffering from hemophilia.

IV.1.3.Inclusion criteria (age, gender, clinical and para-clinical parameters, etc.)

1. Patients suffering from Severe congenital hemophilia (FVIII and FIX congenital deficit)

without inhibitors
2. Patients with an age between 1-18
3. Patients suffering from a severe form of the disease, regardless of the age, with a high risk of bleeding (dental therapy, invasive interventions, physical recovery)

IV.1.4.Treatment (doses, conditions for reducing the dose, treatment period)

Products:
Hemophilia A: Plasma coagulation factor VIII; Recombined coagulation factor VIII
Hemophilia B: Plasma coagulation factor IX; Recombined coagulation factor IX

Dosage:
Hemophilia A: FVIII coagulation concentrates
- 20-40 UI factor VIII/kg, every 2 - 3 days for patients over 6 years of age
- 20-50 UI factor VIII/ kg of the body, administered 3 - 4 times per week for patients with the age < 6 years.

Hemophilia B: FIX coagulation concentrate of
- 20-40 UI factor IX/kg, every 3-4 days.

Administration: intravenously, slowly. The administration interval must be established by the hematology specialist/doctor.

Administration recommendations:
- During prophylaxis in children that were not previously treated (PUPs), it is recommended to administer recombined coagulate factor VIII concentrates.
- For the long term it is generally recommended to use the same biologic product, except for the situations in which it is proven that they are not efficient or complications occur because of the said product
- A personalized, pharma-kinetic guided treatment protocol provides the possibility to establish individual doses and administration interval

IV.1.5. Treatment monitoring (clinical and para-clinical parameters, periodicity)
- Careful monitoring, through clinical examination and laboratory tests, in order to detect the development of antibodies inhibitors.
- Clinical and para-clinical monitoring of the hemorrhage events and articular status

IV.1.6. Treatment exclusion criteria

- Hypersensitivity to the active substance or to mouse or hamster excipients or proteins, with the recommendation of changing the treatment's biologic product
- Development of the anti-FVIII or anti-FIX coagulation inhibitors

IV.2. Curative treatment of hemorrhagic accidents

IV.2.1. Objective: stopping the hemorrhagic accident

IV.2.2. Inclusion criteria (age, gender, clinical and para-clinical parameters, etc.)

1. Patients with congenital hemophilia without inhibitors, with hemorrhagic episode
2. Age: any age group

IV.2.3. Treatment (dose, conditions for reducing the dose, treatment period)

Products:
Hemophilia A: Plasma coagulation factor VIII; Recombinant coagulation factor VIII
Hemophilia B: Plasma coagulation factor IX; Recombinant coagulation factor IX
The dose and duration of the substitution therapy depend on the severity of the factor VIII/ IX deficit, hemorrhage location and degree and the patient's clinical state.

Hemophilia A:

Dose:
Determining the necessary dose of factor VIII relies on the following empiric observation:
1 UI factor VIII/kg increases the plasma activity of factor VIII with 2 UI/dl.
The necessary dose is determined by utilizing the following formula:
Necessary units (UI) = body weight (kg) x desired increase of factor VIII (%) x 0.5.

Hemorrhage severity	Plasma level of the necessary FVIII (% of normal of UI/dl)	Administration frequency (hours) / treatment duration (days)
Early haemarthrosis, muscle or oral hemorrhages	20 – 40	Repeated injections are administered every 12-24 hours (from 8 to 24 hours, for patients with the age under 6), for at least one day, until the hemorrhagic episode is remitted or until the patient is healed, this being indicated by the lack of pain.
Haemarthrosis, muscle hemorrhages or enlarged hematoma	30 – 60 60 – 100	Repeated injections are administered every 12-24 hours (from 8 to 24 hours, for patients with the age under 6), for a period of 3–4 days or more, until pain or acute functional impotence are remitted.
Hemorrhages that endanger life		Repeated injections are administered every 12-24 hours (from 6 to 12 hours, for patients with the age under 6), until the danger is eliminated.

Table 4. Plasma level of the necessary FVIII, depending on the severity of the hemorrhage episode

The dose and frequency of administration must be adapted depending on the individual clinical answer. In certain circumstances (for example the presence of a low titer of anti-factor VIII anti-bodies) it might be necessary to administer higher doses than the ones calculated with the help of the formula.

Hemophilia B:

Dose:

The calculation of the necessary dose of factor IX relies on the empiric observation, in accordance to which 1 UI factor IX per kg increase the plasma activity of IX with 0.9% of the normal activity.

The necessary dose is calculated by utilizing the following formula:

Necessary units = body weight (kg) x desired increase of factor IX (%) (UI/dl) x 1.1

Hemorrhage severity	Plasma level of the necessary FVIII (% of normal of UI/dl)	Administration frequency (hours) / treatment duration (days)
Early haemarthrosis, muscle or oral hemorrhages	20 – 40	Repeated every 24 hours, for at least 1 day, until the bleeding indicated by pain is resolved or healed.
haemarthrosis, muscle hemorrhages or haemarthrosis hematoma	30 – 60 60 – 100	The perfusion is repeated at intervals of 24 hours, for 3-4 hours or more, until the pain and acute invalidity is resolved.
Hemorrhages that endanger life		The perfusion is repeated at intervals between 8-24 ore, until the vital risk is eliminated.

Table 5. Plasma level of the necessary FIX depending on the severity of the hemorrhage episode

Administration: intravenously, slowly. The administration interval must be established by the hematology specialist/doctor.

IV.2.4.Treatment monitoring (Clinical, para-clinical parameters, periodicity)

- Careful monitoring, through clinical examination and laboratory tests, in order to detect the development of antibodies inhibitors.
- Clinical and para-clinical monitoring of the hemorrhage events and articular status
-

IV.2.5.Criteria for being excluded from the treatment

- Hypersensitivity to the active substance or to mouse or hamster excipients or proteins, with the recommendation of changing the treatment biologic product
- Development of the anti-FVIII/IX coagulation inhibitors

IV.3. Substitution treatment in the event of surgeries and major orthopedic surgeries

IV.3.1.Objectives: ensuring hemostasis during surgeries and major orthopedic surgeries

IV.3.2. Inclusion criteria (age, gender, clinical and para-clinical parameters, etc.)

1. Patients with congenital hemophilia without inhibitors, with require surgeries or major orthopedic surgeries
2. Age: any age group

IV.3.3. Treatment (dose, conditions for reducing the dose, treatment period)

Products:

Hemophilia A: Plasma coagulation factor VIII; Recombined coagulation factor VIII

Hemophilia B: Plasma coagulation factor IX; Recombined coagulation factor IX

Hemophilia A:

Dose:

The calculation of the necessary dose of factor VII relies on the empiric observation:

1 UI of factor VIII/kg increase the plasma activity of factor VIII with 2 UI/dl.

The necessary dose is calculated by utilizing the following formula:

Necessary units = body weight (kg) x desired increase of factor VIII (%) x 0.5.

Hemorrhage severity	Plasma level of the necessary FVIII (% of normal of UI/dl)	Administration frequency (hours) / treatment duration (days)
Minor Including dental extractions	30 – 60	Every 24 hours (from 12 to 24 hours in the case of patients with the age under 6), at least one day, until obtaining a scar.
Major	80 – 100 (pre and post operatory)	Repeated injections are administered every 8-24 hours (from 6 to 24 hours, for patients with the age under 6), until a scar is obtained, afterwards the treatment is continued for a period of at least 7 days, in order to maintain a level of the activity of Factor VIII of 30-60% (UI/dl).

Table 6 – Plasma level of necessary FVIII depending on the surgery

The dose and frequency of administration must be adapted in accordance with the individual clinical response.

In certain circumstances (for example a low titer of anti-factor VIII antibodies) it may be necessary to administer higher doses than the ones calculated with the support of the formula.

Hemophilia B:

Dose:

The calculation of the necessary dose of factor IX relies on the empiric observation, in accordance to which 1 UI factor IX per kg increases the plasma activity of factor IX cu 0.9% of the normal activity.

The necessary dose is calculated by utilizing the following formula:

Necessary unit = body weight (kg) x desired increase of factor IX (%) (UI/dl) x 1.1

Hemorrhage severity	**Plasma level of the necessary FVIII (% of normal of UI/dl)**	**Administration frequency (hours) / treatment duration (days)**
Minor Including dental extractions	30 – 60	Every 24 hours, at least one day, until healing.
Major	80 – 100 (pre and post operatory)	The perfusion is repeated every 8-24 hours, until healing, afterwards therapy shall be administered for at least 7 additional days in order to maintain the F IX activity of 30%-60%.

Table 7. Plasma level of necessary FIX depending on the hemorrhagic event's severity

Administration: intravenously, slowly. The administration interval must be established by the hematology specialist/doctor.

IV.3.4. Treatment monitoring (Clinical, para-clinical parameters, periodicity)

- In the event of major surgeries, it is mandatory to have precise monitoring of the substation therapy by analyzing the plasma activity of factor VIII/IX.
- Careful monitoring, through clinical examination and laboratory tests, in order to determine the development of inhibitor antibodies.

Response type	Response definition
Excellent	Intra and post operatory blood losses are similar (10%) to those of the patient without hemophilia • without additional doses of FVIII or FIX • the need of blood transfusions is similar to that of the patient without hemophilia
Good	Intra and post operatory blood loss is slightly elevated as compared to the patient without hemophilia (between 10-25%), yet the difference is evaluated by the surgeon/anesthetist as being clinically insignificant • without additional doses of FVIII or FIX • the need of blood transfusions is similar to that of the patient without hemophilia
Satisfactory	Intra and post operatory blood losses are slightly elevated with 25-50% as compared to the patient without hemophilia and additional treatment is needed: • additional doses of FVIII or FIX • the need of blood transfusions is two times higher than that of the patient without hemophilia
Bad/No response	Intra and post operatory blood loss is substantially elevated (>50%) as compared to the patient without hemophilia and it is not explained by the presence of a medical/surgical emergency, other than hemophilia • hypotension or unexpected transfer of ATI due to bleeding Or • substantial increase of the need of transfusions > twice as big as the expected need

Table 8. Defining efficient hemostatic efficiency of surgeries

IV.3.5. Criteria for being excluded from treatment

· Hypersensitivity to the active substance or to mouse or hamster excipients or proteins, with the recommendation of changing the treatment biologic product
· Development of anti FVIII or antiFIX inhibitors

V. PROTOCOL FOR TREATING HEMOPHILIA WITH INHIBITORS

V.1. Disorder definition

- Administering concentrates of coagulation factors can be acknowledged by the body as foreign protein stimulating the production of antibodies (immunoglobulin IgG).
- Presence of inhibitor anti-FVIII or anti-FIX antibodies is considered the most severe complication associated to hemophilia treatment
- The presence of inhibitors is suspected in every patient that does not react to the treatment with coagulation factors
- The incidence of developing inhibitors is 20-30% in patients suffering from hemophilia A severe form and 5% in patients suffering from hemophilia B
- The inhibitors are different depending on the level of answer
  – High titer (high responder) ≥5 BU; usually anamnestic response to FVIII
  – Low titer (low responder) <5 BU; rare anamnestic response to FVIII
- The transitory inhibitors are those that disappear spontaneously

- V.2. Treatment Principles

- The management of bleeding episodes in patients with inhibitors must be done in

collaboration with a center experienced in the management of inhibitors.
- Patients with low titer inhibitors can be further treated with the substitution factor in a higher dose in order to neutralize inhibitors and control the hemorrhagic event.
- It is recommended to initiate immune tolerance induction (ITI) in patients with high titer inhibitors (> 5BU).
- Patients with inhibitors who have not responded or who are not eligible for the immune tolerance induction therapy should be administered the prophylactic treatment with bypass agents.

V.3. Prophylaxis of hemorrhagic accidents

V.3.1. Objectives: preventing hemorrhagic accidents, improving the joint chronic disease, improving the quality of life for patients that suffer from hemophilia and inhibitory antibodies.

V.3.2. Inclusion Criteria (age, sex, clinical and paraclinical parameters, etc.)

1. Patients with congenital hemophilia and anti-FVIII or anti-FIX inhibitory antibodies.

V.3.3.Treatment (doses, dose reduction conditions, treatment period)

Products:
- Activated prothrombin complex concentrate (APCC) coagulation-anti-inhibitors complex

Doses:
- for patients with high antibody titer inhibitors and with frequent bleeding, and in who the ITI is low or not taken into account:

70-100 U/kg body weight, once every other day.

This dose can be increased up to 100 U/kg body weight daily, if the patient continues to bleed, and the dose can be decreased gradually.
- for patients with high inhibitor antibodies titer and ITI: concomitant administration of factor VIII concentrates.

50-100 U/kg body weight, twice a day until the titer of inhibitory antibodies of factor VIII has been reduced to <2 B.U.

Administration: intravenous slowly. This should not exceed the injection/perfusion rate of 2 U/kg body weight per minute.

V.3.4. Treatment Monitoring (clinical and para-clinical parameters, periodicity)
- Do not exceed a single dose of 100 U/kg body weight and the daily dose of 200 U/kg. Patients who receive a single dose of 100 U/kg body weight should be monitored carefully, particularly in regard to the CID risk or for symptoms of acute coronary ischemia.

V.3.5. Treatment exclusion criteria
- Hypersensitivity to the active substance or to any of the excipients
- Disseminated intravascular coagulation
- Acute coronary ischemia, acute thrombosis and/or embolism

V.4. Curative treatment of hemorrhagic accidents

V.4.1. Objectives: stop the hemorrhagic events in patients that suffer from hemophilia and anti-FVIII or anti-FIX inhibitory antibodies.

V.4.2. Inclusion criteria (age, sex, clinical and para-clinical parameters, etc.)

1. Patients with hemophilia and anti-FVIII or anti-FIX inhibitory antibodies.

V.4.3. Treatment (doses, dose reduction conditions, treatment period)

Products:
- Activated prothrombin complex concentrate (APCC) coagulation-anti-inhibitors complex
- Recombined activated coagulation factor VII (rFVIIa)

Doses:
- Activated prothrombin complex concentrate (APCC)

Bleeding in the joints, muscles or soft tissues

➢ For mild or moderate hemorrhages a dose of 50-75 U/kg body weight every 12 hours

➢ For severe hemorrhages of the muscles and soft tissues, such as retroperitoneal bleeding, the recommended doses are of 100 U/kg body weight every 12 hours.

Mucosal bleeding

➢ 50 U/kg every 6 hours, and close monitoring of the patient (the visible bleeding site, repeated measurement of the hematocrit). If hemorrhage does not stop, the dose may be increased to 100 U/kg body weight, taking care not to exceed the daily dose of 200 U/kg body weight.

Other severe hemorrhages

➢ In the case of severe hemorrhages, such as bleeding of the central nervous system, a dose of 100 U/kg body weight, every 12 hours. In individual cases, it may be administered every 6 hours, until a clear clinical improvement is obtained.

Administration: slow intravenous perfusion. This should not exceed the rate of injection/perfusion of 2U/kg body weight per minute.

- **Recombinant activated coagulation factor VII (rFVIIa)**

Mild or moderate bleeding episodes

➢ Two to three injections of 90 µg/kg administered every three hours; if further treatment is required, an additional dose of 90 µg / kg may be administered.

Or

➢ A single injection of 270 µg/kg.

Serious bleeding episodes

➢ The recommended starting dose is of 90 µg/kg, repeated every two hours until clinical improvement is observed. If continued therapy is necessary, the dose interval can be increased to 3 hours for 1-2 days. Thereafter, the dose interval can be increased successively to 4, 6, 8 or 12 hours, for as long as the treatment is deemed necessary.

Administration: intravenous bolus injection for a 2-5 minute-period.

V.4.4.Treatment monitoring (clinical and para-clinical parameters, periodicity)

- The bleeding severity and the clinical response to treatment should guide the necessary doses
- Patients should be monitored with care, especially for CID risk or thrombotic accidents

V.4.5. Treatment exclusion criteria

- Hypersensitivity to the active substance or to any of the excipients
- Disseminated intravascular coagulation
- Acute coronary ischemia, acute thrombosis and/or embolism

V.5. Substitution treatment in the case of major surgeries and orthopedic interventions

V.5.1. Objectives: to ensure homeostasis during major surgeries and orthopedic interventions

V.5.2. Inclusion criteria (age, sex, clinical and para-clinical parameters, etc.)

1. Patients with hemophilia and anti-FVIII or anti-FIX inhibitory antibodies that require major surgeries or orthopedic interventions.

V.5.3 Treatment (doses, dose reduction conditions, treatment period)

Products:

- Activated prothrombin complex concentrate (APCC) coagulation anti-inhibitors complex
- Recombinant activated coagulation factor VII (rFVIIa)

Doses:

➢ **Activated prothrombin complex concentrate (APCC)**

- - 50 to 100 U/kg body weight can be administered every 6 hours, taking care not to exceed the maximum daily dose.

Administration: slow perfusion, intravenously.

This should not exceed the rate of injection/perfusion of 2U/kg body weight per minute.

- **Recombinant activated coagulation factor VII (rFVIIa)**

- Immediately after the intervention an initial dose of 90 μg/kg should be administered. The dose must be repeated every 2 hours and afterwards every 2-3 hours in the first 24-48 hours, depending on the type of intervention and the clinical condition of the patient.
- During major surgeries, the administration should be continued every 2-4 hours for 6-7 days. Afterwards, the interval between the doses can be increased to 6-8 hours, for another 2 weeks of treatment. The patients undergoing major surgeries can be treated for 2-3 weeks until healing.

Administration: intravenous bolus injection for a 2-5 minute-period.

V.5.4.Treatment monitoring (clinical and para-clinical parameters, periodicity)

- The bleeding severity and the clinical response to treatment should guide the necessary doses
- Patients should be monitored with care, especially for the CID risk or thrombotic accidents

V.5.5. Treatment exclusion criteria

- Hypersensitivity to the active substance or to any of the excipients
- Disseminated intravascular coagulation
- Acute coronary ischemia, acute thrombosis and/or embolism

VI. PERIODIC EVALUATION PROTOCOL FOR PATIENTS SUFFERING FROM HEMOPHILIA

Joint Status

- clinical joint score
- radiological, ultrasound, MRI joint score
- orthopedic consultation

Neuro-psychic status

- neuro-psychiatric examination
- further investigations based on the patient's condition

Dental, ophthalmological, ENT status

Biological status

- coagulation, residual activity of the deficient factor
- titer inhibitors
- hepatic explorations (proteinemia, serum protein electrophoresis, transaminases, lactic dehydrogenase, alkaline phosphatase, bilirubin)
- serological exploration (HBs Ag, anti-HBs Ac, anti-HCV Ac, anti-HIV 1 and 2 Ac, anti-HTLV I and II Ac, anti-CMV IgG Ac and IgM)
- Others: blood count, urinalysis

- Other measures
- genetic advice
- psychological support and socio-professional guidance

Complications Tracking Protocol (annually)

1. Treatment Complications

1.1. Determining inhibitors (anti-factor VIII/IX antibodies infusion)

- the Bethesda metric coagulation method or the Nijmegen modified test
- in children, inhibitor dosing must be administered after the first 5 days of exposure, at 10 -15 days, after 25 days of exposure and 50 days of exposure and afterwards, twice per year; subsequently the inhibitors should be determined at least once per year, before surgery or in case of suboptimal response; inhibitors control is also required after massive substitutions (over 5 days), in those with favorable mutations for inhibitors or post-surgically.
- basal titer and anamnesis titer determination after the administration of the VIII/IX factor (<5 BU inhibitors in low titre ;> 5 BU inhibitors in high titer)

1.2. Determining infectious complications

- hepatic explorations (proteinemia, serum protein electrophoresis, transaminases, lactic dehydrogenase, alkaline phosphatase, imunocantitation)
- serological exploration (HBs Ag, anti-HBs Ac, anti-HCV Ac, anti-HIV 1 and 2 Ac, anti-HTLV I and II Ac, anti-CMV IgG Ac and IgM) twice a

year
- viral level in case of infection (HBV, HCV, HIV)

2. Disease Complications

2.1. Chronic Arthropathy
- clinical joint score - Gilbert and HJHS score (hemophilia joint health score)
- Petterson radiological joint score
- ± ultrasound score , MRI score, ± osteodensitometry score
- orthopedic consultation/physiotherapist
-

2.2. Neuro-sensorial and psychical sequelae
- neuro-psychiatric examination
- further investigations based on the patient's condition (CT, MR, EMG, nerve conduction velocity)

2.3. Dental problems
- dental examination
-

2.4. Others
- ophthalmological examination , ENT, blood count

2.5. Life quality assessment
- HaemoQoL
- Kids'Life Assessment tool
- Hemophilia activities list (HAL)
- Functional independence score in hemophilia (FISH)
- Pediatric hemophilia Activities List (Ped HAL)

VON WILLEBRAND DISEASE

I. Definition of the disease:

The von Willebrand disease is the most common congenital coagulopathy transmitted dominantly autosomally, that is affecting both sexes. The disease is characterized by qualitative or quantitative deficiency of a glycoprotein (called von Willebrand factor or FvW) in the blood, which is needed for thrombocytes adhesion to the vascular wall. Because this protein has also the role of carrier protein and the stabilizing role for the VIII coagulation factor, the VIII factor activity is sometimes decreased in proportion to the reduction

Type	Transmission	Prervalence	Bleeding phenotype
Type 1	Dominant autosomal	Up to 1%	Mild - moderate bleeding
Type 2A	Dominant (or recessive) autosomal	Not very frequent	Varied phenotype - frequent moderate bleeding
Type 2B	Dominant autosomal	Not very frequent	Varied phenotype - frequent moderate bleeding
Type 2M	Dominant (or recessive) autosomal	Not very frequent	Varied phenotype - frequent moderate bleeding
Type 2N	Recessive autosomal	Not very frequent	Varied phenotype - frequent moderate bleeding
Type 3 (severe)	Recessive autosomal	Rare (1: 250,000 to 1: 1,000,000)	Severe bleeding

Table 9. The prevalence and bleeding phenotype in patients suffering froms von Willebrand disease

Test	Type 1	Type 2				Tip 3
		A	B	M	N	
Bleeding duration	prolonged	prolonged	prolonged	prolonged	(N)	prolonged
vWF:Ag	↓	↓	(↓)	↓	↓	ND
vWF:RCoF	↓	↓	↓	↓↓	↓	ND
vWF:CBA	↓	↓↓	↓	↓↓	↓	ND
RIPA	(↓)	↓	()	↓	↓	↓↓
FVIII	(↓)	↓	(↓)	↓	↓↓	↓↓
vWF multimers	N	↓(high GM	↓(high GM) (low GM)	N	N	absent

Table 10. Specific biological features characteristic for the various subtypes of the von Willebrand disease

Legend
N-normal; ↓- reduced; ↓↓ - very reduced, (↓) - normal or reduced; ( ) - normal or increased; ND- not detectable

level of FvW.

II. Treatment Principles for the von Willebrand disease

- The disease treatment depends on the disease subtype and on the severity of the hemorrhagic manifestations.
- The long-term prophylaxis is rarely necessary, but it should be considered in cases of recurrent haem arthrosis or excessive muscle skeletal bleeding that cannot be adequately controlled with other treatments.
- The treatment of the von Willebrand disease consists in the administration of desmopressin (DDAVP) and FVIII concentrates enriched in VWF.

In deciding the therapeutic conduct for patients with von Willebrand disease the following factors should be considered:

- The type of bleeding manifestation
- The serum levels of FvW and FVIII and the von Willebrand disease subtype
- The patient's previous history of bleeding episodes and the response to treatment
- The previous response of FVIII and FVW to treatment with desmopressin (if performed)
- The presence of inhibitors
- The potential risks of treatment

Type of the von Willebrand disease	Elective treatment	Alternative or additional treatment
Type 1	Desmopressin	Antifibrinolytics Estrogen
Type: 2A 2B 2M 2N	 FVIII concentrate with vWF FVIII concentrate with vWF FVIII concentrate with vWF Desmopressin	
Type 3	FVIII concentrate with vWF	Desmopressin platelet concentrate

Table 11 - Treatment of various types and subtypes of the von Willebrand disease

Bleeding type	Dose (IU/Kg)	Number of administrations	Administration intervals	Objective/Duration of administration
Dental extraction	20	1	-	FVIII> 30 UI/dl for a period of at least 6 hours
Posttraumatic bleeding	20	1	-	
Spontaneous bleeding	45 ±12	Until bleeding resolution	24 hours	5±4 days
Major Surgical Interventions	50	1 administration/ day or 1 administration every 2 days	24-48 hours	FVIII>50 UI/dl until complete healing
Minor Surgical Interventions	30	1 administration/ day or 1 administration every 2 days	24-48 hours	FVIII> 30 UI/dl until complete healing

Table 12. dosage of FVIII concentrate enriched with vWF in patients with von Willebrand disease, unresponsive to desmopressin, depending on the type of bleeding

Recommendations regarding the minor bleeding treatment and the prophylaxis in the case of minor surgery interventions

- Minor bleeding should be treated with intravenous or nasal DDAPV
- If the response to DDAPV is inadequate, concentrated vWF should be administered with initial vWF units dosage: RCo and FVIII secondary units
- For minor surgery, prophylaxis should provide a level of vWF activity : RCo and FVIII> 30 IU/dl and preferably> 50 IU/dl for 1-5 days

Recommendations regarding the treatment of major hemorrhages and the prophylaxis in the case of major surgery

- In the case of severe hemorrhages (e.g. intracranial, retroperitoneal) or in the case of prophylaxis for major surgery interventions, the initial target of the vWF activity level: RCo and FVIII must be>100 IU/dl and the level of> 50 IU/dl should be maintained for at least 7 - 10 days
- Patients receiving concentrates with vWF

should be carefully evaluated in what concerns the thrombotic risk and measures preventing the occurrence of thrombosis should be initiated
- In order to reduce the perioperative risk of developing thrombosis, the level of vWF: RCo must not exceed 200 IU/dl and the FVIII activity must not exceed 250 IU/dl.

The therapeutic approach in the case of women with the von Willebrand disease

- For teenagers and adult women who do not want children, hormonal contraceptives are the first line of treatment for menorrhagia
- During childbirth, the serum levels of vWF: RCo and FVIII must be at least 50 IU/dl before birth giving and this level should be maintained for at least 3-5 days, with the subsequent supervision to prevent post-partum bleeding.

Sarcoidosis in a patient with 5q- syndrome

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Abstract

We present the case of a 59-years-old male who was first admitted to our clinic in July 2011 with pallor, weakness, fatigue, dizziness and pain in the left upper quadrant. A clinical and biological diagnosis of Myelodysplastic syndrome- RAEB1 with del(5q) was made. Treatment was initiated with low doses of Cytarabine in association with Epoetinum beta, but with no marrow response. He obtained complete hematological response after one course of "3+7" regimen. There was no need for further therapy. Within one year, the patient returned with cough, dyspneea, pallor, skin lesions, eye dryness and redness. A CT exam of the chest revealed mediastinal lymph nodes. After biopsy, the diagnosis of sarcoidosis was established. He is now under corticotherapy, with good response and just begun treatment with Lenalidomide.

This case illustrates the predisposition for sarcoidosis created by the deletion of key genes on the long arm of chromosome 5. Research on this association is recommended.

Introduction

Sarcoidosis (Besnier-Boeck-Schaumann disease) is a multisystem inflammatory illness that involves abnormal collections (noncaseating granulomas) in multiple organs, most often in the lungs and intrathoracic lymph nodes, but any organ can be affected.

T cells play a central role in the development of sarcoidosis, as they propagate an excessive cellular immune reaction. This picture may explain its association with several blood diseases like lymphoma, leukemia, multiple myeloma and myelodysplasia. 5q- syndrome has been showed to predispose to the development of sarcoidosis due to an imbalance in the cytokine pool and repeated interactions between macrophages, T-cells and B-cells. These phenomenons may be caused by the deletion of genes coding for T helper cell 2 cytokines, situated on the long arm of chromosome 5.

5q- syndrome is a distinct type of myelodysplasia with a medullary blast count less than 5% and a isolated deletion of the long arm of chromosome 5, including bands q31-q33. This region also carries the gene coding for T helper cell 2 cytokines (IL3, IL4, IL5, GM-CSF). A special attention should be given to those patients not correctly defined as 5q- syndrome: they have either excess blasts or additional karyotypic abnormalities. They do not have a good prognosis, unlike the one with isolated del(5q).

In 2005 Lenalidomide was approved by the FDA for the treatment of patients with transfusion dependent anemia due to low/intermediate 1 risk MDS associated with a deletion 5q with or without additional cytogenetic abnormalities.

Case presentation

We present the case of a 59-years-old male, J.E., smoker, with a history of large hiatal hernia, colonic diverticulosis, left renal lithiasis and internal hemorrhoids, who was first admitted to Emergency Military Hospital complaining of pallor, pain in the left upper quadrant, weakness, fatigue and dizziness. The local blood tests revealed the presence of mild anemia (Hb 10.4 mg/dl) and leucopenia (WBC 2900/mm3), a mild rise in serum creatinine and uric acid; an abdominal ultrasound showed hepato-splenomegaly. A bone marrow aspirate was performed and the result suggested the diagnosis of myelodysplasia.

He was referred to our clinic in Jul 2011 for further investigations, with the same symptoms. The only abnormalities at the physical examination were pallor and mild splenomegaly.

The laboratory studies showed no significant changes compared to the patient's previous results, except for high levels of feritin (158.4 ng/dl) and serum EPO (59.4 mU/ml).

A bone marrow aspirat (Fig. 1 and 2) and biopsy confirmed the diagnosis of myelodysplastic syndrome – RAEB1 (WHO), blasts 7-8%. The biopsy also found the presence of multiple, small, paratrabecular granulomatous lesions, without necrosis, one of which containing a giant multinuclear cell. The Ziehl-Neelson test was negative.

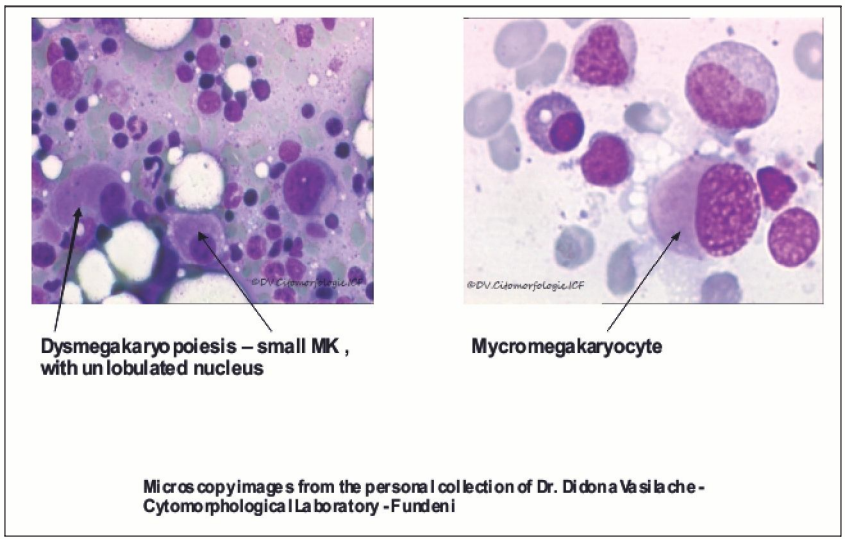


Fig. 1

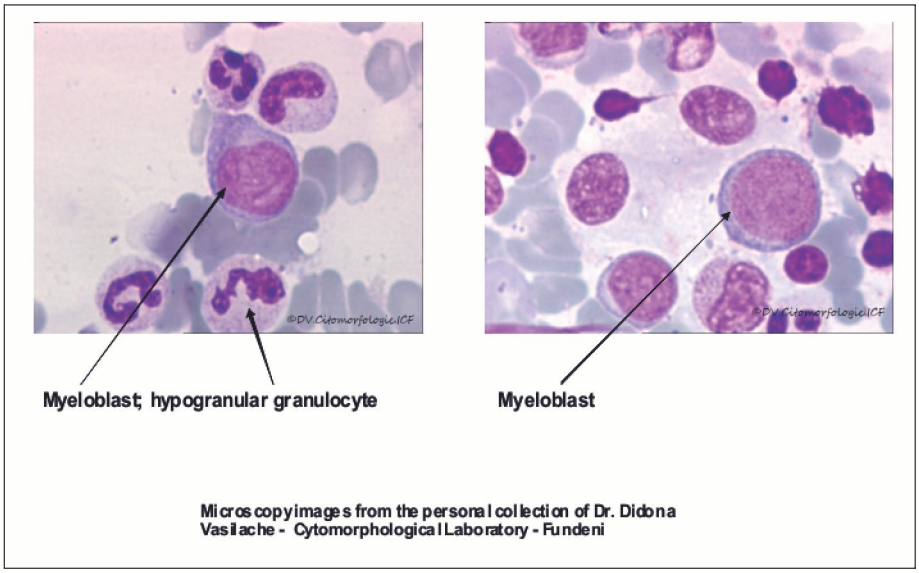


Fig. 2

Due to the temporary unavailability of the karyotype studies at that moment, we tested the patient for the deletion of 5q by FISH, at the Medical Genetic Laboratory of "Victor Babes" Institute. The test was positive for the presence of q31.1 deletion, in 24 of the 56 analised metaphases. (Fig. 3)

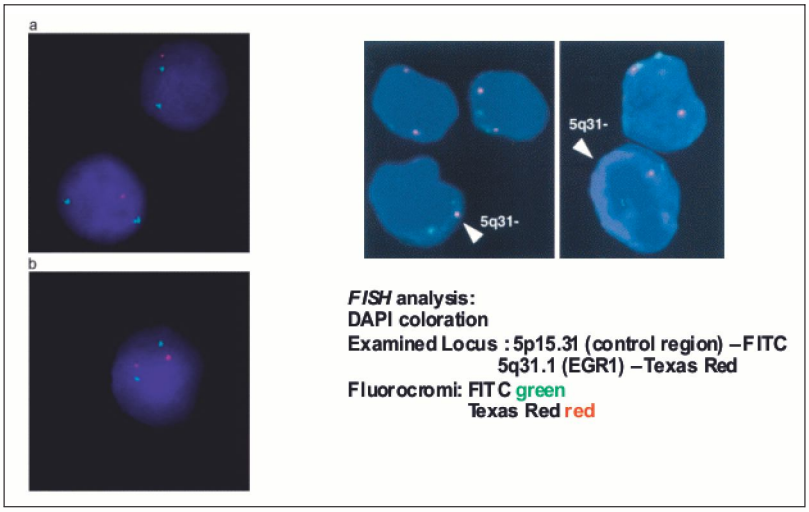


Fig. 3

One month later we performed the cytogenetic analysis and we discovered other abnormalities besides del(5q) (Fig. 4):

46,XY(4)
46,XY,del(5)(q)[3]
92,XXY,…[2]
174,XXXYY,…[2]
146,XXYY,…[2]

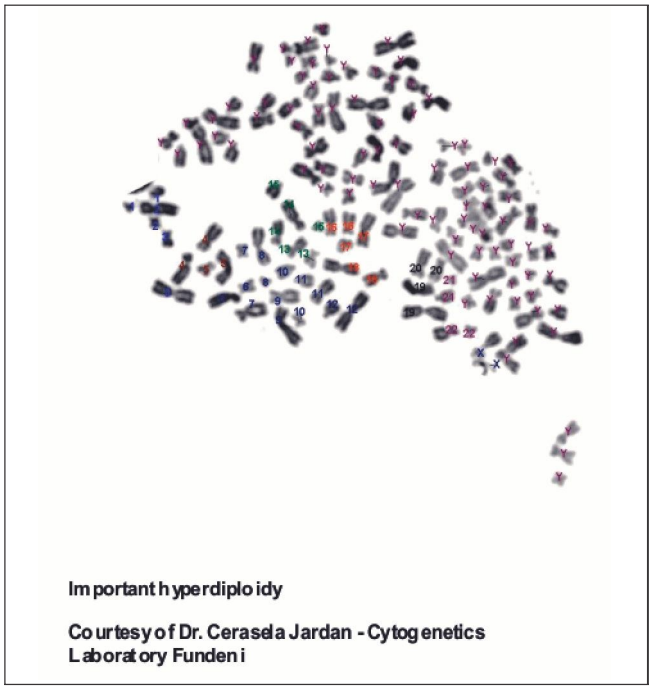
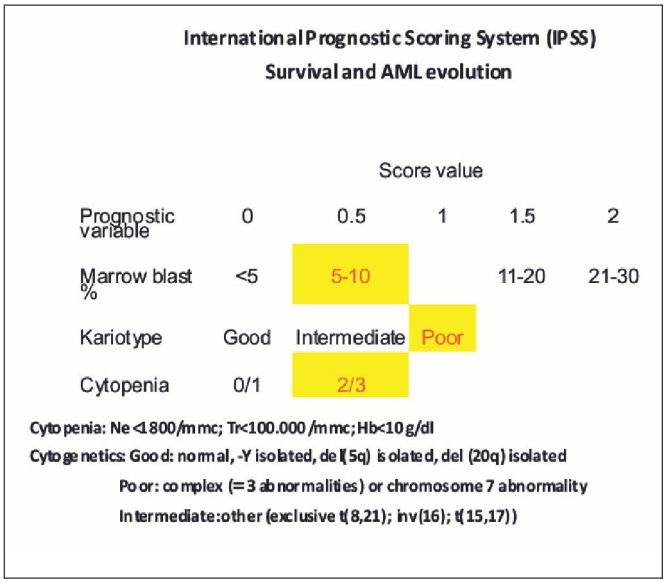
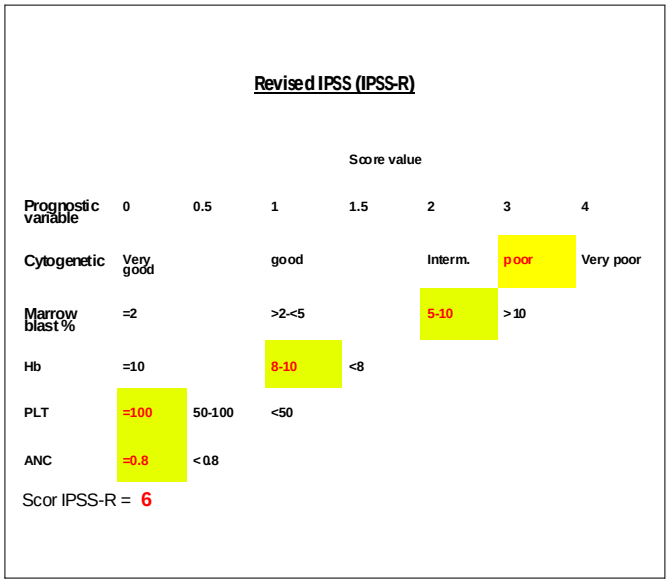


Fig. 4

Due to this findings, we concluded that the patient had MDS-RAEB1, IPSS INT-2, IPSS-R high, with unfavorable prognosis in the absence of therapy.





According to the NCCN Guidelines for the Myelodysplastic Syndrome for not high-intensity therapy candidate, the first line treatment was Azacitidine (preferred) or Decitabine (Fig. 5). But none of them was available in our hospital and the patient did not have the financial possibility to obtain them.

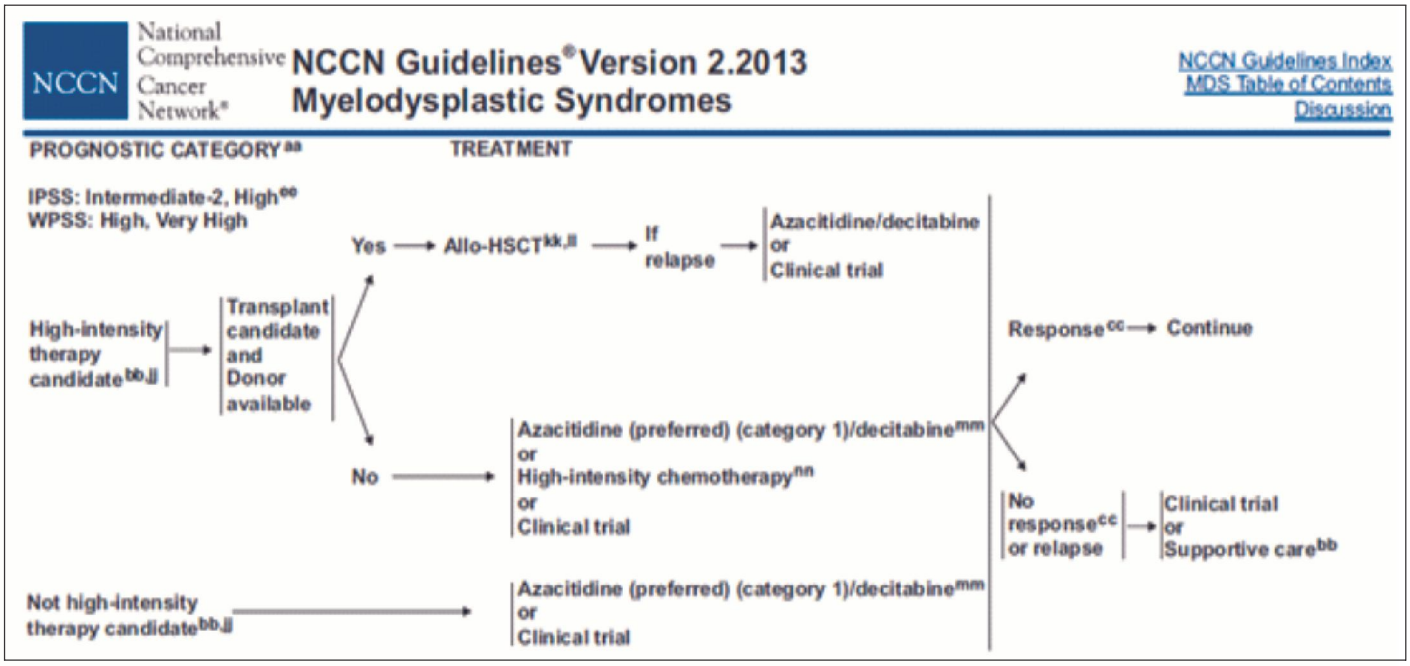


Fig. 5

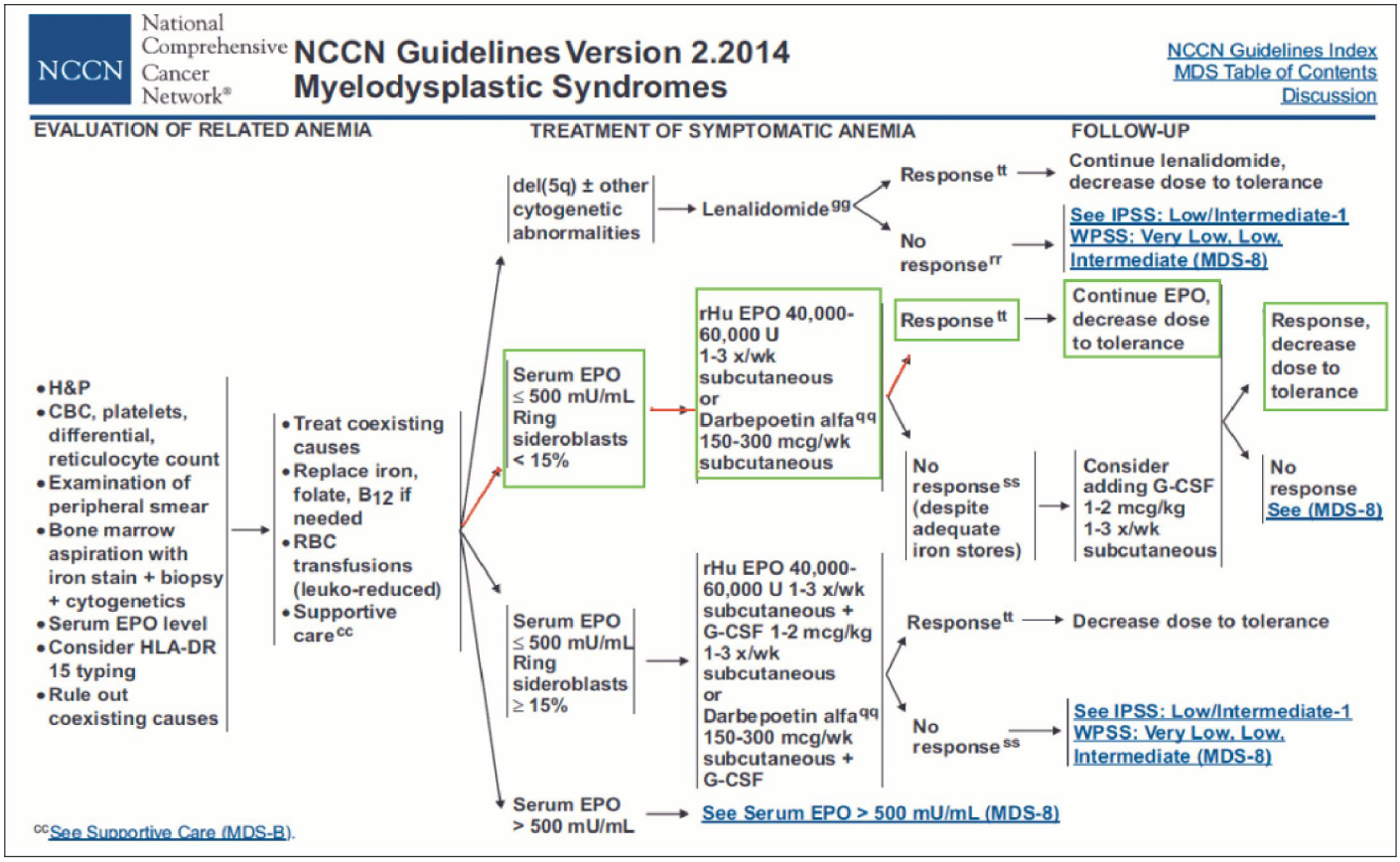


Fig. 6

In this circumstances, we begun to treat him with subcutaneous small doses of Cytarabine (30mg/day, 7 days) along with weekly administration of Epoetinum beta 30.000 UI (Fig. 6); the patient responded well, with a rapid rise in Hb levels (from 6.3 to 12.6 mg/dl in 4 months), but with the persistence of leucopenia and marrow blasts >5%. So, in March 2012 the patient received a more intensive chemotherapy (Cytarabine 100

mg/m2/day 7 days + Epirubicine 45 mg/m2/day 3 days) well tolerated and with no major complications. The marrow response was good, with a residual blast population of 2-3%. The patient remained under a close supervision, with regular bone marrow evaluations and with no further therapy. He no longer needed the administration of rHu EPO, the Hb levels maintaining within normal limits and the general condition of the subject being excellent.

Everything went well until early 2013 when the Hb begun to fall, the patient feeling progressively weak and dizzy. No further cytopenias developed beside anemia (Hb 9.6-8.2 mg/dl). The marrow showed no increase in blast percentage. The cytogenetic examination (Oct 2013) indicated only the presence of del(5q) abnormality (so 5q- syndrome at this moment?):

46,XY[11]
46,XY,del(5)(q)[5].

The WPSS at this stage was 0 (MDS 5q-, good karyotype and no transfusion requirement) (Fig. 7). The NCCN Guidelines recommended treatment with Lenalidomide (Fig.8), also not available in our country. We choosed to keep the patient under close surveillance, weekly Epoetinum beta and monthly evaluation of the hematologic parameters.

The WHO classification -based Prognostic Scoring System (WPSS) for myelodysplastic syndromes (MDS)

Prognostic Variable	Score Value				
	0	1	2	3	
WHO category	RA, RARS, del(5q)	RCMD, RCMD-RS	RAEB-1	RAEB-2	
Karyotype*	Good	Intermediate	Poor	--	
Transfusion requirement†	No	Regular	--	--	
	WPSS Risk Category‡				
	Very Low	Low	Intermediate	High	Very High
Median overall survival, mos	141	66	48	26	9
AML progression, cumulative probability					
2 yrs	0.03	0.06	0.21	0.38	0.80
5 yrs	0.03	0.14	0.33	0.54	0.84

*Good = normal, -Y, del(5q), del(20q); intermediate = other karyotypic abnormalities; poor = complex (≥ 3 abnormalities) or chromosome 7 abnormalities.
†Red blood cell transfusion dependence was defined as having at least 1 red blood cell transfusion every 8 wks over a period of 4 mos.
‡Based on validation cohort.

Fig. 7

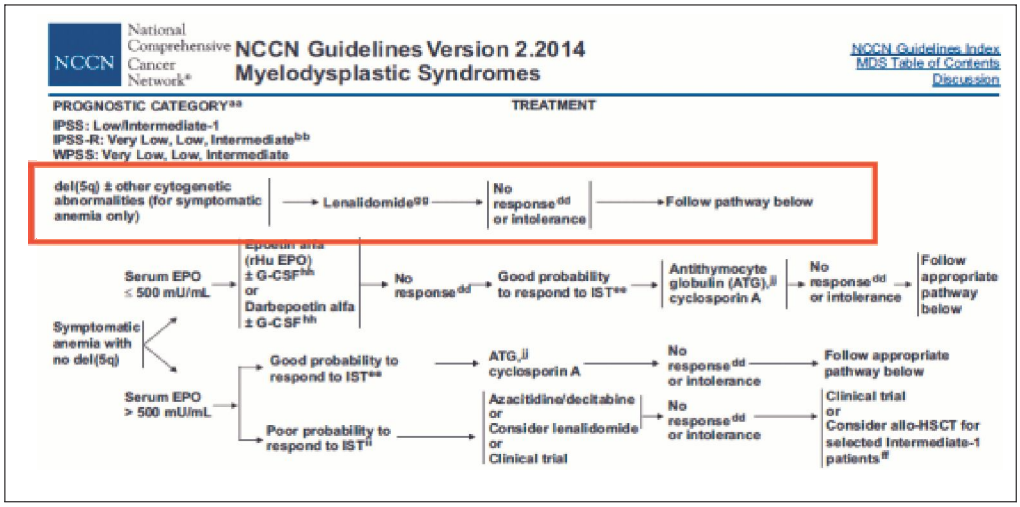


Fig. 8

Since 05.2013 he also begun to experience hacking cough, shortness of breath, eye dryness and redness, lack of energy and several macula-papular eruption on the skin. A dermatologic examination could not determine the cause of the skin lesions; topical ointments were unsuccesfull. The ophthalmologist suspected a more complex explanation for all the symptoms: a systemic inflammatory disease with multiple organ involvement – sarcoidosis.

The chest X-ray detected mediastinal lymph nodes so he was referred to the Pneumophtisiology Institute "Marius Nasta" for further investigations. The chest CT confirmed the presence of meddiastinal adenopaties. The patient underwent anterior mediastinoscopy Carlens type, with lymph node biopsy. The diagnostic of sarcoidosis (with pulmonary, skin and eye involvement) was established.

He begun corticotherapy, with favorable evolution.

But in Jan 2014 he started to experience worsen of the anemia (Hb 6.3 mg/dl) and the related symptoms (asthenia, fatigue, dyspnea). He received transfusions regulary, 1-2 units/month, concomitent with weekly Epoetinum.

In these conditions, we preceeded with the search for Lenalidomide, a very efficient drug for the treatment of 5q- syndrome. The patient managed to obtain it from abroad and on 1st Jul he begun to take 10 mg/day, 21 days/month.

We are looking forward to evaluating the patient's response.

Conclusions

1. 5q-syndrome must be differentiated from other forms of MDS with del (5q) with higher blast percentage in bone marrow and / or other associated cytogenetic abnormalities. They have a worse prognosis and benefit from more aggressive therapies;
2. 5q31 FISH analysis is useful, but not sufficient for diagnosis; it should be made in suspected cases of 5q-syndrome if cytogenetic study is inconclusive or with no metaphases. Cytogenetic testing will be required for confirmation
3. Urgent steps must be taken to introduce on the list of drugs reimbursed by the National Insurance Agency of new molecules that have proven efficacy in treating certain myelodysplastic syndromes (azacitidine, decitabine, lenalidomide) so that they would be available to our patients.
4. 5q-anomaly can create a favorable ground for the development of immune imbalances which in turn can trigger complex disorders. Is also the case of sarcoidosis, a multisystem inflammatory disease that can have major repercussions on the health of the patient
5. Treatment with Lenalidomide may induce cytogenetic remission and consequently an improvement of the disturbance in the cytokine pool and the inflammatory processes that appear in sarcoidosis.

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Human Babesiosis in Romania cause of anemia less known

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Abstract

We present two cases of autochthonous human babesiosis in Romania. The first, documented in 1993, was a case of a splenectomized, 30 year-old woman patient, resident in a country side area of Northern Romania. The infection source in this case was Ixodes, an ectoparasite berried by a buffalo from her personal farm. As a consequence of some aggravating circumstances (included in the highest risk group for Babesia divergens infection as a prior splenectomized, the absence of early diagnosis and treatment), the most severe symptoms and complications are registered leading to death. The second case, detected in 2009, a 36 year-old woman, non-splenectomized, apparently immunocompetent is a German resident, but of Romanian origin. The infection was accidental and the source was Ixodes from a Romanian field where this is present in the environment. The parasites were inoculated into the host by tick bite. The patient presented clinical symptoms of infection after two weeks, although the tick was immediately removed and its hypostome extracted. In both cases, the patients developed: fever, fatigue, headache, arthralgia, hemolytic anemia, thrombocytopenia, splenomegaly, hepatomegaly, but there were differences in the severity of symptoms, and for the splenectomized patient, babesiosis was fatal. The diagnosis was established by microscopic examination of Giemsa-stained thin and thick blood smears, when we noticed typical intraerythrocytic parasites, especially ring and oval forms, and even paired piriform bodies, without parasitic pigment. Blood exam revealed poikilocytosis (crenate erythrocyte or burr cells, codocytes or target cells, spherocytes, ovalocytes, teardrop or pear-shaped cells), anisocytosis, and hypochromic erythrocytes.

Keyword: human babesiosis, Babesia species, Ixodes vectors, intraerythrocytic parasites, tick-borne disease

Introduction

Babesiosis is a parasitic disease caused by protozoan parasites of the genus *Babesia* (Apicomplexa, Babesiidae), which infect red blood cells. First *Babesia* species was described in cattle in 1888 by the Romanian biologist Victor Babeş (1) (after whom the genus and family were named). Due to the pear shaped forms, the parasites are commonly called *Piroplama*, and family, Piroplasmidae. The firsts human babesiosis was identified in 1957, by Skrabalo & Deanovic (2) in Europe, in the former Yugoslavia (near Zagreb), in 1966 in California (3), and in 1969 in Nantucket Island off the coast of Massachusetts.

Worldwide have been reported more than 100 species of *Babesia*, which infect many mammalian and avian species, but only a few infect humans. Most cases of human babesiosis have been reported from the United States (over 500) and Europe (approximately 40 reported mostly in Ireland, the United Kingdom, and France). Sporadic case reports of babesiosis in Japan, Korea, China, India, Mexico, South Africa, and Egypt have also been documented (4).

In many European countries, most human infections with babesiae are believed to be caused by *Babesia divergens* (5), that occur especially in splenectomized individuals (6), with high mortality, and some cases are due to *Babesia venatorum* Herwaldt et al., 2003 (EU-1 strain), identified in asplenic patients from Italy and Austria, reported also from Germany, Netherlands, Switzerland, Slovenia (7), Sweden (8), Poland (9). Some autochthonous cases of human infection with *Babesia microti* (10; 11), or this species presence in Ixodidae, in Slovenia, Switzerland, Germany, Hungary, Poland, Lithuania, Russia, Czech Republic (12) have recently been confirmed.

In USA, babesiosis is due mainly to *Babesia microti*, but other strains have been reported from Washington (WA-1), California (CA-5) and Missouri (MO-1). Based on the characterization of isolates WA-1 and CA-5, obtained from human patients, a new species, *Babesia duncani* Conrad et al., 2006 (13) was described. WA-1 strain *Babesia* is closely related to canine species *Babesia gibsoni*

and MO-1 strain showed affinity to the cattle parasite *B. divergens* (14).

The cases from Africa, Mexico, Japan (15,16,17), Taiwan and India (18) were attributed to *B. microti* and to unidentified *Babesia* species. In Taiwan, the *Babesia* isolate (TW1) is morphologically indistinguishable from and serologically related to the rodent parasite of *B. microti* (19). In Korea was detected a new type of Babesia (KO1), similar to ovine *Babesia* (20).

Babesiosis is a tick-borne disease, the vectors for *Babesia* being the haematophagous Ixodidae species. In Europe, *Ixodes ricinus*, the most common tick species, with the highest prevalence, registered a latitudinal and altitudinal extension, fostered by the global climate warming (21). In Eastern Europe also *I. persulcatus* serve as vectors for *B. divergens*. In the northeastern part of the United States the vectors for *B. microti is Ixodes scapularis (= Ixodes dammini)* and on the West Coast, *I. pacificus*.

Material and methods

Two women patients were investigated, 30 and 36 years old, one in 1993 and the other in 2009, both infected by ticks bite. In the 1993 case, the authors' contribution consisted only in establishing the diagnosis (by microscopic examination of Giemsa-stained thin and thick patient blood smears, and discovering typical intraerythrocytic *Babesia* parasites), studying a lot of specialized literature and suggest to administer chlorochin, clindamycin and quinine treatment. In the 2009 case, hematologic, bacteriologic, biochemical, coproparasitologic exams and ELISA tests for *Toxoplasma gondii, Toxocara canis, Cysticercus, Entamoeba histolytica, Chlamydia pneumoniae, Chlamydia trachomatis, Mycoplasma pneumoniae, Lysteria monocytogenes, Borellia burgdorferi* were made in the Clinical Laboratory, Department of Parasitology and Immunology, of the Fundeni Clinic Institute, in Bucharest. Patient survey and the recommended treatment were made in Romania (at the Center of Hematology and Bone Marrow, of the Fundeni Clinic Institute, in Bucharest) and in Germany.

Results and discussions

By microscopic examination of Giemsa-stained thin and thick blood smears, two human babesiosis cases were detected. In both, typical intraerythrocytic parasites ring, round, oval forms, single pear shaped or binary trophozoites (pyriform bodies), and even very rare tetrad form (Maltese cross) and without parasitic pigment from hemoglobin were discovered (Figs. 1-2).

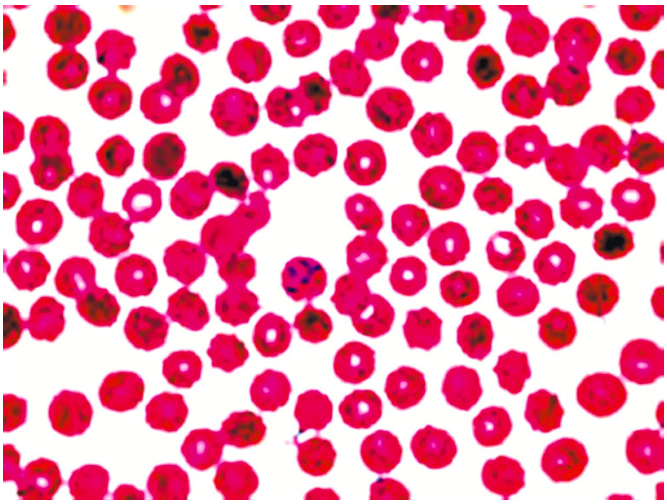

Fig. 1

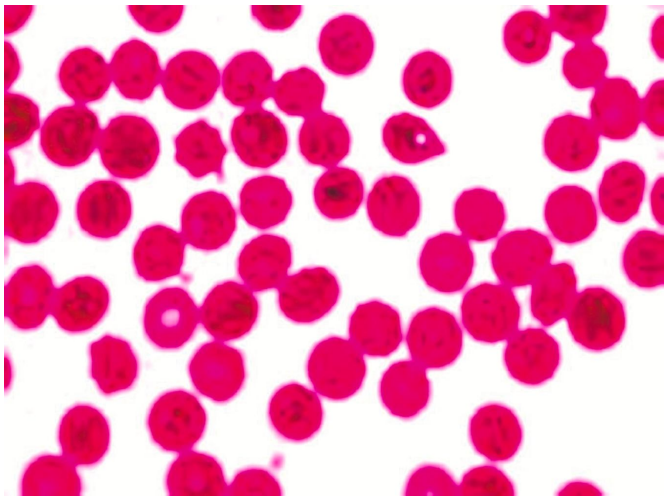

Fig. 2

Figs. 1, 2: Giemsa-stained thin blood smear with Babesia parasites and erythrocytes modifications

The absence of the hemozoin pigment within parasites cytoplasm, serve to not confuse the *Babesia* ring form, with *Plasmodium falciparum* ring form. In most of cases, the parasites positions in human erythrocytes are subcentral.

It was not possible to determine the species of *Babesia*, and to verify the species of *Ixodes*.

Blood exam revealed the same erythrocytes modifications: abnormal variation in shape (poikilocytosis, for example crenate erythrocyte or burr cells, codocytes or target cells, spherocytes, ovalocytes, teardrop or pear-shaped cells), inequality in size (anisocytosis), and erythrocytes paler than normal, with less concentration of hemoglobin (hypochromic erythrocytes) (Figs 3-5).

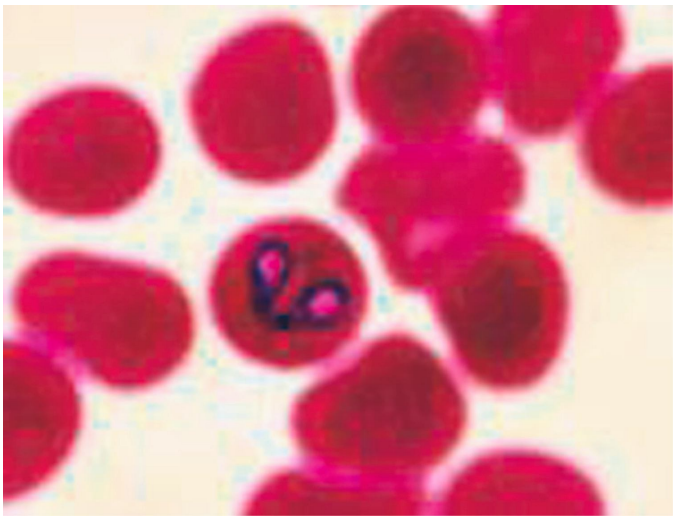

Fig. 3

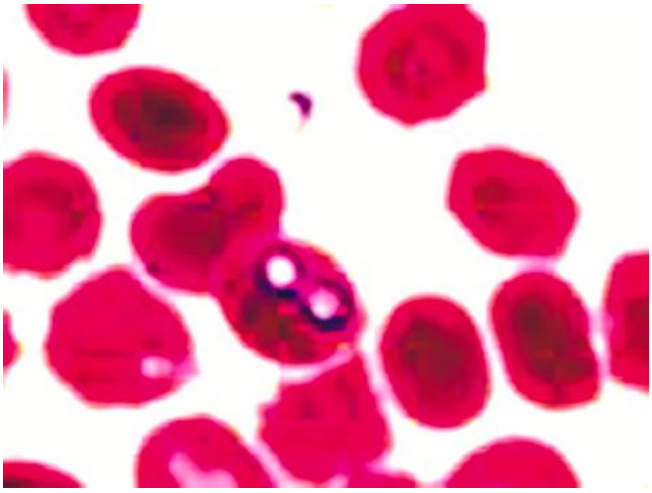

Fig. 4

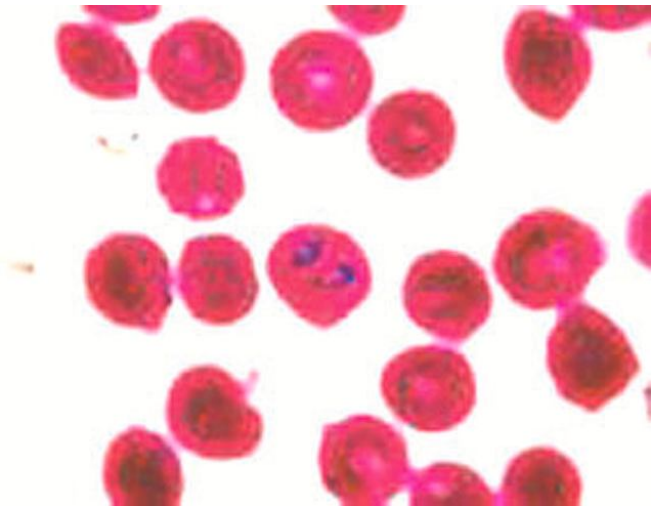

Fig. 5

Figs. 3, 4: Giemsa-stained thin blood smear with binary Babesia trophozoites and erythrocytes modification
Fig. 5: Giemsa-stained thin blood smear with paired pyriform Babesia and erythrocytes modifications

The first case, documented in 1993, was a splenectomized prior to infection, 30 year-old woman patient, resident in Negreşti Oaş, a locality in Maramureş County (side area of Northern Romania), hospitalized successively in Szeged (Hungary), Cluj and Bucharest (Romania). The infection source in this case was *Ixodes* ecto-parasite berried by a buffalo from her personal farm. In this case, *Babesia* species has bovine origin. The authors analyzed Giemsa-stained thin and thick blood smears, and discovered typical intraerythrocytic parasites, especially ring forms but even characteristic paired pyriform bodies, without parasitic pigment As a result of the parasite presence, blood exam also revealed red blood modifications: poikilocytosis, anisocytosis, and hypochromic erythrocytes. In the Bucharest hospital, the attempt to administer chlorochin, clindamycin and quinine, and whole-blood exchange transfusion remained ineffectual.

As a consequence of some aggravating circumstances (the absence of an early diagnosis and treatment and mainly included in the greatest risk group of acquiring clinical infection with *Babesia divergens* as a splenectomized patient), the most severe symptoms and complications registered ended in death.

The correlation between the disease severity

and spleen status was proving by differences in susceptibility of infection from normosplenics members of this woman family that develop only asymptomatic or subclinical forms of babesiosis.

The second case, detected in 2009, was of a 36 year-old woman, normosplenic, previously healthy and apparently immunocompetent, residing in Germany, but of Romanian origin.

In this case the infection was accidental on May 14th 2009 and source was an infected *Ixodes* from a Romanian field, in a touristic zone (Cheia, a mountain resort, sourrounded by Ciucaş and Zăganu mountains, in Prahova county), where the tick was present in the environment.

The *Babesia* parasites were introduced in the host's left foot by the tick bite. The tick was immediately removed by pincers but its hypostome (covered with recurved teeth) remained anchored into the skin and was extracted later at Floreasca Emergency Clinic Hospital in Bucharest.

After the two weeks of the incubation period, the patient was hospitalized in the Clinic of Hematology, of the Fundeni Clinical Institute-Bucharest, with some nonspecific infection symptoms: fever (38°C-38.5°C), fatigue, inapetency, vomiting, sleeplessness, headache, arthralgy, diarrhea, feeble respiratory insufficiency, emotional lability, and photophobia. The physical examination signaled splenomegaly and hepatomegaly.

Laboratory exams revealed: hemoglobin 10.5g/dl; hematocrit 29 %; eosinophils 28 %; monocytes 12 %; lymphocytes 21 %; platelets (thrombocytes) 110,000/mm3; VSH 62/94; urea 22 mg/dl; glycemia 108 mg/dl; cholesterol 98 mg/dl; AST 24 U/L; ALT 31 U/L; amilase 32 U/L; alkalin fosfatasis 210 U/L; Na 132 mEq/l; K 3.98 mEq/l; PT 7.2 g/l; albumin 2.52 g/l; alfa1 0.78 g/dl; alfa2 1.36 g/dl; beta 0.94 g/dl; gama 2.35 g/dl; seric crioglobulines.

The relevant hematologic values were anemia, hypereosinophilia, thrombocytopennia.

Negative results: AgHBs; HVC; ELISA for *Toxoplasma* IgM 0.339 (cut off 0.500); ELISA for *Toxoplasma* IgG 0.761 (cut off 0.500); ELISA for *Toxocara* IgM 0,092 (cut off 0.360); ELISA for Cysticercosis IgM 0.014 (cut off 0.500); ELISA for *Giardia* IgM 0.117 (cut off 0.400); ELISA for *Chlamydia trachomatis* IgM 0.045 (cut off 0.600); ELISA for *Chlamydia pneumoniae* 0.398 (cut off 0.500); ELISA for *Mycoplasma pneumoniae* 0.271 (cut off 0.500); ELISA for *Borrelia burgdorferi* IgM 0.268 (cut off 0.500), coproparazitologic exam, pharingian exudate, uroculture.

HIV infection was absent.

The diagnosis of *Babesia* infection was established by microscopic examination of thick and thin blood smears stained with Giemsa, and detection of intraerythrocytic parasites: ring form, round, oval, pear-shaped, even paired pyriform and very rarely tetrad form ("Maltese cross").

The same red cells modifications (poikilocytosis, anisocytosis, and hypochromic erythrocytes) were detected.

To verify the diagnosis, the examination of the blood smears was repeated in Germany.

The treatment recommended in Romania (clyndamicin and quinine and whole-blood exchange transfusion) was confirmed and applied in Germany.

After one month, new blood exams were made in Romania and Germany, and they registered no parasite presence. Although all analysis and the symptomatology revealed normal values, to avoid the persistence of low parasitemia after acute phase and the infection reappearance and to consolidate the good result, the patient was recommended to apply a new treatment (atovaquone associated with azithromycin) and blood control in the minimum following two years. Very important is also to verify a co infection with other infectious agent, because this additional immunosuppressive factor can explain *Babesia* opportunistic behavior and the infection severity in this normosplenic patient.

In Romania, Olteanu et al. (22) reports that the first case of human babesiosis was diagnosed by Panaitescu in 1972, and subsequently, another two cases were diagnosed in animal caretakers in Mehedinti County, without other specifications.

Conclusions

Two cases of Babesiosis, a rare parasitic disease, potentially fatal, were detected in Romania.

In one case, as a result of aspleny, Babesiosis conducted to fatality.

The species of Babesia was difficult to determine morphologically.

The infections were acquired through an infected Ixodid tick bite.

Diagnosis was based on clinical manifestation, tick bite, spleen status (splenectomy), and especially on examination of stained blood smear and typical intraerythrocytic Babesia trophozoites detection. To correct diagnosis, very important were the presence of pyriform bodies and Maltese cross and lack of cytoplasm pigment.

Both patients developed fever, fatigue, headache, inapetency, vomiting, arthralgia, hemolytic anemia, thrombocytopenia, splenomegaly and hepatomegaly, but in different degrees of intensity.

The differences in the severity of symptoms, complications and disease evolution were the consequences of the difference in their immunity competence, spleen status (aspleny or intact spleen), and the moment when the diagnosis was established and the treatment was applied (early or undue).

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Abstracts - The 10th Romanian Congress of Cytometry
Bucharest, May 21-22, 2014

Dear colleagues,

It is our pleasure with Mihaela Baica and Adriana Dumitrescu to invite you to participate in the 10th Romanian congress of cytometry.
This is an important year for our meeting. As you know we celebrate the tenth year of the Rumanian Association of Cytometry. During these years our Association has grown up, with biologists, scientists from different disciplines and has been very active through the implication of cytometrists in the different cytometry fields. We have accomplished several things. The association has evolved at the same pace as the rapid advancements in cytometry technology and applications. As Flow cytometry forms an integral part of both basic biological research and clinical diagnosis, from the beginning we have always privileged diversity favoring the interaction between biologists from clinical laboratories, scientists and clinicians resulting in an improved knowledge of flow cytometry.

Every year we have incorporated many different subjects in our meeting including:
***Themed sessions** providing a comprehensive view of the state of art in specific areas such. Hematology, Immunology, Basic research.*
***Workshops**: covering different topics of science, clinical applications and practical cytometric aspects.*
***Plenary talks** given by national and international invited speakers who are leaders in their disciplines.*

We always try to focus on the themes of innovation and development of new technologies.
One important objective for us is to encourage the presence of young cytometrists and to give them the opportunity to meet international experts.

Our next goal would be to improve educational sessions and create an official degree in cytometry. This becomes indispensable for the formation of the youngest and for continuous medical education of cytometrists. The new President of the Association Mihaela Baica and the former President Adriana Dumitrescu have prepared us with a very exciting program. In addition, I am convinced that this year we will have a very convivial atmosphere with the 10th Anniversary of our annual meeting. We are looking forward seeing you.
Sincerely yours,

Pr. Lydia Campos
Saint Etienne, France
Honorary President of Romanian Association of Cytometry

C1. **Evolution of flow cytometry over the last ten years**

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In the last 10 years, flow cytometry (FCM) benefited of a tremendous progress in its different aspects. Major contributions have been achieved in the field of **instrumentation, reagent development, and software analysis tools, leading to the** identification of specific subsets of cells with unique biological functions in normal and pathological conditions. The evolution from 3-4 color analysis to 8-10 or more has allowed a better specificity for the desired cells, specially the detection of low frequency populations.

Instrumental technological advances: high powered and smaller lasers provide stable excitation sources in a wide variety of wavelengths (emitting blue, red, green, violet and yellow light). More sensitive measurement allows better resolution of dimly-staining populations from background.

Advances in **affinity reagent technology** have enabled the flow cytometric detection of numerous proteins and other molecules through the development of new monoclonal antibodies, peptides/MHC multimers, recombinant receptor and ligand binding proteins, and aptamers.

Fluorescence chemistry (tandem dyes, nanocrystals), allows fine cell analysis up to 20 parameters. Parallel advances in instrument calibration methodology, have facilitated the application of multiparametric flow cytometry analysis of the biology of quiescent, activated, growing, differentiating, proliferating, dying and dead cells, as well as cell signaling and cytokine production.

All this generates very complex data sets that demand sophisticated tools of analysis, storage and data representation. However, **data analysis strategies** are still relatively underdeveloped. The multitude of data available is rarely analyzed easily. So, a need for plug-and-analyze software has emerged. New analysis tools development remains an important step as they will permit to analyze and compare several parameters in a multi-well format simultaneously and this for several cell types.

In the last few years, new bench-top flow cytometers have appeared. They combined analytic power into ergonomic, ultra-compact and easy- to-use analyzers.

Perspectives

In recent years, there was not only innovation in flow cytometry but also in the field of image-based cytometry. The maturation of multispectral imaging cytometry in flow imaging and the slide based laser scanning cytometers offers the possibility to have real time analysis of cells and tissues. Technological innovations are bringing the next generation of cytometers. In this context, the development of microfluidic, lab-on-a-chip (LOC) technologies is one of the most innovative approaches toward the advancement of cytometry. These technological advances have given rise to new platforms for the characterization of single cells.

These technologies include the:

FISHMAN-R is a Japanese analyzer that enables flow cytometry on a microfluidics chip. It could detect particles sized from 0.5 to 20 μm (bacteria and cells). Sample acquisition and data analysis are performed using the same software (PC). The Celigo is the first adherent cell cytometer, which analyzes cells in their environment with minimal sample manipulation. It analyzes adherent and non-adherent cells in brightfield and 3 fluorescence colors. The high-throughput acquisition is achieved by using an F-theta lens with a high speed galvanometer mirrors. It has a CCD camera. The Celigo software performs both image acquisition and analysis. The LEAP is a microplate-based cytometry system for non-invasive in situ process, allowing the use of various adherent and non adherent cell types.

Imaging flow cytometry: FlowCAM was the first bench top digital imaging analyzer for particle or cell measurements in solution, originally developed for oceanographic research.

The **ImageStream** is a multispectral imaging flow cytometer that combines the strength of FC and fluorescence microscopy in a single platform. It can digitally image millions of cells directly in flow. This technology enables the identification of single cell by fluorescence and morphology (5 excitation lasers and 12 channels of detection).

The new **FlowSight** based on the same technology, combines a brightfield lamp, 4 excitation lasers and 12 channels of detection to analyze simultaneously brightfield, darkfield and ten fluorescence colors per cell at a rate of 2,000 cells /second.

Other **optical developments** appear such as **COST coding**, which is a new way to detect **multifluorescent wavelengths** using a single photodetector. This method of discriminating multiple fluorescent colors with a single photomultiplier holds promise to significantly reduce the cost and size of the total system. User can differentiate 11 fluorochromes in FC by using a single PMT.

Mass cytometry is a completely different, non-fluorescence based approach. The Cytometric Time of Flight (CyTOF) cell analyzer is a high throughput mass cytometer for individual cell analysis based on a novel technology. The instrument detects the stable isotopic tags attached to antibodies using labeling kits. There are > 100 stable isotopes and the mass spectrometer provides highly precise resolution between detection channels (w/o compensation). In the instrument stained cells are nebulized into single-cell droplets

and introduced into the plasma. The resulting charged atomic ion clouds are immediately transferred into the high vacuum of the mass spectrometer. The cytometer is configured as a quadrupole-time-of-flight (qTOF) instrument. The quadrupole acts as a filter allowing only the heavier elemental ions, which consist primarily of the reporter "masses", to be quantitated by TOF mass analysis. A thousand cells are analyzed per second. Mass cytometer allows as many as 45 parameters to be measured for each cell. With phospho-flow cytometry is possible to measure the phosphorylation status of proteins critical to intracellular signaling cascades at the single-cell level.

This new technology allows simultaneously examined internal functional markers and cell surface markers to put together a more complete picture of cell signaling. It has increased understanding of cell expression and maturation pathways during hematopoiesis.

We expect that FCM will continue to decrease in size and energy consumption and will increase in detection and precision measurements.

In conclusion, the evolution of flow cytometry technology, has allowed an important understanding of cell biology. All this new technology is now used in clinical research but it would be applicable in clinical laboratories with adequate standardization inter-laboratories.

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C2. **Evaluation of the B-cell compartment in human peripheral blood as a tool for the diagnosis of primary immunodeficiencies**

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Important technological advances have been achieved in the last decades in flow cytometry instrumentation and reagents, which have increased both the multiparameter capabilities of flow cytometry immunophenotyping and the potential number of cells being analysed per sample. Together, such advances have facilitated the identification of low represented cell populations and their detailed immunophenotypic characterization.

Among other cells, characterization of bone marrow, peripheral blood and tonsil/lymph node B-lymphocytes has specifically benefited from these technological advances. Therefore, at present, multicolor combinations can be built which allow for very detailed characterization of the B-cell populations present in the above types of samples in normal vs. disease (e.g. primary immunodeficiency) states.

For many years it is well-established that B-cell maturation from early CD34+ hematopoietic

precursors to mature immunocompetent B-lymphocytes in human occurs in the bone marrow. As an end result, both immature/transitional and naïve B-lymphocytes are released to the peripheral blood; these cells will circulate in peripheral blood until they will enter the different lymphoid tissues (e.g. lymph nodes). At these lymphoid tissues, usually in association with T-cell help, naïve B-lymphocytes further undergo proliferation, hypersomatic mutation and potentially also immunoglobulin class switch together with maturation to memory B-lymphocytes and/or antibody-producing plasma cells, in case they would encounter their specific antigen. The newly-generated memory B-cells and plasma cells recirculate through peripheral blood to the lymph nodes, spleen, mucosa-associated lymphoid tissues (MALT) and the bone marrow, where they will potentially develop further effector functions. Therefore, during B-cell differentiation, peripheral blood acts as a crossroad where recently produced naïve, as well as memory B-lymphocytes and plasma cells arrive. Therefore, multiple different compartments of B-lineage cells can be identified in such samples, including steady state peripheral blood.

Each B-cell population typically displays unique immunophenotypic features that allow clear cut discrimination among distinct maturation stages. As an example, B-cell precursors frequently express CD10 and CD38 in association with variable amounts of CD20. Immature/transitional B-cells strongly express CD20 and IgM, they are clearly positive for CD38 and CD10 but at lower levels, and they display variable levels of CD5. Naïve B-cells typically lack CD10 and CD38, as well as CD27 and they coexpress SmIgM and SmIgD. Most memory B-cells in normal individuals show positivity for CD27 in association with expression of a single immunoglobulin heavy chain isotype: either IgM or IgD, or IgA, IgG or IgE. In contrast, after the germinal center reaction, plasma cells increase the expression of CD38, they progressively decrease SmIg expression, and acquire plasma cell-associated markers like CD138. Thereby, construction of antibody panels based on the above markers allows for detailed dissection of the different B-cell compartments in peripheral blood. Additional markers are typically required for the dissection of early B-cell (e.g. NuTdT, CD19, CD22, CyCD79a and CD34) and late plasma cell (e.g. CD19 and CD56) maturation in the bone marrow, and to evaluate the germinal center B-cell reaction (e.g. CD44 in addition to CD10 and CD38).

In recent years, the EuroFlow Consortium has initiated a new Working Group aiming at the development of new tools for the assessment of lymphoid maturation and cell compartments, through the characterization of patients with immunological disorders such as primary immunodeficiencies, based on previously developed EuroFlow tools and concepts.

In this presentation, we will review the immuno-phenotypic features of the distinct subsets of B-cells and plasma cells present in bone marrow, peripheral blood and tonsils, as a reference for the detection of altered maturation patterns and immunophenotypic profiles in primary immunodeficiency patients.

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C3. **Diversity for the Big Picture: Complete Solutions in the Flow Cytometry Laboratory**

Andreas Bohmler
Beckman Coulter, Krefeld, Germania

Laboratories are as unique as people. There are differences in patient demographics, required applications, financial possibilities and many more. In addition there is a need to react dynamically to scientific advances while retaining a high level of standardization.

Beckman Coulter has established a portfolio in flow cytometry that addresses these needs and offers you the possibility to individually create your lab. We build upon our long time tradition in dealing with complex processes. Our experts are happy to work with you to analyze your workflow and design a concept that includes flow cytometry analyzers, antibody cocktails, sample prep systems, and solutions for data management.

C4. **Advanced flow cytometry with new Guava easyCyte 12 HT**

Anda Stuparu
Bucuresti, Romania

Abstract not available

C5. **Innovative methods for cellular analysis: Imaging Flow Cytometry and Real Time, Label-Free Cellular assays**

Miloslav Korbel
Praga, Cehia

Imaging Flow Cytometers from Amis combine the speed, sensitivity, and phenotyping abilities of flow cytometry with imagery and funcional insights of fluorescence microscopy. Imaging flow cytometers can digitally image millions of cells directly in flow so you can perform high content assays on rare cell populations and quantitate biological phenomena with incredible accuracy.

Signal translocation of Nf-KB, signal colocalization of pDc´s and Phagocytosis assays on Imaging flow cytometers will be shown.The xCEL Ligence Systems from ACEA Biosciences are

microelectronic biosensor systems for cell-based assays that provide dynamic, real-time, label-free cellular analysis for a variety of research applications in drug development, toxicology, cancer, regenerative medicine, immunology, and infectious diseases. This Real-Time Cellular-Analysis (RTCA) technology allows researchers to increase productivity and exceed the limits of endpoint analysis by capturing data throughout the entire time course of an experiment and obtaining more physiologically relevant data.

C6. **CYTOGNOS: New tools for Flow Cytometry Data Analysis**

Pablo Penalosa
Salamanca, Spania

Abstract not available

C7. **Signaling pathways in cancer cells**

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Background: Signaling pathways from growth factor receptors and integrins are cross-talking at a non-receptor tyrosin kinase, focal adhesion kinase (FAK) and overexpression of integrins was associated with increased phosphorylation of protein kinase B (PKB or Akt) and extracellular-signal-regulated kinases (ERK) in cancer cells. The aim of this study was to provide new insides the signaling pathways in seven cancer cell lines.

Methods: Three suspension cell lines: NB-4 (human acute promyelocitic leukemia), SEM (human B cell precursor leukemia), K-562 (human chronic myeloid leukemia in blast crisis) and four adherent cell lines: HT-29 (human colon adenocarcinoma), NTERA-2 (human embryonal carcinoma/teratocarcinoma), A-594 (human lung carcinoma) and A-431 (human epidermoid adenocarcinoma) were cultured according to their specifications. The samples, 10^6 cells/ sample, were stained for surface- and intracellular markers followed by measurements using Becton-Dickinson FACS Canto II and Beckman Coulter Gallios flow cytometers. The data were analyzed using DIVA and respectively, Gallios software. A-431 cells were treated for 30 minutes and 48 h with epigallocathechin 3-*O* gallate (EGCG) and the effect of the flavonoid was evaluated on the signaling proteins. An Akt-inhibitor (Calbiochem) was applied for 48 h to the leukemic cell lines and its effects were investigated on the signaling proteins and by clonogenic assay.

Results: The following signaling proteins were evaluated by flow cytometry: FAK/pFAK, Akt/pAkt, ERK/pERK, pSTAT3, pSTAT5 and in the leukemic cell lines additionally the surface markers: CD13, CD15, CD33. In the investigated cancer cell lines: pFAK and pAkt displayed an increased expression level. The Akt inhibitor applied in different concentrations 5, 10 and 20 μM modulated the expression level of pAkt and the number of colonies evaluated by clonogenic assay. EGCG administrated to A-431 cell line induced a reduction in pFAK expression level at 30 minutes and 48 h of incubation.

Conclusions: Our results recommend pFAK and pAkt as new biological markers for clinical investigations and support the anti-cancer activity of the natural compounds, like EGCG.

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C8. **Using FACS for new therapeutic targets validation and selective antineoplastic drugs identification**

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Introduction. Therapeutic target validation is one of the greatest challenges in drug discovery. Knowledge generation about the validity of therapeutic targets, via integration of genomics and cell biology data in systems based on cells/tissues, will increase the new medicine success rate.

Sorting and propagating the cells with specific therapeutic targets characteristics provide the chance to validate the target and also to set-up screening systems that have physiologic properties similar to the in vivo targets.

Excessive activation of different signaling pathways is common in disorders therefore we used kinases to prove the concept.

Materials and Methods. Our strategy involved sorting cells transduced with bicistronic retroviral vectors, that express definite targets at predetermined levels, based on CD2, CD4 and GFP levels. These sorted cells were propagated as cell lines and used in cellular systems for target validation and selective inhibitors screening.

Results. We could show that Janus kinases (JAK), that play crucial role in controlling many cellular processes and are excessively activated in different neoplasms, might be more efficiently targeted if used in combination with another kinase inhibitors targeting a pathway downstream of the aberrant JAK2 signaling. Our cell-based assays were able to show a strong synergy between JAK and PI3K Inhibitors. This effect occurs best when the concentration ratio set is in favor of JAK inhibitor over the PI3K inhibitor. This implies that the PI3K signaling is secondary to the JAK2 signaling in our cell models. This effect was confirmed on an animal model developed using sorted engineered Ba/F3 TpoR JAK2 V617F cells and also transplanted mice with JAK2 V617F knock-in bone marrow.

Conclusion. Our therapeutic target validation strategy using sorted engineered cells with specific targets at determined levels, allowed evaluation of the target and also the downstream pathways, target modulation in disease-relevant cells/tissues/animal models, and, at the same time, screening different compounds on systems with physiologic conformation similar to in vivo target.

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C9. **Flow-cytometry investigation of cutaneous melanoma. Correlations with genomic tests**

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Introduction. Cutaneous melanoma (CM) is below 5% of skin cancer cases, but is responsible for 80% of deaths from these cancers. The prognosis worsens as the lesion extends under the skin due to increased tumor tendency of invasion and metastasis. Evaluation by flow-cytometry of melanoma cells positive for MCSP (Melanoma-associated Chondroitin-Sulfate Proteoglycan), coupled with the identification of mutations in genes associated with cell cycle control and highlighting changes in the products of these genes, may represent indices to assess the evolution of the tumor.

Materials and methods. MCSP+ cells in

peripheral blood were identified by flow-cytometry with anti-MCSP–APC antibody (*Miltenyi Biotec*) in 23 MC patients and 5 control subjects. A total of 37 types of mutations in the BRAF, CDKN2A, CTNNB1, GNAQ, HRAS, KIT, KRAS, NRAS, PIK3CA and STK11 genes were analyzed by qPCR-array (Human Melanoma Somatic Mutation qBiomarker - *SABioscience/Qiagen*) on DNA extracted from 8 MC. The products of BRAF, HRAS and KRAS genes were evaluated by ELISA (*MyBioSource*) in the sera of 21 MC patients. Correlations between serum levels of gene products and the presence of circulating MCSP+ tumor cells were performed for 11 patients.

Results. The presence of gene mutations was found in 63% of MC tested cases. The determinations of serum proteins encoded by BRAF, HRAS and KRAS genes revealed increases in 52% patients for BRAF, 10% patients for HRAS and 57% patients for KRAS, representing cases with overexpression of these genes and subsequent increase in blood concentration of gene resulting products. 64% of patients tested for MCSP+ cells showed higher percentages than in controls, indicating the presence of circulating tumor cells. The correlation of this result with BRAF, HRAS and KRAS products concentrations in serum revealed an increase of at least one gene product in 83% of MCSP+ patients, suggesting that mutations in these genes may be associated with metastasis.

Conclusion. The MCSP and ELISA for gene products tests in MC are useful in determining the tumor invasive potential and treatment monitoring in patients with known mutations in genes involved in the development and proliferation of malignant melanocytes.

C10. **Characterisation of mesenchymal stem cells interaction with biomaterials using flow and image cytometry**

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Introduction. Bone regeneration during fracture healing or implant integration is essential for the efficient recovery in orthopaedic pathology. To this end, the efficient direction of proliferating mesenchymal stem cells (MSCs) towards the osteogenic fate is a primary key step. *In vitro*, three differentiation factors are commonly used for osteogenic induction of MSCs: dexamethasone, ascorbic acid, and beta-glycerophospate. Use of growth factors such as bone morphogenic proteins for the facilitation of osteoblast differentiation is well documented. However, few studies reported the use of FGF2 during MSCs differentiation while its effects and mechanism of action are not entirely understood. Its use as component of biomaterial coatings is expected to trigger incresed proliferation[1,2] of osteoprogenitor cells before their commitment to mineralized matrix-producing bone cells. For the design of laser-transferred polymeric thin scaffolds incorporating growth factors, prior investigation of substrates as well as of proteins to be embedded must be thoroughly performed. In the present study, we have screened several polymer combinations for biocompatibility with bone cells using live/dead staining and flow cytometry analysis. To determine the optimal dosage and treatment intervals of FGF2 for enhanced differentiation of bone marrow-derived MSCs, we used a combinatorial approach followed by endpoint mineralization quantification. The effect of FGF2 on MSCs phenotype was characterized using both flow and image cytometry in order to characterize signalling molecules expression during osteogenic induction.

Materials and methods. The readout of MSCs differentiation was the Alizarin Red S staining of mineralized tissue performed at 28 days after induction. We have used the Live/Dead Viability Kit (Invitrogen) to quantify bone cell viability after growth on polymer thin films by flow cytometry using FACSVerse instrument and FACSuite Software (BD Biosciences). Live cells were stained by calcein AM while dead cells were stained by ethidium homodimer-1 following a 15 minutes labelling at room temperature (RT). The expression of signalling proteins was assessed by both flow and image cytometry using Cell Signaling and abcam antibodies. For FACS, cells were fixed and scraped in 1.5% PFA, and incubated for 10 min at RT. Following centrifugation, pellets were permeabilized in cold methanol, washed in 0.5%

BSA-PBS staining media and incubated with primary antibodies 30 min at RT. Alexa Fluor conjugated antibodies (Invitrogen) were used for detection. For all experiments, ten thousand cells were acquired using a BD instrument and data were analysed with Cytobank Software. For image quantifications, a dedicated microscope-based TissueFAXSplus cell analysis system was used (TissueGnostics, Austria) for specimen total scanning, image stitching and cytometric measurements on scattergrams. Cells were fixed in 4% PFA in PBS 15 min at RT and permeabilized in cold methanol after a protocol recommended by Cell Signaling Technology. Finally, cells were mounted in Prolog Antifade Reagent (Invitrogen) and subjected to microscopic analysis.

Results. Polyethylene glycol:polycaprolactone (PEG6K:PCL) was chose for further examinations as a potential scaffold for growth factors controlled release. We have characterized the expression of MAPKs during osteogenic differentiation in the presence of FGF2 and determined the optimal conditions to obtain a positive effect on mineralization.

Conclusions. Flow and image cytometry can be used in complementarity to assess cell signaling triggered by specific molecules and to screen for optimal cell growth and differentiation conditions in order to design smart 3D biomaterials.

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C11. **EuroFlow panels and strategies for the diagnosis of myelodysplastic syndromes**

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In the last two decades, there has been an increased amount of information about the immunophenotypic profiles of normal and myelodysplasia (MDS)-associated hematopoiesis. This includes both detailed knowledge about the immunophenotypic patterns of early committed CD34+ hematopoietic progenitors and precursor cells (HPC) and the features of maturing neutrophils, monocytes, erythroid, basophil, mast cells, dendritic cell, B-cell and T lymphoid precursors, in normal/reactive bone marrow compared to MDS. In order to evaluate different maturational compartments of hematopietic cells, in 2012, the EuroFlow Consortium has proposed a validated 8-color MDS panel of antibodies to be used in combination with standardized sample preparation procedures and new software tools for reproducible analysis of normal vs dysplastic hematopoiesis in the clinical settings. Overall the EuroFlow MDS panel includes up to a maximum of 7 antibody combinations from which the first four are considered essential, while the other three tubes are devoted to obtain complementary information

in selected cases.

In detail, all 7 combinations include a backbone of 4 markers which are common to all of them (CD34, CD117, HLADR and CD45) and that are combined with another 4 markers that vary among the different combinations (tube specific characterization markers). The backbone markers repeated in every combination are devoted to the reproducible analysis per tube of the early stages of hematopoiesis in CD34+ and/or CD117+ immature precursors. Thus, such markers already allow for clear discrimination of the early stages of CD34 commitment toward the neutrophil / erythroid / megakaryocytic / T vs mast cell vs B-lymphoid / monocytic / dendritic cell lineages based on the pattern of expression of CD117 and HLADR; in addition, usage of SSC and CD45 expression permits further discrimination between neutrophil, T-lymphoid and erythroid precursors, at the same time it also contributes to the separation between B-lymphoid vs monocytic/dendritic cell precursors at the CD34+ and/or CD117 stages of maturation.

In tube 1, the four variable markers (CD13, CD11b, CD16 and CD10) are specifically devoted to a highly detailed analysis of the neutrophil maturation, although they are also informative for the assessment of other (e.g. monocytic) hematopoietic cell compartments. Therefore, based on this 8-color combination, neutrophil committed precursors from normal bone marrow are known to sequentially loose expression of CD34, HLADR, CD117 and CD13, followed by acquisition of CD11b, CD13, CD16 and CD10 expression. Similarly, tube 2 is devoted to the specific dissection of the monocytic pathway by combining the CD64, CD35, CD14 and CD300e markers, with the above listed four backbone antigens. CD34+ monocytic precursors can be easily identified by their early acquisition of CD64, followed by loss of CD34 and CD117 and sequential acquisition of CD14, CD35 and finally, CD300e. The third tube in the MDS EuroFlow panel aims at detailed characterization of the erythroid maturation from the earliest CD34+ CD117+, HLADR+ precursors to the more mature nucleated red cells in the bone marrow. Therefore, erythroid maturation is first confirmed by coexpression of CD36 and CD105 on CD34+ precursors that progressively loose expression of CD34, CD33, HLADR and finally CD117, at the same time they show increasing amounts of CD36, CD105 and CD71; subsequently, erythroid precursors loose CD105, and downregulate at the very late stages also CD36 and CD71. With tube 4, assessment of the lymphoid maturation in the bone marrow is achieved through the addition of NuTDT, CD19 and CD7 to the CD34, CD45, CD117 and HLADR backbone markers; in this tube CD56 is used mostly for the assessment of NK-cells and aberrant marker expressions on hematopoietic precursors, monocytic and neutrophil lineage cells.

The remaining three supplementary tubes allow identification of minor myeloid cell lineages (e.g. tubes 6 and 7 permit the identification of basophils, plasmacytoid dendritic cells and megakaryocytic cells and their precursors) whereas tube 5 is devoted to the identification of aberrant phenotypes and immature CD34+/CD38- precursors.

Of note, preliminary data suggests that usage of the EuroFlow MDS antibody panel in combination with the newly developed maturation tools implemented in the INFINICYT software (Cytognos, SL, Salamanca, Spain), allow for sensitive detection of aberrant and altered immunophenotypes among the bone marrow maturing neutrophil monocytic and erythroid cells from MDS patients, with a high accuracy. In addition, it has also proven to be highly efficient for the diagnostic screening of MDS-associated with other lymphoid malignancies e.g. in patients with multiple myeloma. At the same time, usage of the maturation software tools in combination within reference EuroFlow data bases, provides a reproducible basis for the identification of such alterations in suspected patients, provided that highly comparable and reproducible sample preparation procedures and antibody panels had been used.

Altogether, this will most probably facilitate extended usage and application of multiparameter flow cytometry immunophenotyping in routine diagnostic practice for the diagnosis, classification and monitoring of MDS.

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C12. **Adhesion-related abnormalities of mesenchymal stromal cells as part of pathological process evaluation in myelodysplastic syndromes**

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The present work is dedicated to the argumentation of the morpho-molecular roles of mesenchymal stromal cells (MSCs) in myelodysplastic syndromes (MDS) pathogenesis, with regards to their ability to generate a microenvironment suitable to hematopoietic cells development.

The understanding the mechanisms by which neoplasia manage to integrate the stromal components in tumorigenesis represents also a major source of progress. The hematopoietic stem cells (HSC)-MSC relationship is a critical point in the hematopoietic malignancies pathogenesis, and the current technical approaches provide limited and rather slow progresses.

Previous work has shown that adherent layers of stromal cells from bone marrows (BM) of MDS patients achieved confluence at significantly slower rate than normal donor-MSC [Boudard D, Haematologica 2003].

In line with these data, we noticed the growth deficiencies and spontaneous lysis in primary cultures of MDS stromal cells compared to normal controls, especially in refractory cytopenia (RC) cases. Moreover, morphological assessment of Refractory Anaemia with Excess Blasts (RAEB) primary layers depict dysplastic changes related to altered actin organization, such as thin and flat cells, as Ilić et al. have already reported in mouse FAK (-/-) fibroblasts [Ilić D, Nature 1995]. Morphometric evaluation highlights the different distribution of the three morphotypes (rounded-shaped, with the appearance of undifferentiated cells; thin, spindle-shaped cells; and large, flat cells) of MSC in MDS layers compared to normal settings, as well as size differences which could indicate maturation defects.

Thereof we were interested to find whether the MSC maturation abnormalities affect equally the functionality of the hematopoietic compartment, and to evaluate the adhesion-related processes, such as cell proliferation and clonogenic growth. In this order, a first step was to achieve standard-compliant MSC preparations from reduced quantities of BM aspirates and from cells showing significantly reduced abilities to reach confluence.

Thus, we used an initial enrichment in primary cultures and an immunomagnetic double selection based on the different expression of STRO-1 and CD73, two specific markers for MSC. Technically, we noticed that these two fractions could be exploited differently, STRO-1^+ cells being more robust for carrying out *in vitro* MSC growth assays, whereas $CD73^+$ cells have proven their utility in the evaluation of adhesion profiles.

Of note is the fact that under the MDS condition, a higher number of STRO-1^+ cells which co-expressed CD106 and CD31, were noticed between 20 and 30 days in primary cultures, and were persistent until 60 days. Two hypotheses can be evoked from the expression of these molecules in relation to MDS physiopathology: the former is related to CD106 upregulation induced by TNFα stimulation [Xing L, Asian Biomedicine 2012], and the second is related to CD106 function as a major ligand for selective CD29-mediated hematopoietic precursor cells (HPC)-to-MSC adhesions, and thus, to its influence on the HPC mitotic rate and division kinetics [Kohase M, Cell 1986]. In addition, the increased expression of $CD31^+$ could be an imprint of the neoformation of blood vessels in MDS settings, as Boudard D et al. showed in a previous study [Boudard D, Haematologica 2003]. Likewise in MDS settings, the $CD73^+$ fractions of MSC displayed a significant reduction of adhesion markers, CD29, CD54, CD44, and CD49e.

Furthermore, the functional tests revealed MSC growth abnormalities in the absence of any contact with or stimulation by soluble molecules from HPCs and proved the pathological nature of stromal precursors in MDS settings.

Thus, MSC production in STRO-1^+ and $CD73^+$ cell cultures from refractory cytopenia with unilineage dysplasia (RCUD) and refractory dysplasia with multilineage dysplasia (RCMD) marrows was deficient, and, in addition, the clonogenic ability of these fractions was strongly diminished. We have concluded that the relative proliferation in MSC cultures from RC is the result of a division process that is continuous, but occurs at a low rate and without the ability to generate the normal functional progenitors required to form

colonies. By contrast, in RAEB settings, the proliferation rate is moderately improved due to the reduced doubling time (DT) of STRO-1 cells. However, at the end point, this was not accompanied by complete functional maturity as reflected in the CFU-F number. Of note is the fact that MSCs from the RAEB in transformation (RAEB-T) cases shows a highly rate of proliferation, both in rounded, and spindle shaped STRO-1$^+$ MSCs, as well as in large STRO-1$^-$ CD73$^+$ MSCs.

Thereafter, we observed a diminution of CFU-F capacity of CD73$^+$ fractions in MDS settings which directly correlated with the CD44 mitigate on their surface. In addition, the doubling time of MSCs from MDS inversely correlate with their expression for CD49e (α_5-integrin). In conclusion, the MSC proliferation and clonogenic potential are adhesion-dependent processes.

Then, we have explored the focal adhesion (FA) signalling pathways in order to understand whether adhesion-mediated processes contribute to transduction of intrinsic proliferative signals, as well as their impact on HPC-to-MSC interactions.

Thus, we have observed that the large proliferation differences occurring in RAEB-T cultures compared to normal settings can be attributed both to smaller (S-MSCs), as well as to large cells (L-MSCs), which present qualitative defects of FA proteins (focal adhesion kinase [FAK], and paxillin) and of the chaperone heat shock protein 90 (HSP90), such as intensity differences, nuclear localization, and their association in complexes. In normal cells, HSP90 plays a number of important roles, which include assisting protein folding and DNA damage response, transcription, and degradation of proteins as well as facilitating cell signalling. Recent evidences shows that HSP90 control the histone code through regulation of KDM4B demethylase stability [Ipenberg I, J. Biol. Chem. 2013].

The MSCs from RAEB cultures highlight a strong overlapping of FA proteins to HSP90 in nuclear area, which could explain the increases proliferative capacity of these cells. A possible explanation of this is the cessation of proteasome-mediated recycling of the proteins co-located to HSP90, which confer them a proliferative advantage, as Dong JM et al. showed in a previous study [Dong JM, Biochem J 2005]. Moreover, recent evidences support the FAK direct contribution to cell growth both by influencing the proliferation rate as well as apoptosis also. FAK binding at paxillin induce its phosphorylation and conformational modification, blocking its nuclear export [Dong JM, Biochem J 2005]. It has been proven that this particular nuclear localization of paxillin stimulate DNA synthesis and cell proliferation [Dong JM, Biochem J 2005], [Kasai M, Cancer Research 2003], by suppression of H19 (a tumor-suppressor gene) transcription and promotion of Igf2 expression at the translational level [Dong JM, Biochem J 2005].

Another hypothesis that arises from our study is the putative role of FAK [Y397] expression on MSCs in HPC-to-MSC interactions and thus its implication in modulation of HPC clonogenic capacities. There is recent evidence that sustain that FAK regulates integrin expression in human fibroblast [Michael EK, Mol Biol Cell 2009]. In this study we find that the increased levels of pFAK [Y397] reversely correlate with the CD49e expression on MSC cells, and this reduction significantly correlate with the diminution of clonogenical potential of HPCs selected from MDS patients.

In conclusion, these data prove that MSCs selected from MDS patients are intrinsically pathological and that they could influence HSC behaviour by their direct interactions via FA proteins signalling. The perception of the stroma-related disease mechanisms may underlie the development of alternative therapeutic approaches, e.g. if considering that FAK is one of HSP90αβ-client proteins, HSP90αβ inhibitors may be used as adjuvants in MDS therapy.

C13. **Correlations of immunophenotypic, cytogenetic and molecular biology data in acute leukemias: Fundeni Clinical Institute Experience**

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Acute leukemia represents a clinically and biologically heterogeneous malignancy with uncontrolled proliferation of hematopoietic

precursors.
Materials. 90 cases of acute leukemia diagnosed in 2013 at Center of Hematology and Bone Marrow Transplantation Fundeni Clinical Institute. We have two sources of patients: Center of Hematology and Bone Marrow Transplantation and Pediatric Clinical Institute.

Methods. Analysis immunophenotyping, cytogenetic and molecular biology data in patients with acute leukemias.

Results. We analyzed 50 cases of acute lymphoblastic leukemia (ALL) and 40 cases of acute myeloid leukemia (AML) in Hematology Center of Fundeni Clinical Institute. All patients had immunophenotyping, cytogenetic examination and molecular biology at diagnosis.

From those 50 cases of ALL: 31 patients (62%) were < 18 years old and 19 patients (38%) were > 18 years old.

In patients aged <18 years old: 11 patients (35%) had pre B ALL, 15 patients (48%) had common B ALL, 4 patients (13%) had T-ALL and 1 patient was with biphenotypic ALL.

Common B ALL was associated with TEL-AML (7/50), the majority of cases showed normal karyotype and 3/50 were associated with BCR-ABL; all these patients presented at immunophenotype exam the CD66c.

Pro B ALL was associated with the MLL - AF4 (2 of the 3 patients with pro B ALL).
Patients with pre B ALL (2/11) associated E2A PBX1; 2 cases were associated TEL- AML; 3 cases showed hyperdiploidies at cytogenetic examination - the latter involving expression of CD66c.

T- ALL and biphenotypic leukemias showed no abnormalities at molecular biology except for a case that showed E2A PBX at molecular biology.
In acute myeloid leukemias (without maturation, with minimal differentiation and those with maturation) no alterations were found in molecular biology exam, except of 3 cases that presented FLT3 – ITD; the last ones were associated with additional cytogenetic abnormalities. In this group, one single case of acute leukemia with maturation presented AML1 - ETO and chromosomal instability.

6/40 cases of acute myeloid leukemias showed PML- RARα at molecular biology - one patient associated expression of CD56 and a high number of leucocytes, with unfavorable outcome.
Cases of acute leukemias with monocytic component did not associated MLL - AF9 nor the cytogenetic abnormalities and only 3 of these patients presented NG2; one case of acute myelomonocytic leukemia presented CBFB - MYH11 type A; also 5 cases associated FLT3 – ITD.

Conclusions. In all cases, the immunophenotype diagnosis was correlated with molecular abnormalities, according to the literature data. CD66c expression was found in all patients diagnosed with hyperdiploidies and in cases of acute lymphoblastic leukemia with BCR- ABL. MLL - AF9 mutation was not found in any case of acute monocytic leukemia. NG2 expression in acute leukemias was associated with monocytic component, but with a low frequency.

C14. **Acute leukemia with ambiguous triphenotypic lineage – a challenge for diagnosis and treatment**

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Background. Undifferentiated acute leukemias were best analyzed by immunophenotypic methods and in EGIL classification for the first time were found leukemias with more than one lineage. The scoring system of EGIL was integrated in the 2001 and 2008 edition of the WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues, which recognized a special category called "leukemias of ambiguous lineage." The vast majority of these rare leukemias are classified as mixed phenotype acute leukemia (MPAL), and acute undifferentiated leukemias and natural killer lymphoblastic leukemias are also included in this category.

The major immunophenotypic markers used by the WHO 2008 to determine the lineage for these proliferations are myeloperoxidase, CD19, and cytoplasmic CD3. However, extensive immuno-

phenotyping is necessary to confirm that the cells indeed belong to 2 different lineages or coexpress differentiation antigens of more than 1 lineage. Specific subsets of MPAL are defined by chromosomal anomalies such as the t(9;22) Philadelphia chromosome BCR-ABL1 or involvement of the MLL gene on chromosome 11q23.

Other MPAL are divided into B/myeloid NOS, T/myeloid NOS, B/T NOS, and B/T/myeloid NOS. MPAL are usually of dire prognosis, respond variably to chemotherapy of acute lymphoblastic or acute myeloblastic type, and benefit most from rapid allogeneic hematopoietic stem cell transplantation.

A diagnosis of triphenotipic acute leukemias also an extremely rare diagnosis and requires strong signs of three lineages in blastic cells.
Coexpression of B- and T-lineage associated antigens or antigens of all three lineages is exceedingly rare, accounting for <5% of MLLs.

Material and method. Results. Current diagnosis in our department is done based on ELN panels, and immunophenotyping on a FACS Calibur cytometer. We present a difficult case of 31 years male who was diagnosed with ambiguous lineage acute leukemia in 2014, February.

Clinical presentation was typically for acute leukemia with anemia, thrombocytopenia and leukocytosis with 49% blasts in peripheral blood and 95% in bone marrow, with medium size, fine chromatin, rare nucleoli, small amount basophilic cytoplasm, positive at myeloperoxidase (MPO) 30% and periodic acid Schiff (PAS) 9%. Immunophenotyping by flow cytometry on bone marrow aspirate identified 85% blasts with CD45 medium/low, SSC medium with expression of lineage markers cCD3+ (60%) cMPO- cCD79a+ (64%) TdT-/+ and stem cell markers CD34+ CD38+; other surface markers were found for myeloid lineage CD33+/- CD13+ CD117+ CD15- CD36- CD11b- CD14- CD64-, B cell lineage CD19- CD10+/-, T cell lineage CD2+ CD3- CD7+ CD5- CD1a- and NK lineage CD56- CD16-. Immunohistochemical staining on bone marrow biopsy described the same blasts with expression of CD34, MPO, CD33, cCD3, negative for cCD79a and CD68, suggestive for MPAL T/myeloid.
Based on scoring system for MPAL, we considered the diagnosis for trilineage ambiguous acute leukemia, T/B/myeloid.

Cytogenetic and molecular analysis detected only a few monosomias and no specific fusion gene.

Treatment choice was difficult but regimen for ALL, **UKALL XII/ECOG 2993,** was done, with good response at interimar analysis after first month of induction therapy. Further treatment will include consolidation with allogeneic SCT.

Conclusion. Ambiguous lineage acute leukemias are rare cases, associated with cytogenetic high risk changes. Powerfull diagnostic tools by multiparametric immunophenotyping are extremely important in diagnosis and aplying scoring system developed by EGIL could be a chalenge. Most MPAL are biphenotypic, but MPAL with three lineages could be found and treament choice is difficult, but ALL regimen have been more efficient.

C15. **Minimal Residual Disease in Multiple Myeloma**

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Introduction. The achievement of minimal residual disease (MRD) negativity is proved to be a powerful predictor of favorable outcomes in multiple myeloma (MM). Recent technical developments in flow cytometry (FC) afford for a MRD detection with high sensitivity. So far the 0.01% threshold for MRD detection has consistently proven to bear prognostic relevance in MM.

Materials and methods. Between January 2011 and May 2014, 52 MM patients were evaluated in our unit by FC for MRD detection, following induction therapy. A 6-color antibody panel testing for CD19, CD56, CD38, CD138, CD45, kappa,

lambda was used to distinguish abnormal plasma cells from normal plasma cells. Between $5x10^5$ and $1x10^6$ events were acquired and 50 to 100 events were considered the cutoff for MRD positivity.

Results. The limit of FC-mediated detection depends on the number of events used to define a population as well as the total number of events acquired. The detection limit may render reproducible when the assay contains sufficient antigens to reliably discriminate the neoplastic cells in virtually all cases.

Conclusion. Coordinated efforts from international groups are currently working for defining consensus criteria for the minimum requirement for MRD testing in MM, as well as for the optimal timing of MRD detection in order to implement this technique in treatment trials and in clinical practice.

P1. **Comparative study between EGIL and WHO 2008 classification in mixed phenotype acute leukemia**

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Introduction. The majority of acute leukemias (AL) can be classified as myeloid, B, or T lymphoid. In some cases this is not possible because of the evidence of expression of both lymphoid and myeloid lineage-specific antigens in the blast cells. These cases were defined previous as biphenotypic or biclonal AL by EGIL classification. Based on the classification of WHO (2008) biphenotypic and biclonal AL were redefined as the mixed phenotype acute leukemia (MPAL) assigning new criteria for this group of diseases. The purpose of this study was to compare EGIL and WHO (2008) classifications of MPAL and to present importance of immunophenotyping by multiparametric flow cytometry in their diagnosis.

Material and method. In our report we present 10 cases diagnosed initially with biphenotypic acute leukemia from a total of 272 acute leukemia patients. We performed immunophenptyping of bone marrow samples. Four-color immunofluorescence staining was used. The initial diagnosis was established according to EGIL classification. The same cases were reviewed according to WHO criteria for mixed phenotype acute leukemia.

Results. Based on GEIL scoring system, immunophenotypic analysis identified 6 cases of biphenotypic acute leukemia with B-lymphoid + myeloid lineage, 3 cases with myeloid and T-lymphoid lineage + 1 case of B+T lymphoid lineage. One patient was diagnosed with biclonal AL, both morphologically and immunologically two distinct population of blasts were identified, one with B lymphoid lineage and one with myeloid lineage. After reviewing these cases, it was found that in 2 cases (previously diagnosed as biphenotypic acute leukemia with B-lymphoid and myeloid lineage) did not fulfill the diagnostic criteria of WHO for MPAL. The final diagnosis in one case was AML with aberrant lymphoid markers and in one case was B-lineage ALL with aberrant myeloid markers. The other 8 cases were defined as MPAL according to WHO 2008 criteria.

Conclusions. By applying the WHO 2008 criteria, much stricter than the previous classification of EGIL, we can avoid overestimation of biphenotypic AL, some of them being redefined as ALL with aberrant myeloid markers or AML with aberrant lymphoid markers. This has particular implications for the choice of therapeutic strategy in patients with MPAL.

P2. **Dendritic cells acute leukemia – a rare disease with cutaneous onset**

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Background. Dendritic cell leukemia (DCL) is a rare subtype of acute leukemia and is characterized by clonal proliferation of plasmacytoid dendritic cell precursors. Diagnosis by immunophenotyping

allowed identification of this form of acute leukemia that was extracted from undifferentiated acute leukemia group. It is framed in the WHO classification of tumors of hematopoietic and lymphoid tissue editions in 2001 and 2008 as blast plasmacytoid dendritic cell neoplasm (BPDCN).
This disease has a characteristic immunophenotype with expression of following markers CD4, CD56, CD123, CD43. The skin lesions identified CLA expression. Also described is the expression of TCL1 (T-cell leukemia 1).

BPDCN is manifested by aggressive behavior that responds well to initial chemotherapy, but relapse is the rule and a poor prognosis. Clinical manifestations include cytopenia, particularly thrombocytopenia. In the literature there are few data about this subtype of leukemia, which makes it problematic to define the biological characteristics and clinical therefore appropriate therapeutic approach.

Material and methods. Results. We present a patient 84 years male who has been presented at diagnosis with maculopapular lesions posterior and anterior thorax and abdomen and splenomegaly.

Examination of bone marrow cellularity describes about 44% rich medium cells, the nucleus incised, fine chromatin, rare nucleoli, reduced quantity of basophilic agranular cytoplasm. Immuno-phenotypic analysis performed with FACS Calibur cytometer describes blasts population with CD45 low, SSC medium, with absence of stem cell markers CD34 and intracytoplasmic lineage markers, cCD3- cCD79a- cMPO- TdT-, myeloid markers, CD33- CD117- CD64-, lymphoid markers CD7- CD3- CD19- CD10- CD8-, except CD2+ CD4+/- CD56-/+ and coexpression of CD123+, CD36+ CD38+. Immunophenotypic markers were identified as meaningful for diagnosis of dendritic cell leukemia, according to the diagnostic score.

The diagnosis was supplemented by biopsy of skin lesions, which described rich cellular infiltrates perivascular and perianexial, consisting predominantly of lymphocytes in the dermis, with extension into the superficial portion of the hypodermis, concluding to the dermal inflammatory reactive nature in the context of a cutaneous pseudolymphoma.

During the outcome, the patient was followed clinically and by immunophenotyping, with favorable response to chemotherapy cures type CVP (cyclophosphamide, vincristine, prednisone), with the disappearance of splenomegaly and significant improvement of skin lesions while reducing component of dendritic cells from peripheral blood.

Conclusion. Dendritic cell leukemia is a rare group of acute leukemias and association with cutaneous lesions is suggestive of the diagnosis. Immunophenotyping is the main diagnostic tool that allows the diagnosis of this rare form of leukemia.

P3. **The role of immunophenotyping by flow cytometry in diagnosis of acute myeloid leukemias HLA-DR negative**

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Acute myeloid leukemia (AML) is diagnosed on the basis of cytomorphology, cytochemistry, flow cytometric immunophenotypic studies and the identification of recurrent cytogenetics and molecular genetics abnormalities. The purpose of the work was to assess the contribution of the flow cytometry to determine the subtype of acute myeloid leukemia at a number of patients hospitalized in Coltea Hematology between 2013-2014. The tests taken in question were carried out in the Laboratory of Coltea Hematology Departament with BD FACS CANTO II equipment. IVD optimized reagents were use. The immuno-phenotype has been identified to debut in bone marrow samples by means of marking, lyse, washing, acquisition, analysis.

Acute promyelocitic leukemia with t(15;17)(q22;q12) often has the following phenotype: CD34 negativ or only partially positive, HLA-DR negative or only partially positive, CD11b negative, CD13 heterogeneous, CD117+, CD33+, CD15 negative or only partially positive. Recently, a similar CD34- HLA-DR negative phenotype has been described in a subset of AML with myeloblasts, normal cytogenetics and

FLT3 gene internal tandem duplication. Additional phenotype include aberrant expresion of CD56+ and Cd123+.
Conclusion: flow cytometric immunophenotyping is an important diagnosis tool, but is they lack specificity and sensitivity for the detection of somme cytogenetic and molecular genetics abnormalities in nonpromyelocytic leukemias.

P4. **Sperm chromatin structure assay by flow cytometry**

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Introduction. Sperm chromatin structure assay (SCSA) is a reliable test for sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) and offers information predictive for male infertility, including for those patients with normal semen parameters that would otherwise be assigned with idiopathic infertility. SCSA also indicates whether the changes are reversible, thereby assisting clinical decisions. A high technical variability comes with a worldwide use of such a tool, therefore there is need for further studies validating clinically useful threshold values. Additional information may be provided by the complex SCSA assay, with potential implications on male infertility diagnosis and associated therapy options.

Materials and methods. Semen samples from 35 patients referred to the Origyn Fertility Center, Iasi, Romania (October 2013-January 2014), were SCSA tested. DFI, HDS and three additional FCM-derived parameters were evaluated: CVcomp (CV% of the signal from compact sperm DNA), %mod, and %high (% of sperm with fragmented DNA, having a moderate/ high fluorescence). Samples were processed based on a standardized protocol (SCSA Diagnostics Inc.). Internal assay validation (intra- and inter-assay coefficients of variation of 5,57 and 8,02 %, respectively) was performed on a FACS AriaIII (BD Biosciences) flow cytometer. The FlowJo (TriStar Inc.) software was used for FCM data processing.

Results. Semen samples from normozoospermic subjects (n=12) showed a statistically decreased CVcomp (p=0.019) when compared with those with an altered spermogram, while DFI and HDS were variable. Other significant data showed an association between high (>11) values of CVcomp with a decreased %high (p=0,045), a male/mixed-related cause of infertility (p=0,034), and a declared patient exposure to heat (p=0,047). There was a negative correlation between DFI and vitality (r=-0.76, p=0.021) and mobility (r=-0.53, p=0.028). No other statistically significant data were obtained for SCSA parameters evaluated.

Conclusions. Studies on larger cohorts are necessary to confirm these findings. Some of the patients within the study group are in the process of pending for clinical decision, therefore, there is still too early for correlation with the outcome of assisted reproductive procedures.

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P5. **The role of *Pseudomonas aeruginosa* culture fractions and purified quorum sensing signaling molecules on human mesenchymal stem cells morphophysiology**

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Mesenchymal stem cells (MSCs), the symbol of regenerative medicine, are a major tool for the therapy of lung damaging diseases. The opportunistic pathogen *Pseudomonas aeruginosa* is the most encountered etiologic agent of lung

infections, severely affecting patients with cystic fibrosis and also representing the main hitch in the damaged tissue repair and lung transplant related complications.

The aim of this study was to investigate the effects of *P. aeruginosa* culture fractions and purified quorum sensing signaling molecules (QSSMs) on human MSCs death signaling pathways and cytokine profile. The bone marrow isolated MSCs, incubated for different periods of time with one of the seven *P. aeruginosa* PAO1 culture fractions or QSSMs (low density whole cultures, heat inactivated bacterial cultures sediments, sterile supernatants, N-(3-Oxododecanoyl)-L-homoserine lactone (OdDHL), N-butanoyl-L-homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) and 2-heptyl-4-quinolone (HHQ)) were subjected to the following assays: i) fluorescence microscopy evaluation of cellular morphology and viability; ii) flow cytometry analysis of apoptotic/necrotic MSCs, iii) apoptosis related genes expression analysis by qRT-PCR; iv) quantification of the level inflammatory cytokines released in the MSCs supernatants determined by ELISA. Results were statistically analyzed using the GraphPad In Stat software. The PAO1 whole cultures exhibited the most relevant influences, impacting on MSCs morphology and viability, interfering with apoptotic pathways and significantly stimulating the production of IL-1β and IL-10, while decreasing the production of IL-6 and IL-8. Our results demonstrate that all tested QSSMs significantly impacted on several core signaling mechanisms of MSCs in a specific and time dependent manner. Even if all tested autoinducers interfered with the MSCs apoptotic genes expression, only OdDHL and HHQ significantly promoted MSCs apoptosis, this aspect being confirmed by the flow cytometry assay. These results demonstrate that *P. aeruginosa* infections may complicate the success of tissue repair by interfering with the normal function of the MSCs in the human body, and eventually, impair or abolish the success of the stem cells therapy.

P6. **Histone acetylation regulates endothelial differentiation of fetal stem cells**

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Introduction. Epigenetic changes in the genome include DNA methylation, histone modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation), and recently discovered miRNAs, three mechanisms that are often tightly linked in the regulation of gene expression and involved in many cellular processes. Histone acetylation was seen as a phenomenon correlated with an open chromatin conformation that allowed the expression of different genes involved in differentiation. Currently it has been observed that in acetylated state many genes are repressed and thus differentiation to a specific cell line is blocked, maintaining the pluripotent state.
Our aim was to investigate the role of histone acetylation in differentiation of endothelial progenitor cells.

Materials and methods. Characterization of EPCs was performed by flow cytometry and neovascularisation potential was achieved by western blot, qRT-PCR, wound-healing assay, matrigel assay.

Results. Flow cytometry analysis showed that histone acetylation reduces the expression of endothelial markers such as CD31, CD105, CD117, CD133, CD144, and VEGFR2. Furthermore, histone acetylation inhibited neovascularization *in vitro*, acting in the processes of proliferation, adherence, migration and in the formation of vascular network structures.

In conclusion, the discovering of acetylation patterns involved in the differentiation of stem cells to different cell types open new opportunities at the interface between chemistry and stem cell biology and can improve applications of stem cells in tissue engineering and regenerative medicine.

P7. **Assessment of perilipin expression by microscopy, flow cytometry and molecular biology techniques**

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Introduction: Perilipin is involved in the energetic metabolism as it functions as a lipid gatekeeper that controls access to of the lipases to the intracellular lipids in a phosphorylation-dependent manner. The expression of perilipin is correlated to the adipose phenotype and it is traditionally assessed as an adipogenic marker for mature adipocytes. In this context, the aim of our study was the *in vitro* genic and protein evaluation of perilipin in induced human adipose derived stem cells (hASCs) for developing knowledge regarding the adipogenesis kinetics. Additionally, the original adipogenic medium was designed to modulate the differentiation of hASCs in the view of tissue engineering applications.

Materials and methods: Third passage hASCs were induced towards adipogenesis using an original differentiation medium. The protein expression of perilipin was qualitatively and quantitatively evaluated by immuno-cytofluorescence techniques, namely fluorescence microscopy and flow cytometry respectively. In this view, cells were fixed with paraformaldehyde, permeabilised and incubated over night at 4°C with the primary antibody, washed with PBS buffer, incubated with the secondary antibody labelled with rodamine, washed again and stained with DAPI. In order to evaluate the genic expression of perilipin the following procedures were performed: i) RNA extraction, ii) determination of RNA concentration, purity and integrity, iii) revers transcription and iv) qRT-PCR. Quantitative data were analysed using Prism 3.03 Software.

Results: Perilipin wasn't detected at any level at the initiation of the adipogenic process in hASCs. The first detection in genic expression of perilipin was registered after one week of adipogenesis, while the protein expression was detected for the first time at 10 days post induction. In addition, the expression of perilipin increased in time, during the adipogenesis, probably due to the increasing number of cells undergoing the process and to the increasing volume of the intracellular lipid deposits inside the mature cells.

Conclusions: The adipogenic medium formulated sustained the adipogenic process and could be used for further tissue engineering applications.

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C16. **Flow cytometry support in diagnosis of hypersensitivity etiology**

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Hypersensitivity (HS) is a common, clinical event that can have life threatening issue, every year responsible for death of people at any age, most of the time healthy and with rare predictive alerts. Causes of sever HS are multiple: vegetal, food, chemicals, insect stings and most of all medicine (including antibiotics, anesthetic drugs, anti-tumor chemotherapy, steroids...). The frequency of allergy is rising every year and is much more frequent in western countries raising the "hygiene hypothesis", with several possible mechanisms.

It is nowadays generally accepted that there are different types of HS: one NON immune HS (involving specially drugs and chemical) through pharmacological activities and the other ones, highly specific immune HS that correspond to a normal sensitization but a highly exaggerated response. From the immune HS we can clearly distinguish

- immediate hypersensitivities (IHS) occurring within 20 min of exposure, that involves Immunoglobulin E, and possibly some IgG on mast cells and basophils and
- delayed type hypersensitivity (DTH) that needs at least 2 to 3 days and involve T cells with antigen presentation.

Immune HS is a 2 step process, needing first a sensitization process and then the exacerbated crisis itself. The first step is most of the time silent and not diagnosed. The HS can only be diagnosed through effector crisis. There are many symptoms associated with HS. The most frequent are asthma, eczema, urticaria, rhinitis but also anaphylactic shock, sudden death, angio-oedema, Stevens-Jonhson, Lyell, Toxic necrotic epidermolysis syndrom. HS crisis are repeated at each new contact with most of the time increasing gravity but the first reaction can already be life threatening while other HS decline may with time.

Diagnosis of allergy etiology is frequently difficult. A strict inquiry should lead to possible candidates that must be confirmed. The most robust confirmation tools are clinical provocation tests through skin tests and possible challenges. This may be dangerous and is not always possible to perform safely. Dosage of immunoglobulin specific for allergen is a precious, objective and quantitative confirmation of the sensitization but does not cover all types of HS and are not absolutely predictive of the clinical risk. The most predictive, and safe tool for allergy is then the ex-vivo provocation tests by challenging effector cells with the possible allergens and Flow cytometry is ideal for this.

Cell tests for the DTH are then T cell activation tests challenged with allergens. The reading need 2 to 7 days incubation and the read out can be cell activation (CD25, HLA-DR...), cytokine production (IFN□, IL-17...) and cell proliferation. IHS cell tests are performed on peripheral blood basophils, with immediate (10-30 min). Few procedures have been proposed to identify basophils and the read out is measuring membrane expression of internal proteins externalized during degranulation

The diagnosis role of these tests are: confirmation of HS, identification of the allergen and its component involved, identification of possible cross-reacting allergens, well tolerated molecules and possible follow up on time, especially in case of spontaneous or therapeutic decline.

The aim of this talk will be to overview the different flow cytometry tools in diagnosis of immune HS with a special focus on IHS.

C17. **Basotest and techniques for immune function analysis**

Mattias Engele
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Abstract non available

C18. **Importance of flow cytometry to characterize the cellular state of microorganisms of industrial interest**

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Flow cytometry represents a remarkable investigative tool for microbiologists. Indeed, progress in resolution of cytometers one hand, and the emergence of new fluorescent markers on the other hand, make now possible to analyze microorganisms and viruses. Flow cytometry may be used in many areas of microbiology. The speed of analysis and results, and its statistical value, make it an ideal tool in the diagnostic field: for exemple, microbial contaminants in food can be made in maximum 24 hours for total germs, and 48h or 72h for specific germs like *Salmonella* against respectively 72 hours and 5-6 days by the conventional culture methods. This shortening response times is critical for companies from the food industries, allowing them to release the products earlier, thus reducing storage times.

Flow cytometry is also used in the field of environmental microbiology, the study of micro-algae or viruses water, or research of *Legionella* in cooling towers, as well as medical microbiology and basic microbiology with the study of cell cycle and molecular biology with the use of fluorescent proteins as a marker gene (GFP, RFP, YFP .....).

In my laboratory, we study the microorganisms used as tools for the food ferments or microorganisms of interest in white biotechnology (production of biomolecules for chemistry or

health). Flow cytometry allows in these cases to assess the physiological state of the cells.
- First by a global viability parameter that can be compared to cultivability on petri dish.
- But also by analysis of cytomic parameters such membrane integrity, membrane potential, membrane fluidity, intracellular pH, and level of oxidative stress.
- It is also possible to evaluate the expression of antigens at the cell surface in accordance with the progress of culture.
- We have also developed a measure of cell activity or vitality.

We characterize the cellular state of the microorganisms used in industrial processes to understand the mechanisms of cell damage caused by the culture conditions, and also in the case of ferments, by the processes of stabilization. Understanding these mechanisms can propose some change to the culture conditions and stabilization to minimize cell damage. Thus it is possible to improve the cell state for the maintenance of activity during fermentation, or harvest the cells in a cellular state that will enable better conservation and good ability to recovery growth after stabilization. It is this use of flow cytometry for the characterization of cellular state that is developed by using some examples on yeasts and bacteria.

Evaluation of cellular state by flow cytometry during fermentation

In winemaking, a first fermentation is performed by yeast: it must transform the entire grape sugar into ethanol (except sweet wines). In the great majority of cases, musts are now inoculated with selected yeasts. To facilitate their establishment and organoleptic expression, the level of contamination by wild yeasts should not be greater than 10^5 cells/mL. Flow cytometry is used to control the level of contamination before inoculating selected yeasts. In some cases, the fermentation proves slow, or does not end, leaving a residual abnormal sugar in wine. Flow cytometry is used to assess cell state of yeast during the fermentation, to measure cell viability, membrane potential and vitality, and so provide fermentation stops. These measures may also be performed on the yeast fermentation for the production of industrial ethanol. The vitality measurement is also used for lactic acid bacteria.

After the alcoholic fermentation, the malolactic fermentation is often required. This bacterial fermentation is not easy to master, and *Oenococcus oeni* bacteria grow slowly in the petri dishes in the laboratory. Cytometry will allow to detect and enumerate quickly these bacteria and to assess their cellular state to predict the start of malolactic fermentation in wine. We have shown that the *O. oeni* cellular state and its activity depend on his intracellular pH. The measurement of intracellular pH will therefore help to master this fermentation.

Using flow cytometry to predict the behavior of microbial starters after stabilization

The first example concerns a yeast cheese ripening. This yeast is produced and stored in liquid form at 4°C until use. Enumeration by culture petri dish is insufficient to predict the behavior of yeast in cheese. On the contrary, we have shown that the membrane potential is relevant for predicting recovery of activity of this yeast.

The second example concerns the interest of measuring membrane fluidity. We have shown that the cell membrane fluidity at harvest affect bacterial cells survival after lyophilization and storage. Membrane fluidity is strongly related to the composition of membrane fatty acids. The membrane fluidity was measured by fluorescence anisotropy, after adapting the flow cytometer for this measurement.

Then, flow cytometry reveals a very interesting and efficient tool for microbial studies and its use will grow in the years to come.

C19. **Flow cytometry applications in antimicrobial and antipathogenic activities investigation of *Amorpha fruticosa* essential oil**

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Introduction. Desert false indigo (*Amorpha fruticosa* L.) is an invasive alien species, its fruits contain essential oil rich in compounds known for their antimicrobial properties. The aim of the study was to investigate the antimicrobial and antipathogenic activity of *A. fruticosa* essential oil through microbiological and flow cytometry methods.

Materials and Methods. The essential oil was obtained by steam distillation of water and its chemical composition was identified by GC -MS. For testing the antimicrobial activity were studied reference and clinical microbial strains belonging to the species *Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and *Candida albicans*. The screening of antimicrobial activity was made by adapted disk diffusion method and the values of MIC (minimum inhibitory concentrations) were determined by the binary microdilution method in liquid medium. In order to investigate the antimicrobial effect by flow cytometry were used as parameters the cellular membrane integrity (propidium iodide) and the efflux pump activity (Nile red). The antipathogenic properties of *A. fruticosa* essential oil were studied by determining the adhesion capacity of microbial strains to inert and cellular substrate (microtitration method, modified Cravioto method).

Results. *A. fruticosa* fruits contain 0.57 % essential oil rich in δ-cadinene (20,09 %), γ-muurolene (12,79 %), α-muurolene (12,54 %) and γ-cadinene (7,86 %). The volatile oil of *A. fruticosa* showed an antimicrobial activity against all microbial strains studied at concentrations ranging from 1.41-22.5 mg/mL, being most active against Gram-positive strains. The analysis on the essential oil influence on cellular membrane integrity by flow cytometry led to comparable results with those obtained by binary microdilution method in liquid medium and the efflux pumps' activity was disrupted in the presence of essential oil inhibitory concentrations. The *A. fruticosa* essential oil inhibited the microbial adhesion at concentrations ranging from 0.7-22.5 mg/mL, and at sub-inhibitory concentrations, the adhesion index decreased from 96.504 % to 36.932 %.

Conclusion. The antimicrobiological analysis concerning the investigation of the antimicrobial and antipathogenic activity of the *Amorpha fruticosa* essential oil showed microbial growth inhibition and the capacity of tested strains to colonize different substrates. The flow cytometry confirmed the antimicrobial effect of the essential oil to the proper concentration of the MIC value, indicating the disruption of the efflux pump activity as possible mechanism of action. In conclusion, the *A. fruticosa* essential oil may represent an alternative or aid to antibiotics therapy for infections caused by resistant and adhered microorganisms to various substrates and the flow cytometry can be a valuable tool in investigating these properties in real time.

C20. **Evaluation by microbiological and flow cytometry methods of antifungal activity of cinnamon essential oil incorporated in alginate**

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Introduction: Due to the numerous active biological properties, including antimicrobial activity, Cinnamon essential oil (SCO) has many uses in the pharmaceutical, medical and food industry. However, practical applications of SCO are limited due to the volatility of the active components. To overcome this limitation, we developed SCO embedded in alginate beads. This system represents both a way of protection and the controlled release of active the principles. Alginates (Alg) are natural polymers, with hydrophilic and biocompatible properties, and because of their ability to form hydrogels (by reaction with polyvalent metal cations) they are very often used as controlled release systems for

therapeutic agents. The purpose of this study was to obtain alginate beads embedded with SCO and to demonstrate antimicrobial activity of the obtained system by microbiological and flow cytometry classical methods.

Material and methods: Chemical composition of the cinnamon essential oil (*Cinamomum cassia L.*) used in the experiment (provided by COZAC PLANT SRL, Bucharest, Romania) was analyzed by GC- MS. Antimicrobial properties of SCO were evaluated against Gram-positive, Gram-negative bacteria and microfungi by disk diffusion and the successive binary microdilution methods. Antimicrobial activity results obtained for *Candida albicans* ATCC 10231 strain have been correlated with results obtained from flow cytometry method (assessed at 3h, 6h and 24h of incubation), using the parameters of cell membrane integrity (viable cells) and permeability (cell death). The cytotoxic effect of cinnamon essential oil was evaluated using HEp-2 cell line, by CellTiter (cell viability) and flow cytometry (cell cycle quantification) methods.

Manufacturing of beads (AlgSCO) was achieved by emulsion extrusion method. Different volumes of SCO (10, 20, 50, 100 and 200 µL) were embedded in the sodium alginate solution (3%) and the obtained emulsions were homogenized by magnetic stirring (for 15 min. at 40°C and 300 rpm). Beads formation was possible by cross-linking emulsion in $CaCl_2$ solution (5%). Quantification of the antifungal activity against *Candida albicans* ATCC 10231 was performed by three methods, spectrometry, determination of colony forming units (CFU) and flow cytometry.

Results: GC-MS analysis revealed that SCO contains cinnamaldehyde (86.27%), cinnamic acid (1.93%), o-methoxycinnamic aldehyde (2.57%) and other compounds in concentrations of below 1%. All tested strains were susceptible to SCO, correlating the results obtained for antifungal activity by classical microbiological methods with the results obtained by flow cytometry we observed changes of membrane integrity, occurring after 24 h of incubation in the presence of SCO. SCO toxicity at dilutions higher of 1:3,000 is reduced but the results showed a a noticeable change in the G2 phase of the cell cycle. Among all beads variants obtained, only AlgSCO-100 µL and AlgSCO-200 µL variants demonstrated antifungal activity, confirmed by all three methods used. Antifungal activity quantification by flow cytometry proved the advantage of reduced analysis time and the possibility to make certain determinations in temporal dynamics.

Conclusions: The SCO has a broad spectrum antimicrobial action and low toxicity at decreased concentrations. The inhibitory activity of the SCO can be attributed to membrane changes, according to analysis by flow cytometry. Alg beads with higher concentration of SCO show great antifungal activity, demonstrating the possibility of subsequent use for the development of new biomaterials with antifungal properties.

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