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Immunophenotyping of the Blast Cells in Correlations with the Molecular Genetics Analyses for Diagnostic and Clinical Stratification of Patients with Acute Myeloid Leukemia: Single Center Experience

Irina Panovska-Stavridis
*University Clinic of Hematology, Skopje
Republic of Macedonia*

1. Introduction

1.1 Acute leukemias

Acute leukemias are a heterogeneous group of malignancies that result from the malignant transformation of immature hematopoietic cells followed by clonal proliferation and accumulation of the transformed cells. They are characterized by aberrant differentiation and maturation of the malignant cells, with a maturation arrest and accumulation of more than 20% of leukemic blast in the bone marrow (Lichtman et al., 2010).

The natural history of acute leukemia and the response to therapy varies according to the type of blast involved in the leukemic process. Although in many instances the lineage assignment of the different types of blast cells may be recognized by simple morphological and cytochemical stains, it is necessary to employ immunological analyses with monoclonal antibodies and cytogenetic or molecular biological techniques to identify their particular differentiation features (Haferlach et al., 2007).

Acute leukemias are primarily characterized according to their differentiation along the myeloid and lymphoid lineage and they are divided into two main groups: acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). In 10% to 20% of patients, the leukemic cells have characteristics of both myeloid and lymphoid cells (Lichtman et al., 2010).

The classification of the acute leukemias underwent many changes in recent years. The French-American-British (FAB) classification of AML and ALL was based on cytomorphological and cytochemistry details only. Since then, the diagnostic of acute leukemias had undergone a complete change and the routine diagnostic work-up incorporated immunophenotyping by multiparameter flow cytometry, classical cytogenetics, molecular cytogenetics (comprising diverse fluorescence in situ hybridization techniques and comparative genomic hybridization) and molecular genetics (mostly polymerase chain reaction (PCR)-based techniques and sequencing) (Bennet et al., 1997; First MIC Cooperative Study Group, 1985; Haferlach et al., 2007).

According to the proceedings in diagnostic methods and the improved understanding of the diversity of acute leukemia subtypes, the latest World Health Organization (WHO) classification of acute leukemias incorporates and interrelates morphology, cytogenetics, molecular genetics and immunologic markers and pays major attention on the importance of genetic events in the classification, prognosis and therapy of the AMLs. Its prognostic relevance is most clearly demonstrated in the AMLs characterized by recurrent chromosome translocation: t(15;17), t(8;21) and inv(16)/t(16;16) which generally have a favorable prognosis when treated with appropriate therapeutic agents. All other genetic events identified among AMLs had strong prognostic meaning but did not influence on the therapeutic decision (Swerdlow et al., 2008).

In the WHO classification of precursor B-cell and T-cell neoplasms, immunophenotyping of the malignant cells plays a decisive role in the diagnosis, prognosis and clinical stratification of patients (Swerdlow et al., 2008).

Correct diagnosis of the diverse subtypes of AML and ALL play a central role for individual clinical risk stratification and therapeutic decisions.

1.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is one of the most common types of leukemia in adults. It is characterized by limited myeloid differentiation of the malignant cells. The malignant cells characteristically undergo maturation arrest at the level of the early myeloblast or promyelocyte, although varying proportions of mature hematopoietic cells are leukemia derived. The cells that display myeloid markers include morphology, Auer rods (aberrant primary granules), cytochemistry (Sudan black, myeloperoxidase, or nonspecific esterase), and cell surface antigens (Lichtman et al., 2010).

AML encompasses a family of hematologic malignancies that can be categorized according to their cytogenetic and associated genetic abnormalities, which have major prognostic importance. During recent years, considerable progress has been made in deciphering the molecular genetics and epigenetic basis of AML and in defining new diagnostic and prognostic markers. A growing number of recurring genetic changes have been recognized in the new WHO classification of AML. Furthermore, novel therapies are now being developed that target some of the genetic lesions and the treatment increasingly is being individualized by prognostic groups, with a goal of developing treatment tailored to the molecular basis of the patient's malignancy (Swerdlow et al., 2008).

1.2.1 WHO classification of AML

The current WHO classification of AML reflects the fact that an increasing number of new clinico-pathogenetic entities of AML are categorized based upon their underlying cytogenetic or molecular genetic abnormalities (Swerdlow et al., 2008). A number of recurrent genetic abnormalities are adequately defined and are recognized as entities of AML. The subgroup "AML with recurrent genetic abnormalities" is comprised of entities that are defined with seven recurrent balanced translocations and inversions, and their variants. Two entities from this group: "AML with t(8;21)(q22;q22); AML1/ETO" and "AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBF β /MYH11" are considered as AML regardless of bone marrow blast counts. In "APL with t(15;17)(q22;q12); PML/RAR α ," RAR α translocations with other partner genes are recognized separately. The former category "AML with 11q23 (MLL) abnormalities" is redefined in "AML with

t(9;11)(p22;q23); MLLT3/MLL”, and now is a unique entity. Three new cytogenetically defined entities also are incorporated: “AML with t(6;9)(p23;q34);DEK-NUP214”, “AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1”; and “AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1,” a rare leukemia most commonly occurring in infants (Vardiman et al., 2008).

Moreover, two new provisional entities defined by the presence of gene mutations between the group of cytogenetically normal AML (CN-AML) were added, “AML with mutated NPM1 (nucleophosmin),” and “AML with mutated CEBPA [CCAAT/enhancer binding protein(C/EBP), alpha].” There is growing evidence that these two gene mutations represent primary genetic lesions (so-called class II mutations), that impair hematopoietic differentiation, and when present alone in AML have favorable prognostic meaning. Mutations in the FMS-related tyrosine kinase 3 (FLT3) gene are found in many AML subtypes and are considered as class I mutations conferring a proliferation and/or survival advantage. AML with FLT3 mutations are not considered as a distinct entity, although determining the presence of those mutations is recommended by WHO because they have prognostic significance. The former subgroup termed “AML with multilineage dysplasia” is now designated “AML with myelodysplasia-related changes.” Dysplasia in 50% or more of cells, in 2 or more hematopoietic cell lineages, was the diagnostic criterion for the former subset (Schhlenk et al, 2008; Vardiman et al.,2008)

However, the clinical significance of this morphologic feature has been questioned. AMLs are now categorized as “AML with myelodysplasia-related changes” if (1) they have a previous history of myelodysplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasm (MDS/MPN) and evolve to AML with a marrow or blood blast count of 20% or more; (2) they have a myelodysplasia-related cytogenetic abnormality; or (3) if 50% or more of cells in 2 or more myeloid lineages are dysplastic (Swerdlow et al., 2008).

“Therapy-related myeloid neoplasms” has remained a distinct entity; however, since most patients have received treatment using both alkylating agents and drugs that target topoisomerase II for prior malignancy, a division according to the type of previous therapy is not often feasible. Therefore, therapy-related myeloid neoplasms are no longer subcategorized. Myeloid proliferations related to Down syndrome are now listed as distinct entities (Döhner et al., 2010).

A previous FAB classification is recognized in WHO classification as the entity AML, not otherwise specified. In this subgroup one can find the morphologic separation of AML according to the immaturity of leukemic cells as well as according to the hematopoietic lineage involved. This subtype of AML is reserved for the patients without the known cytogenetic or molecular genetic abnormalities. In some of them, there are markers associated with prognostic significance (Swerdlow et al., 2008; Vardiman et al, 2008).

The rare forms of AML and acute leukemia of ambiguous lineage recognized in WHO classification are also associated with poor prognosis (Döhner et al., 2010; Vardiman et al., 2008).

1.3 Diagnostic procedures

The diagnosis of the diverse subtypes of AML is a major challenge for modern hematology. Modern therapeutic concepts of AML are based on individual risk stratification in diagnosis and during follow-up. In the 1970s cytomorphology and cytochemistry represented the only available diagnostic tools. Nowadays, the routine diagnostic setting is completely changed

and it consists of classic cytogenetics, molecular cytogenetics, molecular genetics and immunophenotyping by multi-parameter flow cytometry (Döhner et al., 2010).

1.3.1 Cytomorphology

First steps in the diagnostic work-up of a patient with suspected AML is a morphological evaluation of a classical bone marrow aspirate and a peripheral blood smear by using a May-Grunwald-Giemsa or a Wright-Giemsa stain. It is recommended that at least 200 leukocytes on blood smears and 500 nucleated cells on marrow smears to be counted. For a diagnosis of AML, a marrow or blood blast count of 20% or more is required, except for AML with t(15;17), t(8;21), inv(16) or t(16;16), and some cases of erythroleukemia. Myeloblasts, monoblasts, and megakaryoblasts are included in the blast count. Erythroblasts are not counted as blasts except in the rare instance of pure erythroid leukemia (Bennet et al,1997; Döhner et al., 2010; Panovska-Stavridis et al., 2008).

1.3.2 Cytochemistry

Lineage involvement could be identified with cytochemistry by using myeloperoxidase (MPO) or Sudan black B (SBB) and nonspecific esterase (NSE) stains. Detection of MPO (if present in > 3% of blasts) indicates myeloid differentiation, but its absence does not exclude a myeloid lineage because early myeloblasts and monoblasts may lack MPO. SBB staining parallels MPO but is less specific. NSE stains show diffuse cytoplasmic activity in monoblasts (usually 80% are positive) and monocytes (usually 20% positive). In acute erythroid leukemia, a periodic acid-Schiff (PAS) stain may show large globules of PAS positivity (Bennet et al,1997; Döhner et al., 2010, Panovska-Stavridis et al., 2008).

1.3.3 Immunophenotyping

Application of immunophenotyping together with the cytomorphology and cytochemistry has a crucial role in the initial diagnosis of all cases with a suspected or proven diagnosis of acute leukemias. Immunophenotyping allows the discrimination of different cell population on the basis of their size, granularity, and antigen expression patterns. Flow cytometry is a powerful technology for characterization and analysis of cells. It simultaneously measures and analyzes multiple physical characteristics of single particles, usually cells, as they move in a fluid stream through a beam of light through an optical and/or electronic detection apparatus. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5nm to 40nm diameter (Panovska-Stavridis et al., 2008).

The applied methodology detects cell surface antigens in a suspension of viable cells and cytoplasmic and nuclear antigens in previously fixed and stabilized cell suspension with the application of monoclonal antibodies conjugated with different fluorochromes. It permits simultaneous detection (multiparameter analyzes) of more than two membrane and nuclear or cytoplasmic antigens by means of double or multiple immunostaining (Bain et al., 2002; Bene et al., 1995; Döhner et al., 2010; Panovska-Stavridis et al., 2008).

1.3.4 Cytogenetics

Chromosome abnormalities are detected in approximately 55% of adult AML. Conventional cytogenetic analyses are part of the standard diagnostic approach of a patient suspected with AML. This allows the identification of genetics entities that deserve targeted treatments

like acute promyelocytic leukemia (APL). Also, it allows the distinction of the disease with widely different prognosis. For example AML t(8;21)(q22;q22) with favorable risk versus AML abn 3q26 with adverse risk (Döhner et al., 2010; Grimwade D., 2001).

1.3.5 Molecular genetics

Numerous genetic abnormalities that escape cytogenetic detection like gene mutations and gene expression abnormalities are more recently discovered among CN-AML. Molecular diagnosis by reverse transcriptase- polymerase chain reaction (RT-PCR) for the frequent gene fusions, such as AML1/ETO, CBF β /MYH11, MLLT3/MLL, DEK/NUP214, can also be useful in certain circumstances. RT-PCR, for which standardized protocols are already published, is also an excellent option to detect recurrent cytogenetic rearrangements, if chromosome morphology is of poor quality, or if there is typical marrow morphology but the suspected cytogenetic abnormality is not present. (Gabert et al., 2003; Beillard et al, 2003).

1.4 Prognostic factors

Prognostic factors of an AML case may be subdivided into those related to patient characteristics and general health condition and those related to characteristics particular to the AML clone. The former subset usually predicts treatment-related mortality (TRM) and becomes more important as patient age increases while the latter predicts resistance to, at least, conventional therapy.

1.4.1 Patient-related factors

Age, comorbidities, performance status and genetic variation in the drug metabolism are the main prognostic factors related to patients with AML. Increasing age is an important independent adverse prognostic factor (Appelbaum et al., 2006). Nonetheless, calendar age alone should not be a reason for not offering potentially curative therapy to an older patient because age is not the most important prognostic factor for either TRM or resistance to therapy. Currently all patients under the age of 60 are candidates to receive standard intensive chemotherapy and according to prognostic factors stem cell transplantation or intensive chemotherapy as postremission therapy. It has to be stressed that age as a factor is not only dependent on so-called „calendar age“. Recently many older patients with a good clinical status have been successfully treated with intensive chemotherapy. Attention should be given to a careful evaluation and documentation of comorbidities. Comorbidity scoring is a current field of investigation and should contribute to a better definition of the patient considered “unfit” for intensive chemotherapy (Piccirillo et al., 2004; Sorrow et al., 2005).

1.4.2 AML-related factors

According to the AML working party of European Leukemia Net, several important and independent prognostic factors have been recognized: white blood cell counts, existence of prior MDS or AML with MDS features, previous cytotoxic therapy for another malignancy, and cytogenetic and molecular abnormalities in leukemic cells (Döhner et al., 2010).

1.4.2.1 Cytogenetics

Chromosome abnormalities are detected in approximately 55% of adult AML. Although, there is a diversity of cytogenetic entities of AML, the karyotype of the leukemic cells is the strongest prognostic factor for response to induction therapy and for survival for AML patients

(Swerdlow et al., 2008). Younger adult patients are commonly categorized into 3 risk groups, favorable, intermediate, or adverse. The favorable group is represented by the reciprocal translocations $t(15;17)/PML/RAR\alpha$, $t(8;21)/AML1/ETO$, and $inv(16)/CBF\beta/MYH11$, which are associated with a favorable prognosis. In contrast, AML associated with $t(9;11)(p22;q23)$ shows an inferior prognosis. These subgroups represent the first hierarchy of the WHO classification of AML, emphasizing that these subtypes represent distinct biologic entities. The second subgroup of AML patients shows a normal karyotype and an intermediate prognosis. However, from molecular aspects, this subgroup is very heterogeneous. Lately, there are emerging data suggesting that two genetic entities from this group, the AML with mutation of NPM1 gene without FLT3 mutations and AML with mutation in CEBPA gene should be moved from the intermediate prognosis group to the favorable group of AML. Those two entities are also added to the new WHO classification (Schlenk et al., 2008). The third (prognostically unfavorable) subgroup includes mostly unbalanced karyotypes characterized by a gain or loss of larger chromosomal regions. Within these, an especially complex aberrant karyotype, which occurs in 10% to 12% of patients and that is defined by ≥ 3 chromosomal anomalies shows a very unfavorable prognosis. Cytogenetics is further helpful to delineate patients with therapy related AML (t-AML), who are classified as third hierarchy in the WHO classification, and developed either after treatment with alkylating agents often associated with cytogenetic aberrations involving 5q-, -7, or p53 and complex aberrant karyotype or are showing MLL/11q23 or other balanced cytogenetic aberrations which are in frequent association to previous treatment with topoisomerase II inhibitors. One striking observation is the increasing incidence of adverse versus favorable cytogenetic abnormalities with increasing age. This, at least in part, contributes to the poorer outcome of AML in older adults. (Byrd et al., 2002; Schlenk et al., 2008, Swerdlow et al., 2008)

1.4.2.2 Molecular genetics

Nowadays, considerable progress has been made in elucidating the molecular pathogenesis of acute leukemias that resulted in identification of new molecular diagnostic and prognostic markers. Gene mutations and deregulated gene expression have been identified that allow us to interpret the genetic diversity within defined cytogenetic groups, in particular the large and heterogeneous group of patients with CN-AML. Risk stratification by molecular markers in patients from the former group of AML plays an increasing role at diagnosis. The most relevant markers, which can be detected alone or in coincidence with other mutation are NPM1 mutations that is detected in approximately 40% of cases, MLL-PTD in 6%, NRAs in 8-10%, CEBPA in 10% and FLT3-TKD mutations in 6% of CN-AML. Thus, a rather of limited number of markers further subclassifies more than 85% of CN-AML. Data from the literature suggest that those markers have different prognosis regarding the outcome of the disease and they all indicate that in the near future molecular screening may allow “targeted allogeneic stem cell transplantation (alloSCT)” in AML patients with normal karyotype (Schlenk et al., 2008; Koreth et al, 2009).

There is a growing list of the new genetic abnormalities with clinical value that are being investigated. These genetic events perturb diverse cellular pathways and functions, and they often confer a profound impact upon the clinical phenotype of the disease and treatment response.

It is anticipated that advances in molecular technology will reveal additional markers that will result in more precise classification of this heterogeneous complex of disorders (Löwenberg B., 2008b; Haferlach T., 2008)

1.5 Treatment

The parallel progress of the development of the diagnostic techniques improvement of the classification and therapeutical approach lead acute leukemia which were for the first time described before 150 years, and in the 1970s were still fatal disease nowadays to have overall 5 years survival rates of up to 40%. (Appelbaum et al, 2001).

Despite heterogeneity of the disease, with the exception of acute promyelocytic leukemia, this disease has been treated with a “one size fits all” approach. Although, the vast majority of AML patients can be individually characterized on the basis of the distinct chromosomal aberrations and molecular markers, treatment of AML is still based on quite unspecific cytotoxic therapy. For almost 40 years, the use of continuous infusion of cytarabine combined with another agent, usually an anthracycline, the “3+7” regimen, has been the mainstay of therapy (Yates et al, 1973). Response rates for induction with standard chemotherapy ranged from 70% to 80% for adults aged less than 60 years which are enrolled in clinical trials and average in 50% for patients older than 60 years (Lichtman et al.,2010) .

Alternative consolidation therapies by applying additional chemotherapy, autologous stem cell transplantation (autoSCT) or alloSCT based on the initial cytogenetic and molecular studies are available (Cornelissen J.J.et al.(2007); Fernandez H.F.,2010; Koreth et al, 2009)

As more sophisticated molecular techniques have become available, it is clear that it is still possible to detect residual disease when all morphological and functional criteria for remission are met. Techniques such as “real-time”-quantitative polymerase chain reaction (RQ-PCR) are capable of detection at a level of 1 in 10^4 or 1 in 10^5 residual cells, but such markers are available for only a minority of cases in which the molecular lesion has been characterized(Freeman et al., 2008).

AlloSCT is the most effective antileukemic modality that is characterized by immune mediated graft-versus-leukemia effect of the transplanted cells that reduce the risk of relapse considerably and improve the relapse-free survival but also is associated with the increased risk of death and morbidity. Therefore, the alloSCT advantage has to be carefully balanced against the excess mortality (ranging between 10% and 40%) and morbidity due to transplant-related complications, such as infection and graft-versus-host disease that are typically connected with alloSCT and can diminish all of the benefit of a reduced risk of relapse. (Cornelissen et al., 2007). For this reason, allogeneic stem cell transplantation is usually avoided in a type of AML that has a pattern of cytogenetics with a relatively favorable prognosis, such as AML with the chromosomal translocations $t(8;21)$ or $inv(16)/t(16;16)$ (Marcucci et al., 2000; Perea et al.,2006). In the latter subtypes the risk of relapse is in the order of 35% to 40% or less. By contrast, a transplant is treatment of choice for all other patient whose leukemia cells bear a cytogenetic or molecular abnormality that predicts a high or intermediate risk of relapse after chemotherapy (Löwenberg et al., 2008b; Koreth et al, 2009). Exception could be done for two additional genotypically defined subsets of AML that are categorized as low-risk within the large category of CN-AML. Each of those entities has a risk of relapse of about 35%. The first genotype is defined by the presence of ‘favorable’ mutations in *NPM1* and the absence of concurrent ‘unfavorable’ *FLT3*-internal tandem duplications (*NPM1*mut /*FLT3*-ITDneg).⁶¹ This genotype accounts for approximately 16% of all newly diagnosed patients younger than 60 years old. There is no demonstrable benefit from transplantation in patients with *NPM1*mut/*FLT3*-ITDneg AML. The second subset of AML with ‘favorable’ mutations in the transcription factor gene *CEBPA* (*CEBPA*mut) could not be analyzed in this way because of a lack of statistical power due to a limited number of cases. The latter low-risk subtype *CEBPA*mut accounts for 8% of all AML. Nevertheless, the

available body of evidence suggests that these AMLs are unlikely to profit from an alloSCT (Schlenk et al., 2008).

Thus, it could be concluded that patients with AML with t(8;21), AML with inv(16)/t(16;16), AML with *NPM1*mut/ *FLT3*-ITDneg and AML with *CEBPA* mutations should not be considered for alloSCT. Nevertheless, it is important to stress that sufficient evidence so far exist only for the AML with recurrent cytogenetic abnormalities and the benefits for the two additional genetic low-risk AML entities should be validated in the larger studies in the future (Schlenk et al., 2008).

In the near future these results will also need to be considered more specifically in the light of the extended scale of allogeneic stem cell transplantation strategies with respect to reduced-intensity conditioning regimens and in relationship to different transplant sources and donor types (matched unrelated and haploidentical donors, umbilical stem cell grafts). (Löwenberg et al, 2008a)

2. Motivation and aim of the study

Correct diagnosis of the diverse subtypes of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) play a central role for individual clinical risk stratification and therapeutic decisions. Modern therapeutic concepts of AML are based on individual risk stratification at diagnosis and during follow-up. The ultimate test of any disease diagnostic algorithm approach is its usefulness in guiding the selection of effective treatment strategies. (Haferlach et al., 2007, Löwenberg et al, 2008b) As discussed above, cytogenetic as well as various genomic markers (gene mutations, gene overexpression) may provide input for algorithms for remission induction and post-remission treatment decisions. At the same time, it remains appropriate to realize that prognostic factors in fact remain a moving target and they are only relevant to therapies available at a given time. Algorithms that provide a basis for risk-adapted therapeutic choices may include immunological markers, cytogenetic factors, molecular markers as well as clinical parameters (e.g., age, attainment of an early or late complete remission) and hematological determinants (e.g., secondary AML, white blood cell count at diagnosis).

On the other hand, the more carefully AML is studied, the clearer it becomes that there is considerable heterogeneity between cases with respect to morphology, immunological phenotype, associated cytogenetic and molecular abnormalities and, more recently, patterns of gene expression. This is reflected in the substantially different responses to treatment. Some entities are becoming so distinct that they are regarded as different diseases with specific approaches to treatment.

In order to improve and simplify the diagnosis and management of AML patients that are diagnosed and treated at the at the University Clinic of Hematology-Skopje we conducted a prospective study to establish and standardize a diagnostic algorithm based on minimal screening tests which will facilitate risk adapted therapy for each single AML patient. The aims of our study were: first, to establish the correct lineage assignment of the blast cells, second, to evaluate the incidence of the favorable genetic markers PML/RAR α , AML1/ETO and CBF β /MYH11 among the AML cases, then to correlate the obtained results with the patient age, comorbidities, and performance status and consecutively to select the effective treatment strategy for each single acute leukemia patient.

3. Material and methods

3.1 Patients and samples

A total of 76 adult (>15 years) patients (from initially 77 tested) with acute leukemia who were consecutively admitted at the Clinic of Hematology-Skopje from January through December 2008 were enrolled in this study. The median age of the patients (41 men, 35 women) was 52 ± 18.66 years (range 16-80), and most of the patients 37 (48.7%) were between 55 and 75 years old. The diagnosis was made by standard morphological examination and cytochemical analyses of bone marrow smears according to the criteria established by the FAB Cooperative Study Group (Bennet et al, 1997) and confirmed by immunophenotyping of bone marrow aspirates and/or peripheral blood samples (Bain et al., 2002; Bene et al., 1995) following the criteria of the European Group for the Immunological Classification of leukemias (EGIL) and the British Committee for Standards in Hematology (BCSH) (Bain et al., 2002; Bene et al., 1995). Consecutively, patients were further stratified in the adequate genetic AML entities according to the results of the molecular analyses. The samples contained more than 20% of blast cells (most of which had more than 50%). All patients were tested for the presence of the fusion transcript of the mayor recurrent cytogenetic abnormalities in AML (PML/RAR α , AML1/ETO, CBF β /MYH11) by RT-PCR, according to standard procedures. (Gabert et al.2003; Beillard et al, 2003).

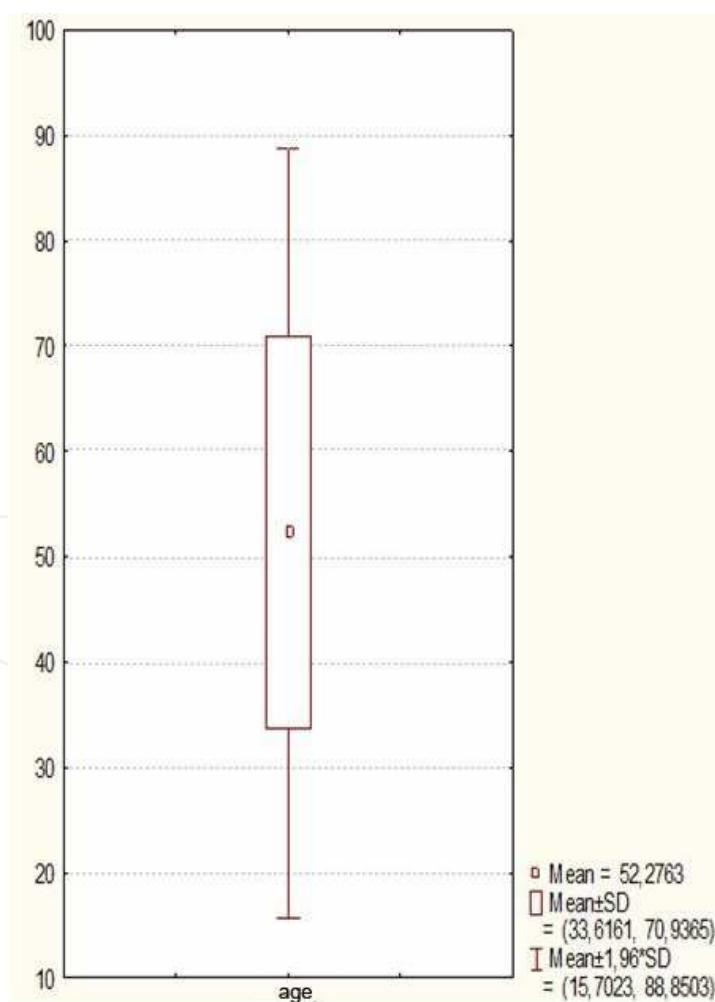


Fig. 1. Average age of the patients in the study group

3.1.1 Clinical data from the patients

Clinical data from the patients were collected according to the internal protocol that was approved by the University Clinic of Hematology Institutional Review Board. All included patients had to sign a written consent.

3.2 Morphology

The morphology was analyzed by microscopic examination of >500 nonerythroid cells on May Gruenwald Giemza stained air-dried bone marrow smears (Bennet et al,1997; Döhner et al., 2010; Panovska-Stavridis et al., 2008).

3.3 Cytochemical analysis

Air-dried bone marrow smears were stained for MPO, non specific esterase (NSE) and periodic acid-Schiff (PAS) according to the manufacturers guidelines. The percentage of positive cells for either stain was assessed by microscopic examination of 200 nonerythroid cells (Bennet et al, 1997; Döhner et al., 2010; Panovska-Stavridis et al., 2008).

3.4 Immunophenotyping

The multiparameter flow cytometry (MPF) was performed at the beginning at the Institute for Immunobiology and Human Genetics, Faculty of Medicine-Skopje and then continued at the University Clinic of Hematology-Skopje. Immunophenotyping was done first, by using Cytomation (DAKO-Cytomation) flow-cytometer and then by BD FACSCanto™ II analyzer, on whole blood and/or bone marrow specimens using lysing solutions (BD-Biosciences, San Jose.CA. USA)(Bain et al., 2002). We prepare the samples for simultaneous detection of three cytoplasmic/nuclear and membrane antigens. The first step involved immunostaining of cell suspension with multiple panels of tree monoclonal antibodies (McAb) labeled with fluorescein (FITC), phycoerythrin (PE) and phycoerythrin-Cy5 tandem complex (Pe-Cy5) as third color (Panovska-Stavridis et al., 2008). The slightly modified panel of monoclonal antibodies (McAb) against myeloid- and lymphoid-associated antigens as suggested by the EGIL was utilized (Bain et al., 2002; Panovska-Stavridis et al., 2008). The antibodies and their manufacturers are listed in Table 1.

	B-lineage	T-lineage	Myeloid markers	Non-lineage restricted
First line	CD19, cyt CD79b, cyt CD22	CD 2, CD7, cyt CD3	CD117,CD13, CD33,CD14, CD15, anti-MPO, anti-lisosome	TdT, CD34, HLA-DR, CD 56
Second line	SmIg (kappa/lambda)**, cytIgM ² ,CD138	CD1a,mCD3 ¹ , CD4,CD5,CD8, anti TCR α/β, anti TCR γ/δ***	CD41, CD61, CD42,CD71 Anti-glycophorin A	CD38

All markers were manufactured by BD-Biosciences, except antilysozyme (DAKO); 1.cyt: cytoplasmic; 2.m: membrane *MPO: myeloperoxidase;** SmIg:surface immunoglobulin;*** TCR:T cell receptor

Table 1. Panel of monoclonal antibodies (McAb) for diagnosis of acute leukemias

For the detection of cytoplasmic and nuclear antigens we used commercially available permeabilization/fixation solutions (FACS Permeabilization solution BDBiosciences; San Jose, CA, USA) (Bain et al., 2002).

We incubated 100ml of specimens (peripheral blood or bone marrow) and appropriate McAb for 15min at room temperature, then added the permeabilization solutions and/or lysing solution and repeated the incubation procedure. After the incubation we washed the samples three times with PBS-A, then re-suspended the cell with isotones solution and acquired the data. As control we used a lysed, but unstained sample (Panovska-Stavridis et al., 2008)

Acquired data were analyzed with software by using CD45 gating strategy (Borowitz et al, 1993). This technique involves incubation of all samples with fluorochrome - labeled CD45 McAb and with the McAb for which reactivity needs to be established with an alternative fluorochrome). In the initial step of the analyses, gating was set up on a CD45-positive versus light side-scatter dot plot. The procedure allowed the discrimination between the blast cell population and normal cells and the exclusion of platelets and debris. Thereafter, if necessary, it was possible to perform another gating, to separate the cells positive with the McAb under study (Borowitz et al, 1993). Leukemias were first screened by the primary panel and, if necessary, further characterized by the McAb of the secondary panel (Bain et al., 2002).

Antigen expression was considered positive if 20% or more blast cells reacted with a particular antibody, except reactivity of blasts cells with MPO. It was considered positive if 10% or more of mononuclear cells were MPO positive (Bain et al., 2002; Döhner et al., 2010).

3.5 Molecular analysis

Mononuclear cell preparation, RNA isolation, and cDNA synthesis were performed at the Department of Molecular Biology, Immunology and Pharmacogenetics, Faculty of Pharmacy-Skopje, according to standard procedures. Aliquots of 5 µL of cDNA (100 ng RNA equivalent) were used for Real-time Quantitative polymerase chain reaction (RQ-PCR) with primers and dual-labeled probes as described by Gabert et al. Positions and nucleotide sequences of the primers and probes are shown in Table 2.

The RQ-PCR reaction was performed in a 25-µl reaction volume using 12.5 µl (1x) Master Mix (Applied Biosystems), 300nM primers and 200nM probes on a Mx3005P^(TM) QPCR System (Stratagene) under the following conditions: 95°C for 10min, followed by 50 cycles of 95°C for 15s, 50°C for 1min. In order to correct variations in RNA quality and quantity and to calculate the sensitivity of each measurement, a control gene (CG) transcript was amplified in parallel to the fusion gene (FG) transcript. Since ABL (Abelson) gene transcript expression did not differ significantly between normal and leukemic samples, ABL was used as a control gene in this study (24). Positions and nucleotide sequences of the primers and probe are shown in Table 2.

3.6 Statistical analysis

Statistical analyses were performed by using the statistical analyses software SPSS 18.0 and by applying the Descriptive statistics (cross tabulation, frequencies, descriptive ratio statistics), bivariate statistics (means, t-test, correlation (bivariate, partial, distances), nonparametric tests and linear regression statistical methods. The Level of probability for obtaining the null hypothesis, in accordance with the international conventions for biomedical sciences was 0.05 or 0.01 (Armitage et al., 2002).

Transcript	EAC code ^a	Primer/probe localization, 5'-3' position (size)	Sequence
AML1 /ETO	ENF701	AML1, 1005-1026 (22)	5'-CAC CTA CCA CAG AGC CAT CAA A-3'
	ENR761	ETO, 318-297 (22)	5'-ATC CAC AGG TGA GTC TGG CAT T-3'
	ENP747	AML1, 1049-295 (30)	FAM 5'-AAC CTC GAA ATC GTA CTG AGA AGC ACT CCA-3' TAMRA
PML /RAR α	ENF905	PML, 1198-1216 (19)	5'-CCG ATG GCT TCG ACG AGT T-3'
	ENF906	PML, 1642-1660 (19)	5'-ACC TGG ATG GAC CGC CTA G-3'
	ENF903	PML, 1690-1708 (19)	5'-TCT TCC TGC CCA ACA GCA A-3'
	ENR962	RARA, 485-465 (21)	5'-GCT TGT AGA TGC GGG GTA GAG-3'
	ENR942	RARA, 439-458 (20)	FAM 5'-AGT GCC CAG CCC TCC CTC GC-3' TAMRA
CBF β /MYH11	ENF803	CBFB, 389-410 (22)	5'-CAT TAG CAC AAC AGG CCT TTG A-3'
	ENR862	MYH11, 1952-1936 (17)	5'-AGG GCC CGC TTG GAC TT-3'
	ENR863	MYH11, 1237-1217 (21)	5'-CCT CGT TAA GCA TCC CTG TGA-3'
	ENR865	MYH11, 1038-1016 (23)	5'-CTC TTT CTC CAG CGT CTG CTT AT-3'
	ENPr843	CBFB, 434-413 (22)	FAM 5'-TCG CGT GTC CTT CTC CGA GCC T-3' TAMRA
ABL	ENF1003	ABL, 372-402 (31)	5'-TGGAGATAAACACTCTAAGCATAACTAAAGGT-3'
	ENF1063	ABL, 495-515 (21)	5'-GATGTAGTTGCTTGGGACCCA-3'
	ENF1043	ABL, 467-494 (28)	FAM 5'-CCA TTT TTG GTT TGG GCT TCA CAC CAT T-3' TAMRA
^a ENF = forward primer, ENR = reverse primer, ENP = TaqMan probe			

Table 2. Sequences and positions of the RQ-PCR primers and probes

4. Results

In the period of twelve months, between January and December 2008, 77 patients were submitted and tested for acute leukemia at the University Clinic of Hematology-Skopje. Cyto-morphological analyses showed that the average rate of the blast cells in the differential blood counts and in the bone marrow was 54.6%(3-99.0%) and 73,5%(20-98.0%) respectively. In 7 patients blast cells were not detected initially.

4.1 Results from the cytochemical analyses

Results from the cytochemical analyses and the images of peripheral smears with positive examples from different cytochemical staining are presented at Figure 2 and Photo 1 respectively.

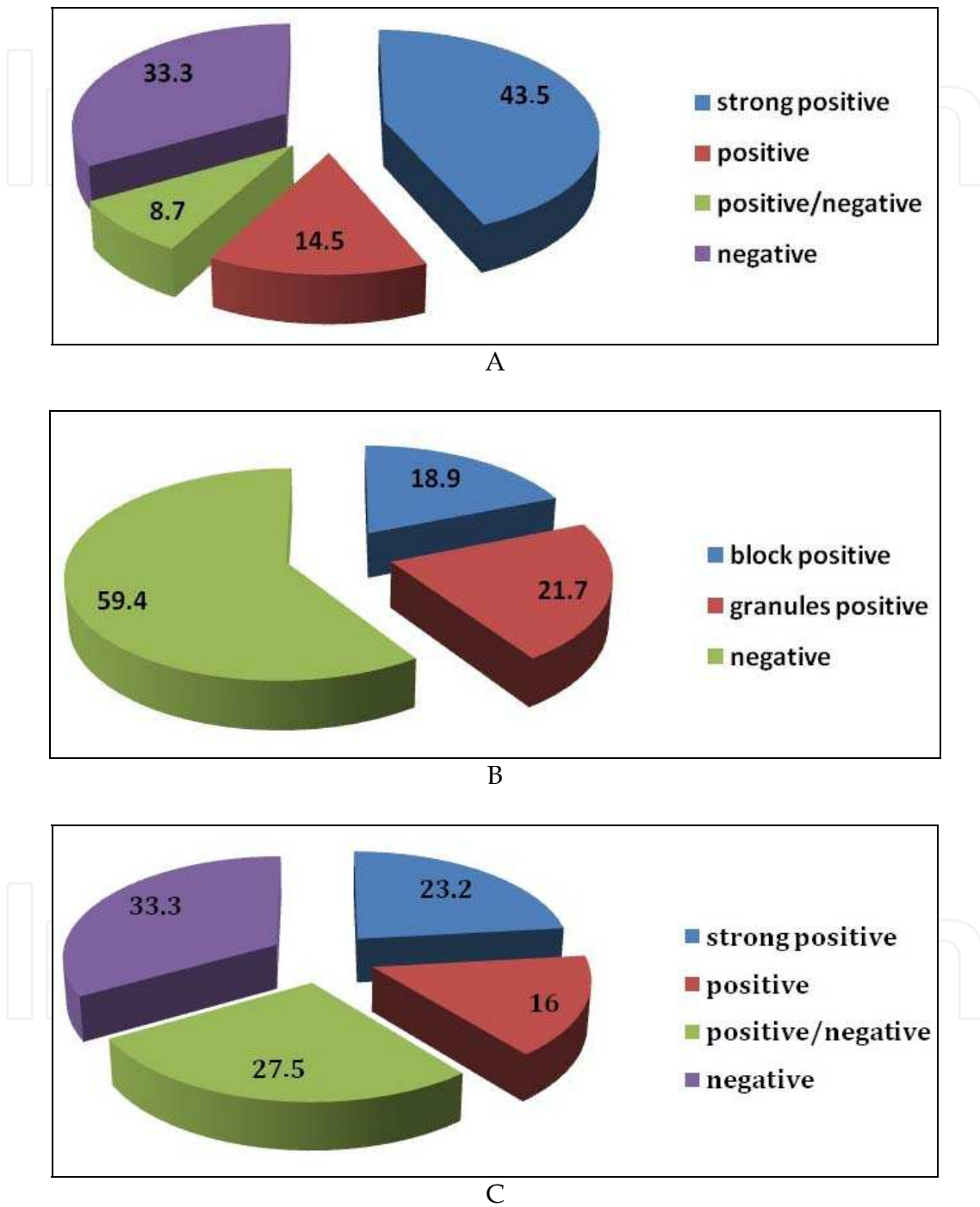
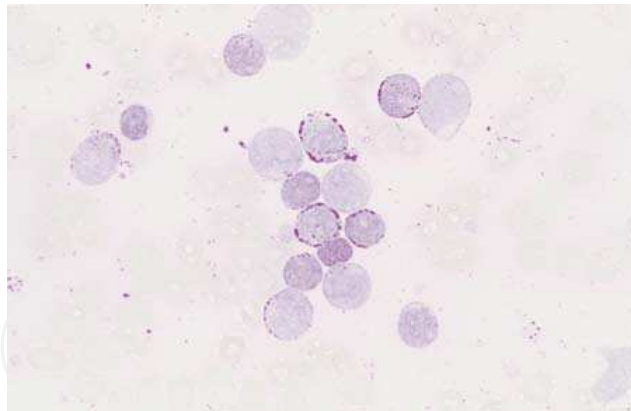
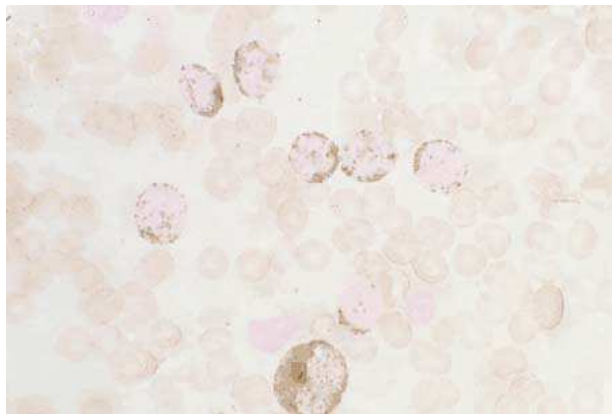


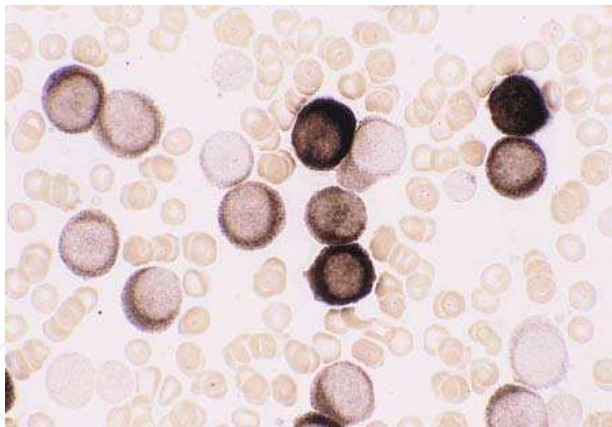
Fig. 2. Distribution of patients according to reactivity with different cytochemical staining. A: Patient distribution according to MPO reactivity of the blasts cells. B: Patient distribution according to PAS reactivity of the blasts cells. C: Patient distribution according to NSE reactivity of the blasts cells.



A



B



C

Photo 1. Images from positive reactivity of peripheral blast cells with different cytochemical staining , A: PAS reactivity, B: POX reactivity, C: NSE reactivity

Statistical analyses showed that there is a statistically significant correlation between the AML and MPO positivity (X^2 with Yates's correction=16.628 $p<0.01$)(Figure 2A). According to cross reaction ratio, MPO positivity presents statistically significant risk which improves the chance of AML diagnosis for 39 times (OR=392.1603 (4.177<OR<305.9796, CI 95%)). Also, a statistically significant correlation was noted between the PAS positivity and ALL (X^2 with Yates's correction=5.514 $p<0.01$)(Figure 2B). Strong PAS positivity was registered in 75% of ALL cases. Regarding the cytochemical stain NSE and AML no statistical correlation was

observed (χ^2 with Yates's correction=2.456 $p=0.117$), expect for AML M4 and M5 entities which showed statistically significant correlation ($p<0,05$) (Figure 2C).

Morphology and cytochemistry established myeloid lineage in 57 (74.0%) cases and lymphoid differentiation in 11 (14.3%) cases (Figure 2). Morphology and cytochemistry did not establish lineage involvement in 9 (11.7%) cases. Basic morphological and cytochemical analyses established the lineage assignment of the blasts cells in 68 (88.3%) patients.

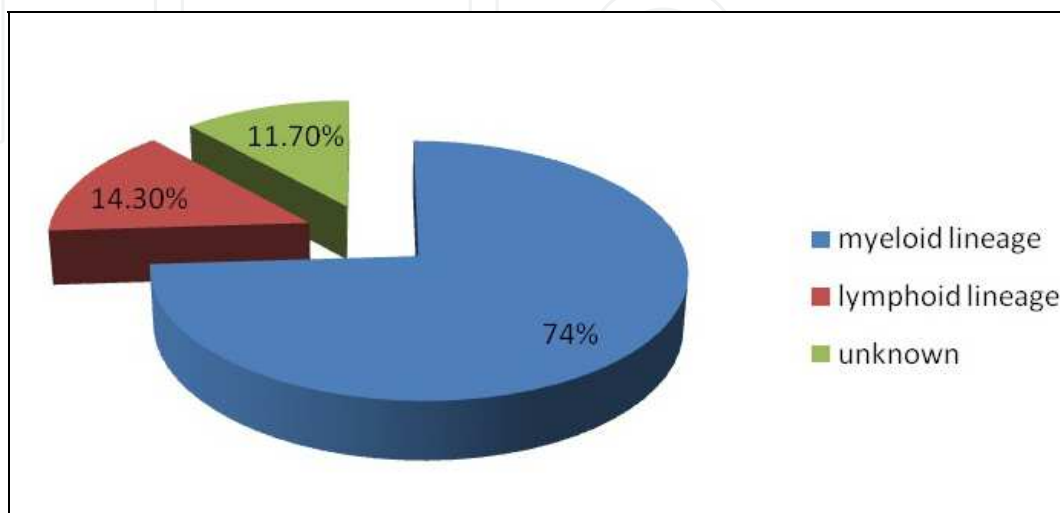


Fig. 3. Distribution of cases based on lineage assignment of blasts cells detected with basic morphological and cytochemical analyses

4.2 Results from the immunological analyses

Immunological analyses with multi-parameter flow-cytometry were performed in all 77 patients. Further immunological analyses of cases in which lineage could not be assigned based on morphology and cytochemistry established myeloid lineage in 4 patients, (AML-M0) and one case indicated nonhematopoietic malignancy. In this case, immunophenotype of the malignant cells (CD45-/CD56+/CD9+) indicated a neuroectodermal origin of the malignant cells (Bain et al., 2002). Later neuroblastoma was diagnosed in this patient. Immunological analyses change the assigned lineage based on morphology and cytochemistry in 3(3.8%) of the patients from lymphoid to myeloid. The results of our study showed that routine immunophenotyping improved diagnosis in 12 (15.5%) cases.

Consequently, based on FAB and immunologic criteria of EGIL and BTSH 64 acute leukemias were classified as myeloid. Correlation between the antigen expressions with FAB morphology of AML cases which confirmed the AML diagnosis in 83.1% of the cases is presented at Table 3. According to FAB criteria, the leukemias were classified as M0 (n=4), M1 (n=8), M2 (n=20), M3 (n=5), M4 (n=17), M4-Eo (n=1), M5 (n=8), M6 (n=1).

Multivariate Cox-proportional regression analyses showed that in 89.7% of AML cases lineage assignment is defined with the following five markers: CD13, CD33, CD117, HLA-DR and anti-MPO. Most frequently detected maturation myeloid marker which was expressed in 42.9% of AML case was CD15. Twenty three (35.9%) of AML patients showed expression of lymphoid antigens. Co-expression of two lymphoid markers was detected in eleven (17.1%) cases, and most frequently co-expressed lymphoid marker was CD7 and was detected in 28.5% of the cases.

	CD45	CD117	CD13	CD14	CD15	CD33	CD10	CD34	HLA-DR	MPO	Lysosime	TdT	CD2	CD7	CD19	CD56
Number of tested patients	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64	64
Number of positive patients (%)	63 (98.4)	53 (82.8)	57 (89.2)	17 (26.7)	28 (43.7)	57 (89.2)	5 (7.8)	40 (62.5)	55 (85.9)	46 (71.8)	30 (46.8)	8 (12.5)	10 (15.6)	18 (28.1)	3 (4.6)	15 (23.4)
FAB morphology																
M0	4	2	1	0	0	2	0	4	4	4	0	2	0	1	0	0
M1	8	7	7	0	0	8	2	6	7	6	0	3	1	4	0	1
M2	21	20	19	1	6	18	1	16	17	18	6	1	0	0	2	7
M3	4	4	4	0	4	4	0	2	1	4	4	0	1	2	0	2
M4	17	14	15	8	8	16	4	9	16	12	12	0	5	6	0	3
M4E-0	1	1	1	1	1	1	0	0	1	1	1	2	0	1	1	0
M5	9	4	7	7	9	7	1	2	8	1	7	0	3	4	0	2
M6	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0

Table 3. Correlation between the antigen expressions with FAB morphology of AML cases

4.3 Results from the molecular analyses

Molecular evaluation of AML cases demonstrated presence of the three major recurrent genetic abnormalities as follows: 5 patients were positive for the fusion transcript PML/RAR α (Figure 5), 3 patients were positive for AML/ETO1 and 7 for the fusion transcript CBF β /MYH11 (Figure 4).

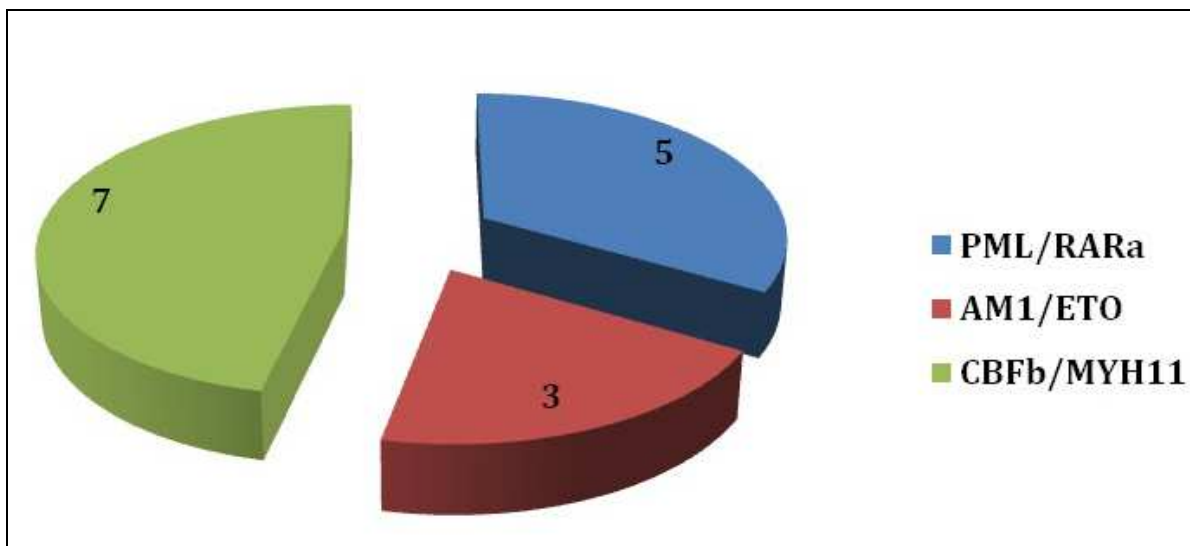


Fig. 4. Distribution of molecular abnormalities detected with the RQ-PCR assay in the group of AML patients;

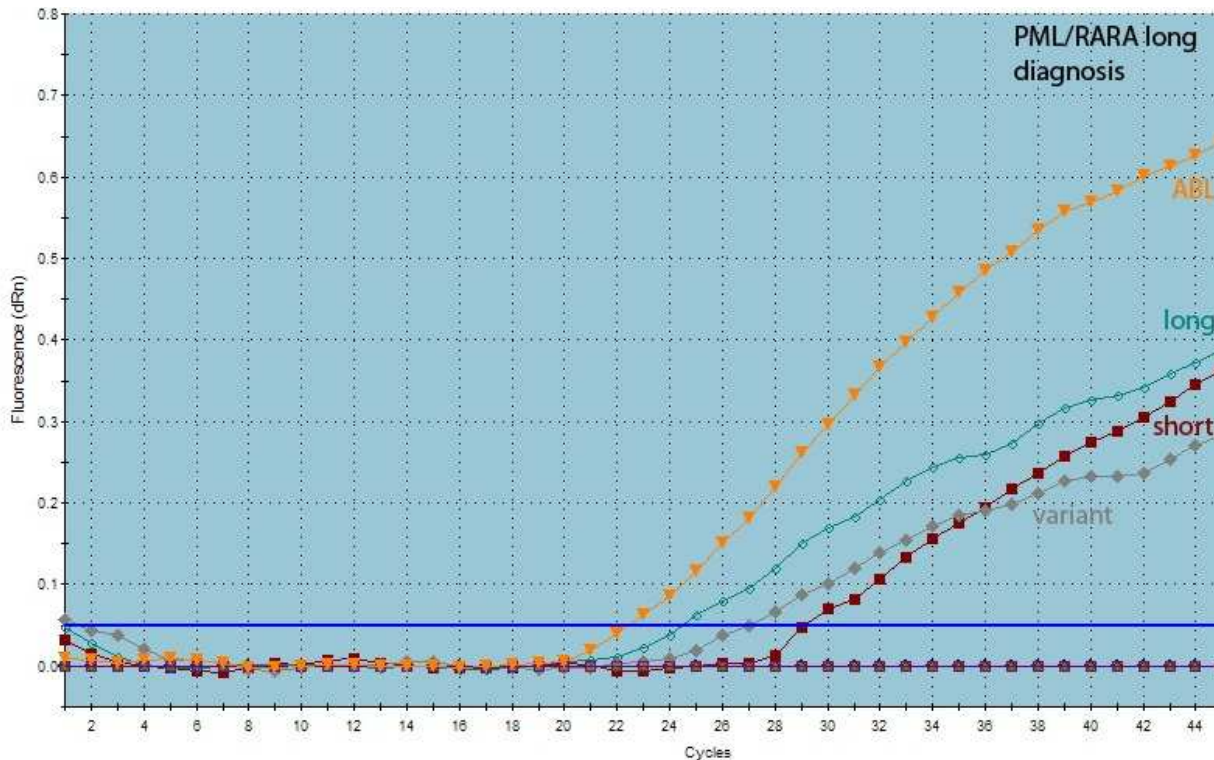


Fig. 5. Detection of fusion transcript PML/RAR α using the RQ-PCR method

In 5 of the AML case PML/RAR α fusion transcript was detected. Four of those patients had morphology and immunophenotype that correlate with AML-M3 diagnosis. The five patients were first diagnosed as AML-M2 and only after the positive result for PML/RAR α the diagnosis was revised as AML-M3. In all those five patients target therapy with ATRA was initiated.

RT-PCR analysis also detected molecular abnormalities in the Core binding factor (CBF) in 10 AML patients; the presence of the AML/ETO1 fusion gene was confirmed in 3 patient and CBF β /MYH11 in 7 patients.

Molecular analyses enabled 23.7% of the cases from our study to be classified in the adequate genetic entities of AML with different prognosis requiring different therapeutic approach.

4.4 Results from the analyses of the clinical data of the patients

We evaluate the distributions of the patients from our study group in the different grades of the Eastern Cooperative Oncology Group (EKO) performance status scale (Oken et al. 1982)(Figure 6). All the patients with EKO performance status higher than grade 2 and patients with serious co morbidities were not suitable candidates for alloSCT.

Furthermore, all the obtained results were correlated and consecutively effective treatment strategy for each single acute leukemia patient was selected.

5. Discussion

Modern diagnostic approach for acute leukemias combines cytomorphology, cytochemistry, multiparameter flow cytometry, chromosome banding analysis, accompanied by diverse fluorescence in situ hybridization techniques, and molecular analyses. The correct diagnosis is essential for classification of this heterogeneous complex of disorders and plays a central role for individual risk stratification and therapeutic decisions (Haferlach et al., 2007; Lichtman et al., 2010)

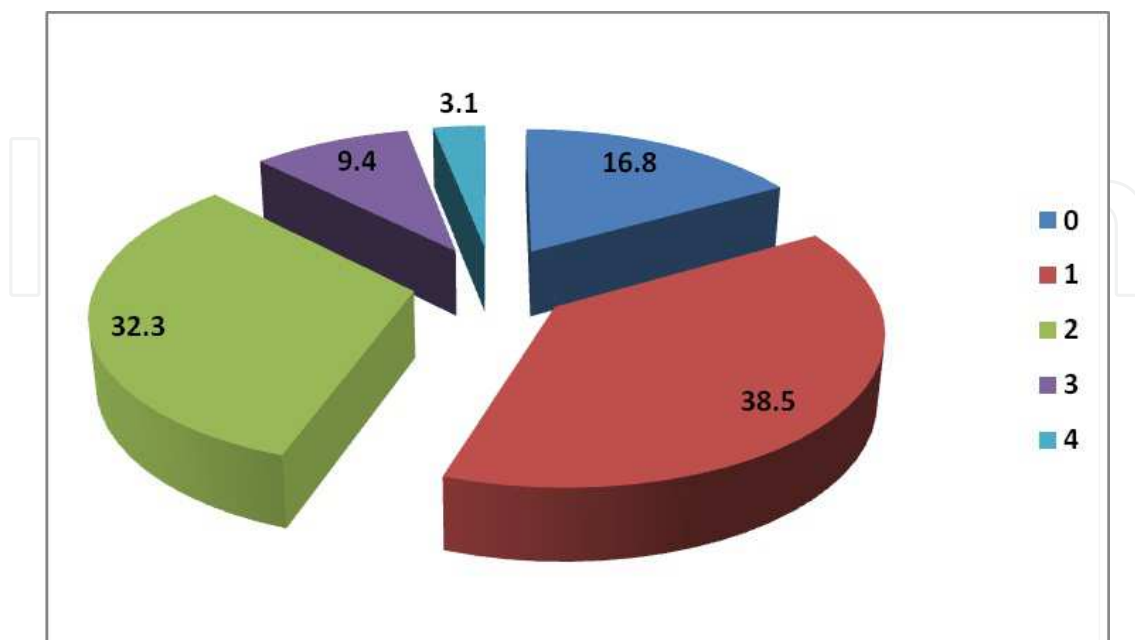


Fig. 6. Distribution of patients according to the grades of the EKO performance status

5.1 Immunophenotyping in modern diagnosis of AML

Assignment of lineage is critical in the diagnostic evaluation of acute leukemia, as treatment for AML and ALL markedly differs. Myeloid and lymphoid lineage may be distinguished based on cellular morphology, cytochemical staining, and expression of lineage-specific antigens (Döhner et al., 2010). Analyses of diagnostic evaluation of acute leukemia in our study showed that immunophenotyping was necessary for lineage assignment in 4 (8.9%) cases that were morphologically and cytochemically undifferentiated, and also for correction of the lineage that was assigned based on morphology and cytochemistry in 3(3.8%) additional cases.

Flow cytometric immunophenotyping is a powerful technological tool that aids in the diagnosis, classification and monitoring of hematological malignancies. It is essential in the diagnosis of AML, as it demonstrates a particular lineage involvement and has a prognostic significance in the majority of AML cases. We used the panel based on the recommendation of the EGIL group and BTSH. Our analyses comprised of a two step process with the first panel of markers being applied to all cases of acute leukemia and the second only in patients with AML that did not demonstrate a clear myeloid commitment. We also evaluated further lymphoid antigen positive AML cases by using the second panel of McAb (Stelzer & Goodpasture, 2000).

The second panel of McAb was aimed at identifying uncommon types of AML, such as those with megakaryocytic or erythroid differentiation and the exclusion or confirmation of diagnosis of non-hematological malignancy. In our study, we applied the second AML panel in 27 cases; 4 which did not demonstrate clear myeloid commitment and 23 cases with lymphoid antigen positive AML cases. With our primary McAb we were able to differentiate AML from ALL in 96.8% of cases. Only in one patient (AML-M6-1.5%) lineage differentiation was assigned after staining with the secondary McAb panel and one case of non-hematological malignancy was confirmed.

Immunophenotyping was crucial in all cases of poorly differentiated myeloid leukemia (AML-M0), megakaryoblastic leukemia (AML-M7) and in some case of monoblastic leukemia (AML-M5) and those with primitive erythroid cells as predominant leukemic cells (AML-M6). This is also important for recognizing an AML case that co-expresses lymphoid-associated antigens. In addition, immunophenotyping enables recognition of unusual forms of acute leukemia: designated acute biphenotypic or acute mixed lineage leukemia. The leukemia associated immunophenotype (LAIP) of the blast cells is a useful tool for detection of minimal residual disease in AML cases (Döhner et al., 2010; Stelzer & Goodpasture, 2000; Swerdlow et al., 2008).

In order to classify AML cases in the different AML entities we correlated the immunological data from immunophenotyping with the FAB morphological and cytochemical classification. One of the difficulties in knotting the flow cytometric data with traditional morphology is the lack of routine flow cytometric data analyses that would ensure correlation of the immunophenotyping data to abnormal morphologic counterpart. Our approach was based on the fact that complete eight-part differential of the myeloid lineage in the normal bone marrow could be done with correlations of the expression of CD45 expression versus light side scatter (SSC) characteristics of the cells. Extension of this technique to the analyses of leukemias allows abnormal cell to be recognized as independent clusters in CD45/SSC histograms with pattern of CD45 and SSC expression that correlate to the same pattern of the morphologically similar cells in normal bone marrow. Flow-cytometric analyses by using CD45 gating strategy revealed that leukemic

myeloblasts demonstrate very similar CD45/SSC characteristics to normal myeloblasts, especially the early myeloblasts. In fact, leukemic cells generally demonstrate CD45/SSC characteristics that closely resemble their nearest normal morphological counterparts in the bone marrow. In the histograms the leukemic cells can be easily recognized as an abnormal cluster of cells in CD45/SSC „space” which is usually occupied by normal myeloid blasts. Using this method we define in our study group each category of AML as defined by FAB system (Stelzer & Goodpasture, 2000). Early myeloblasts are presented at Figure 6. Flow cytometric analyses show that those leukemic cells demonstrate low light forward and side scatter (FSC&SSC), which means that those cells are small and don't contain any granules. They also have low CD45 expression.

The predominant immunophenotype characteristics of early myeloblasts are an expression of all pan-myeloid antigens: CD13, CD33, CD117 and HLA-DR and lack expression of more mature myeloid antigens such as CD15 and CD14. Also, high proportion of the leukemic cells expresses CD34. With the maturation process of the blast cells, CD34 expression becomes more heterogeneous and weaker. The densest is at AML-M0 blasts, which typically express only one myeloid-associated antigen plus. Myeloid blasts that arise as a result of the genetic change in more mature myeloid progenitor lose the characteristics of the early myeloblasts. For example, AML-M3 blasts usually lack the expression of CD34 and HLA-DR.

At Figure 7 are presents more mature myeloblasts, M5 blast. It is obvious that they are bigger when compared with the AML-M1 blasts presented on Figure 6, and contain some more granules (have higher FSC&SSC). Immunophenotypically, they are characterized with expression of some more mature myeloid markers as CD14 and CD15, and in most of the case with lost CD34 expression.

The results from our study showed that routine immunophenotyping improved the diagnosis in 12 (15, 5%) cases with acute leukemia which was essential for more appropriate individual clinical stratification of the patient with acute leukemia. Our data demonstrate that flow cytometry in correlation with the morphological classification criteria for each subtype of AML can be used for initial classification of each FAB AML entities and and justify routine implementation of flow cytometry analyses in the diagnostic evaluation of AML cases.

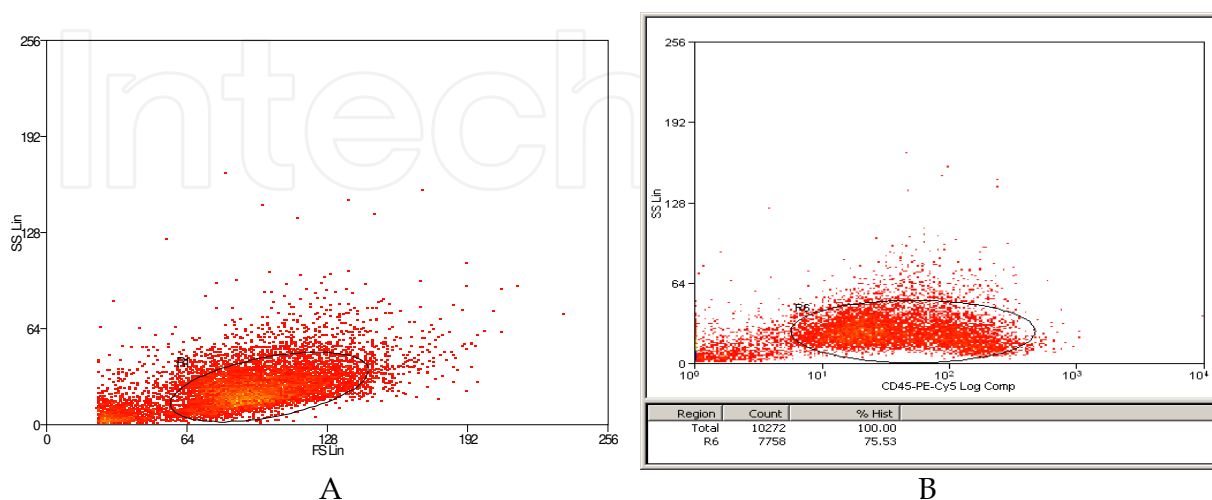


Fig. 6. Histogram analyses of early M1 myeloblasts ; A: Blast cells demonstrate low FSC&SSC, B: Blast cells have low CD45 expression

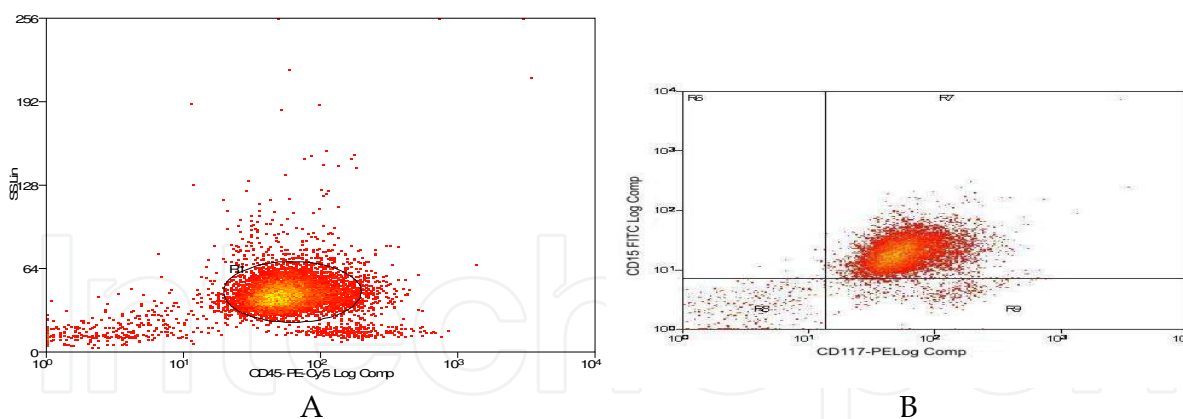


Fig. 7. Histogram analyses of M5-myeloblasts; A: Blast cells are bigger and more granular and have higher CD45, B: Blasts cells express CD117 and the myeloid expression maturation marker CD15

5.2 Molecular analyses in the modern diagnosis of AML

Results from the immunophenotyping guided us to perform exact molecular analyses and further define some specific genetic entities of AML. We tested our AML patients for the presence of PML/RAR α , AML1/ETO and CBF β /MYH11 fusion transcripts, by using RT-PCR method. Results from those analyses additionally improved the exact classification of the AML subtypes and made target and specific therapy available for some of the specific AML subtypes (Marcucci et al., 2000; Perea et al. 2006; Wang et al., 2008)

Nonrandom chromosomal abnormalities are identified at the cytogenetic level in approximately 55% of all adult primary or de novo AML patients and have long been recognized as important independent prognostic indicators for achievement of complete remission (CR), duration of first CR, and survival after intensive chemotherapy treatment. Two of the most prevalent cytogenetic subtypes of adult primary or de novo AML are t(8;21)(q22;q22) and inv(16)(p13q22). These abnormalities result in the disruption of genes encoding subunits of the CBF, which is a $\alpha\beta$ heterodimeric transcriptional factor involved in the regulation of normal hematopoiesis and are collectively referred as CBF-AML. At the molecular level, t(8;21)(q22;q22) and inv(16)(p13q22) result in the creation of novel fusion genes, AML1/ETO and CBF β /MYH11. Detection of t(8;21)(q22;q22) or inv(16)(p13q22) in adult patients with primary AML is a favorable independent prognostic indicator for achievement of cure after intensive chemotherapy or bone marrow transplantation, and may serve as a paradigm of the risk-adapted treatment in AML (Döhner et al. 2010 & Marcucci et al., 2000).

In almost all studies of adult primary AML, the highest CR rate (approximately 90%) and the longest disease free survival (DFS) at 5 years (approximately 50%) have been associated with CBF-AML cases. It has been suggested that the superior outcome of AML patients with CBF gene rearrangements compared with other AML patients may be attributed to an increased sensitivity of the leukemic blasts to cytarabine that in combination with anthracyclines represents the backbone of AML treatment. The collective analysis of all data regarding the treatment of CBF-AML indicates that incorporation of multiple courses of high dose of cytarabine as consolidation therapy should be considered as the therapeutic standard for primary adult CBF-AML (Marcucci et al., 2001; Perea et al. 2006).

The prognostic impact of the CBF gene rearrangements appears equally significant in the setting of BMT of AML patients in first CR. However, the iatrogenic morbidity and mortality of BMT suggest that patients with CBF-AML should not receive this therapeutic modality as initial treatment. The collective analysis of all data regarding the implementation of all SCT in AML treatment suggest that although allo SCT and auto SCT may have a potential role in the initial management of AML other than CBF-AML, the treatment-related morbidity and mortality of SCT represent a therapeutic limitation for treatment of CBF-AML patients. Considering the high probability of cure that these patients can achieve with intensive chemotherapy, it is reasonable to spare them the toxicity of SCT as consolidation in first complete remission (Löwenberg et al., 2008a; Koreth et al., 2009; Marcucci et al., 2000; Marcucci et al., 2001 & Perea et al. 2006).

APL or AML-M3 is a distinct subtype of AML which is characterized by a t(15;17) translocation leading to a *PML-RARA* fusion gene. Historically, recognition of this form of AML as a separate entity was important for the clinicians, not because the chemotherapy used as treatment differed substantially from that used for the other subtypes of AML, but because the relatively common occurrence of life-threatening coagulopathy mandated special supportive maneuvers, including the use of low-dose heparin and aggressive blood product support. In the past, induction mortality was often significant, with some older series reporting an incidence approaching 50%. APL was typified with the worst features associated with leukemia: a fulminant disorder that struck primarily young people, had devastating effects on an individual's life, and resulted in death for a large number of patients during the initial phases of treatment. The last two decades have seen a fundamental shift from this paradigm, with APL now recognized as one of the most curable forms of acute leukemia. Introduction of the differentiation therapy with ATRA into the treatment of APL completely revolutionized the management and outcome of this disease, and presents the first model of targeted therapy for cancer. This agent represents one of the most spectacular advances in the treatment of human cancer, providing the first paradigm of molecularly targeted treatment. Treatment of APL with ATRA combined with anthracycline-based chemotherapy yields a CR rate of approximately 90% for newly diagnosed APLs. The relapse rate is approximately 20%, and with the development of new molecular target therapies such as arsenic trioxide, a cure can now be expected even for relapsed patients. After the advent of ATRA, the introduction of arsenic trioxide (ATO), probably the most biologically active single drug in APL has provided a valuable addition to the armamentarium and may have contributed to further improvements in the clinical outcome of this disease. Several treatment strategies using these agents, usually in combination with chemotherapy, have provided excellent therapeutic results with survival rates exceeding 70% in multicenter clinical trials. Cure of patients with APL depends not only on the effective use of combination therapy involving differentiating and classical cytotoxic agents, but also, critically, upon supportive care measures that take into particular account the biology of the disease and the complications associated with molecularly targeted therapies. Moreover, it is important to consider diagnostic suspicion of APL as a medical emergency (uncommon in AML) that requires several specific and simultaneous actions, including immediate commencement of ATRA therapy, prompt genetic diagnosis, and measures to counteract the coagulopathy (Grimwade et al., 2002; Wang et al., 2008).

5.2.1 Why we used RT-PCR method for detection of the genetic abnormalities?

RT-PCR methods for detecting the PML/RAR α fusion transcript also provide the “gold standard” approach for confirming a diagnosis of APL. In addition to its high specificity and sensitivity, it is essential for defining PML breakpoint location thereby establishing the target for reliable monitoring of the minimal residual disease (MRD).

Standard cytogenetic analysis is currently the most common method for identifying the most common recurrent cytogenetic abnormalities t(8;21)(q22;q22) and inv(16)(p13q22) or t(16;16)(p13;q22) in AML patients. This technique also allows detection of secondary chromosomal abnormalities such as del(9q),-X and -Y which are frequently associated with t(8;21)(q22;q22), or +8 and +22 more often found with inv(16)(p13q22). Still, despite the recent improvements in the cytogenetic methodology and the use of complementary techniques such as fluorescence in situ hybridization and comparative genomic hybridization to increase the rate of successful karyotyping, the possibility that subtle structural chromosomal aberrations are missed remains (Marcucci et al., 2000). In t(8;21)(q22;q22) or inv(16)(p13q22), failure to detect submicroscopic (cryptic) rearrangements of the involved genes leads to false-negative results that may ultimately impact the correct stratification of this patient population in prognostic and risk-adapted therapy groups. Sensitive molecular methodologies such as RT-PCR have been successfully used to detect cryptic CBF abnormalities in diagnostic samples of AML patients with karyotypes that are otherwise negative for t(8;21)(q22;q22) or inv(16)(p13q22). Andrieu et al., 1997 reported detection of AML1/ETO fusion transcripts by RT-PCR in cases with unsuccessful cytogenetic studies, normal karyotypes, or karyotypes other than t(8;21)(q22;q22) such as del(8q), i(8)(q10) or del(9q). Langabeer et al., 1997 used a two-step RT-PCR to screen for the AML1/ETO and CBF β /MYH11 fusion transcripts in AML patients entered into the U.K. MRC AML 10, 11, and 12 trials and compared the results with conventional cytogenetics. All patients with cytogenetic evidence of t(8;21)(q22;q22) or inv(16)(p13q22) were positive by RT-PCR for the corresponding fusion transcripts. RT-PCR was also positive in 31 cases (19 cases of AML1/ETO and 12 cases of CBF β /MYH11) without CBF cytogenetic abnormalities, increasing the overall detection rate of “t(8;21)(q22;q22)” and “inv(16)(p13q22)” from 8.1% to 12.9% and from 6.5% to 10.3%, respectively. The authors of these studies concluded that all primary AML should be routinely tested for the presence of the CBF fusion genes by molecular screening to improve genomic stratification of AML patients in risk-related treatment groups (Marcucci et al., 2000).

Although patients with CBF-AML have a relatively good prognosis, a substantial number of them relapse and eventually die of their disease. Because relapse after intensive treatment is likely to occur as the result of failure to completely eradicate the leukemic blasts, evaluation of MRD by a sensitive molecular technique such as PCR based techniques has been proposed to detect persistence of malignant clones and predict disease relapse in AML patients in CR. This strategy has been successful in CML (Radich et al., 1995) and acute promyelocytic leukemia (Grimwade et al., 2002; Wang et al., 2008), but its clinical applicability to other subgroups of acute leukemia remains controversial.

RQ-PCR provides the most sensitive parameters for AML with reciprocal gene fusions PML/RAR α , CBF β -MYH11, AML1-ETO (Haferlach et al., 2007). The score of gene expression of the respective fusion transcripts after consolidation therapy in relation to gene expression at diagnosis correlates significantly with prognosis [Haferlach et al., 2007]. The

prognostic value of quantitative PCR in MRD diagnostics in these subgroups was demonstrated in several studies. Patients with an MRD level <1% after induction chemotherapy in relation to initial diagnosis had a relapse rate of 8% in contrast to 91% in the patients with MRD levels of $\geq 1\%$ in the investigation on the CBF-leukemias by Krauter et al., 2003. Marcucci et al, 2000, were able to define a distinct transcript copy number in inv(16)/CBFB/ MYH11 below which relapse was unlikely and above which relapse occurred with high probability. A Gruppo Italiano Malattie Ematologiche Maligne dell' Adulto (GIMEMA) trial showed that molecular switch from CR to PML/RAR α positivity was followed by hematologic relapse after a median time of 3 months in 95% of all APL cases (Diverio et al. 1998). Data from different studies indicate that of all AML subtypes, quantitative PCR monitoring could be best established for the reciprocal gene fusions. Molecular analyses enabled 23.7% of the cases of our study to be classified in the adequate genetic entities of AML with different prognosis requiring different therapeutically approach.

5.3 Analyses of the clinical characteristics of the patients in the era of modern diagnosis of AML

5.3.1 Analyses of the EKO performance status

Several studies support the use of the ECOG performance status as a measure of physical functioning and prognosis in patients with AML (Oken et al. 1982). A retrospective study of data from five Southwestern Oncology Group (SWOG) trials that included 968 patients with AML found that the mortality rate within 30 days of initiation of induction therapy is dependent upon both the patient's age and ECOG performance status at diagnosis. A second retrospective analysis of 998 patients age 65 or greater (range 65 to 89; median 71 years) who underwent intensive induction chemotherapy reported eight-week mortality rates of 23, 40, and 72 percent for patients with ECOG PS of zero to 1, 2, and 3 to 4, respectively. One-year overall survival rates for the same groups were 35, 25, and 7 percent, respectively. A third retrospective study of 2767 patients with non-APL AML from the Swedish acute leukemia registry also reported that older patients with an ECOG PS of zero to 1 had 30 day death rates after intensive chemotherapy of less than 15 percent, while patients with a PS of 3 or 4 had higher early death rates regardless of patient age ranging from 26 to 36 percent. Seventy percent of patients up to age 80 years had a PS of zero to 2. A prospective trial of induction chemotherapy with cytarabine plus daunorubicin in 811 older adults (median age 67 years, range 60 to 83 years) with ECOG PS of zero to 2 reported a 30-day mortality rate of 11 percent (Appelbaum et al, 2006). We used the EKO performance status score in initial randomization of our AML patients for different induction and consolidation approaches, in order to avoid intensive induction and consolidation treatment for patient with EKO performance status higher than 2. Only 8 (12.5%) patients from our study group have EKO performance status higher than 2, but only 3 of those patients were younger than 60 years of age.

5.3.2 Analyses of the comorbidities

Comorbidity is an also a distinct additional clinical entity that exists or may occur during the clinical course of patient with a primary disease (i.e. AML). Comorbid conditions are poor prognostic factors especially in older patients with AML. Patients with age-related

chronic cardiac, pulmonary, hepatic or renal disorders or diabetes suffer greater acute toxicity from chemotherapy. Older patients may also have decreased bone marrow regenerative capacity, even after successful leukemia cytoreduction. Inability to tolerate long periods of pancytopenia and malnutrition or the nephrotoxicity of drugs such as amino glycosides or amphotericin remains a major barrier to successful treatment (Piccirillo et al. 2004; Sorror et al, 2005). Six (9,3%) patients from our study had serious comorbidities which limited the application of intensive chemotherapy in their treatment. Five of them were older than 60 years.

5.4 Approach to AML diagnostic algorithms

Modern diagnostic approaches in the AML should be created as integral and basic parts of optimized treatment concepts for the benefit of the each individual patient. The ultimate test of any disease diagnostic algorithm approach is its usefulness in guiding the selection of effective treatment strategies. Algorithms that provide a basis for risk-adapted therapeutic choices may include immunological markers, cytogenetic factors, molecular markers as well as clinical parameters (e.g., age, attainment of an early or late complete remission) and hematological determinants (e.g., secondary AML, white blood cell count at diagnosis) (Haferlach et al., 2007).

A comprehensive approach in diagnosis, classification, and treatment follow-up in patients with acute leukemias can, therefore, be suggested by diagnostic algorithms, which also show the relationship and the hierarchy between single methods. These standard guidelines for AML mostly result from a combination of different methods and intend to add prognostic information (Lowenberg 2008).

Our diagnostic algorithm for AML (as shown in Fig. 8) starts with cytomorphology and cytochemistry. These methods should be performed in combination: cytomorphology and cytochemistry allow rapid classification of the acute leukemias and further enables the choice of the antibody panel for flow cytometric analyses. In case cytomorphology gives indices for characteristic aberrations—in the FAB subtypes M3/M3v for t(15;17)/PML/RAR α , in FAB M4eo for inv(16)/CBF β /MYH11, or in M1/2 with the characteristic long Auer rods for the t(8;21)/AML1/ETO, PCR analyses for these rearrangements should be promptly applied.

Especially in case of suspicion for APL due to clinical symptoms or due to the morphological findings, RT-PCR for PML/RAR α should be initiated as soon as possible (Schoch et al., 2002). We think that, regardless of the morphological, cytochemical and immunological features of the blast cells, RT-PCR for the fusion oncogene PML/RAR α are recommendable in all AML cases. The RT-PCR method is the most sensitive and rapid technique for detection of this oncogene and provides an optimal basis for MRD analyses. Further we suggest all AML cases which are PML/RAR α negative to be tested for the presence of the reciprocal fusion genes that describe CBF-AML, AML1/ETO and CBF β /MYH11. Those analyses provides the basis for sub-classification of the AML cases in prognostically relevant subclasses. This is the prerequisite for adequate individual clinical risk stratification and therapeutic decisions.

Moreover, we correlated the obtained result for the applied multimodal diagnostic approach with the patient age, ECOG performance status and comorbidities, which allowed us to optimize the individual risk stratification for each AML patient.

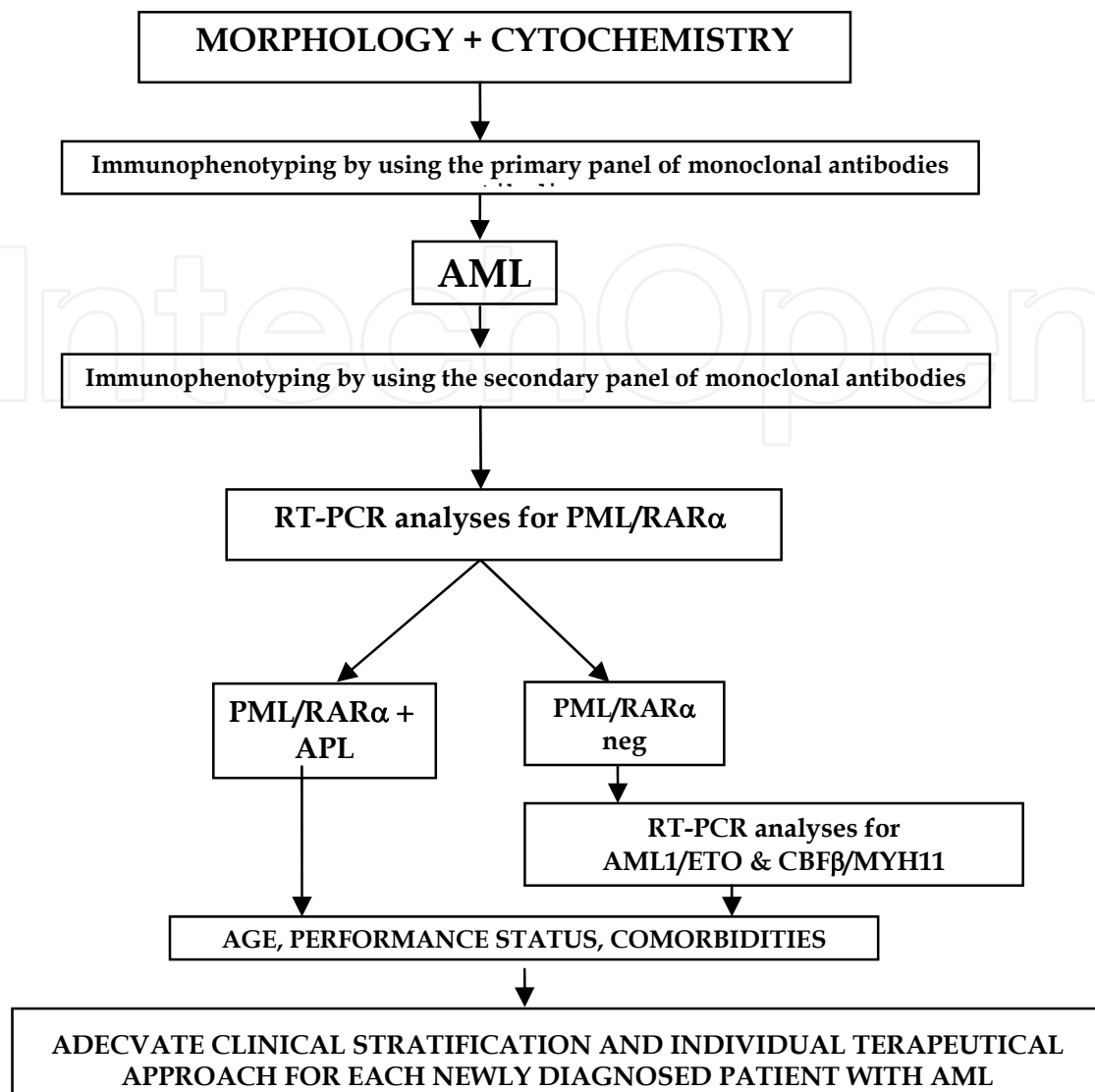


Fig. 8. Diagnostic algorithm and algorithm for risk adapted therapy in AML patients

6. Conclusions

A basis for every therapeutic decision in AML cases should be provided by a multimodal diagnostic approach. The optimal therapeutic conditions are based on exact classification and prognosis of the AML subtype at diagnosis and to delineation of sensitive markers for MRD studies during the complete hematologic remission. MRD methods have many potential applications in the clinical management of patients with acute leukemia. Today, a multimodal diagnostic approach which combines different diagnostic techniques is needed to meet these requirements. The diagnostic process is becoming more demanding with respect to experience, time and costs due to the expansion of methods and algorithms, which guide the diagnostic procedure from basic to more specific methods and which finally lead to results that are essential for modern diagnostics and therapeutic concepts. There are numerous overlaps between different diagnostic methods. These can be used for optimal pathways in the complex diagnostic proceedings and for validation of the results of single methods, which can be summarized in diagnostic algorithms. Basic morphological

and cytochemical analyses performed in our study group established the lineage assignment of the blasts cells in 68 (88.3%) patients. Routine immunophenotyping improved the diagnosis in 12 (15.5%) more cases. Molecular analyses enabled 23.7% of the cases from our study to be classified in the adequate genetic entities of AML with different prognosis requiring different therapeutical approach.

The applied multimodal diagnostic approach consisted of minimal number of cytomorphological, cytochemistry, immunological and molecular analyses enables improved and more precise diagnosis and clinical stratification in 38.2% acute leukemia patients from our study. Moreover, when we correlate those results with the results obtained from the analyses of the EKO performance status and the incidence of the serious comorbidities in our study group, an additional 12.5% of the patients were stratified to a different risk adapted therapy.

Our initial results are consistent with literature data and indicate that our applied multimodal diagnostic approach improved the diagnosis of the specific genetic entities of AML in which specific treatment approach is indicated and allows individual clinical stratification in treatment protocols of the patients.

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Phone: +86-21-62489820
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