

A3.2 AML diagnosis: objectives and general methodology

The early diagnostic work must be **completed in 24-48 hours**, in order to allow rapid recognition of eligible cases and registration into study, and must provide the clinician with the following informations:

- Diagnosis of AML with exclusion of ALL/other neoplasms
- Diagnosis of AML subtype
- Diagnosis of high-risk MDS
- Diagnosis of myeloid sarcoma (MS) or extramedullary AML
- Diagnosis of undifferentiated and biphenotypic acute leukemia

Diagnostic methodology at presentation is based on joint BM/PB analysis through morphology, cytochemistry and immunophenotype. Cytogenetics and molecular biology studies are of course carried out, but their results are known at a later stage and therefore do not assist in the early diagnostic evaluation. These tests must be applied in an integrated and perfected fashion as indicated by major consensus papers of **FAB**, **WHO** and **EGIL** groups, supplemented by others in selected instances:

1. JM Bennett et al. Proposed revised criteria for the classification of acute myeloid leukaemia. *Ann Intern Med* 1985;103:626
2. JM Bennett et al. Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-M0). *Br J Haematol* 1991;78:325.
3. MC Bene et al. Proposals for the immunological classification of acute leukemias. *Leukaemia* 1995;9:1783.
4. Consensual European immunophenotyping panels for leukaemia – 6th PCRDT, april 23, 2005. Obtainable at URL www.leukemia-net.org, select “diagnostics” in the upper menu bar, select again “ diagnostics” in the new page, left column: standards/SOP’s of Project 10 will appear automatically.
5. ES Jaffe et al. WHO classification of tumours - Tumours of haematopoietic and lymphoid tissue. IARC Press. Lyon 2001.
6. JW Vardiman et al. The World health Organization (WHO) classification of the myeloid neoplasms. *Blood* 2002;100:2292.
7. BD Cheson et al. Revised recommendations of the International Working Group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukaemia. *J Clin Oncol* 2003;21:4642.

Because widely employed and promptly applicable to newly diagnosed patients with AML, FAB terminology will be retained in the current study for general diagnostic purposes and study registration. The new WHO diagnostic terminology will be added to each case following the results of cytogenetics etc.

A3.3 How to obtain/organize AML diagnosis

Diagnostic material and tests:

- **BM aspirate and core biopsy; PB.** The core marrow biopsy is necessary to obtain unstained sections for the NPM study (1).
- AML if $\geq 20\%$ of nonerythroid cells are myeloid blasts, according to WHO classification system. This limit is lower than with the FAB classification (30%), and is accepted in this study. Here we shall use the WHO criteria for the primary diagnosis of AML or MDS, reserving both WHO and FAB systems for further disease subclassification.
- For correct identification of morphocytochemical AML features, 500 BM cells and 200 PB cells should be enumerated (WHO).
- In most instances AML blast cells are recognized at a glance by **morphology** and **cytochemistry**. Sudan black B (SBB) or myeloperoxidase (MPO) stains, and NaF-inhibited alpha-naphthyl butyrate/acetate esterase (ANBE/ANAE) stain are necessary in order to identify properly non-M0 AMLs (SBB/MPO reactivity $\geq 3\%$, $< 3\%$ in M0) and cases with monocytic component (which is not always identifiable through immunophenotype), respectively.
- The **immunophenotype panel** includes all necessary reagents to obtain the relevant informations and to discriminate AML from ALL/other.

By adhering to these procedural rules, true biphenotypic leukemias and true undifferentiated/unclassifiable leukemias become rare. When these entities are identified, as they originate from BM stem cells and show no or uncertain/multiple lineage differentiation, they will be treated as AML and included in the current study under the caption of HR subsets. *Table A3.1* reports the suggested immunophenotypic diagnostic protocol (mandatory tests are in bold style, others as second step in order to detect rarer diagnostic and prognostic subsets of disease), and *Table A3.2* specifies the exact rules for the diagnosis of acute biphenotypic leukemia. The cytofluorimetric diagnosis needs to be integrated with light microscopy and cytochemistry studies for SBB/MPO and ANAE/ANBE with NaF inhibition.

Table A3.1 Immunophenotypic diagnosis of AML (bold=mandatory; other markers may be needed to refine diagnosis and/or to define biphenotypic leukemia)

| Purpose | CD antigens (c=cytoplasmic; Tdt=nuclear) |
|--|--|
| Gating of non-erythroid cells and differential vs. nonhematologic | CD45 (pos) |
| Differential vs. ALL | cMPO, CD117 (pos) vs. cCD3, cCD79a, cCD22 (neg) |
| AML subsets: myeloid monocytic erythroid megakaryocytic stem-cell | CD13, CD33, CD56, CD65 CD4, CD11b, CD14, CD64 CD36, CD238 CD41, CD42, CD61 CD34, TdT, CD7, CD38, DR |
| Additional markers (see <i>Table A3.2</i>) | clgM, CD10, CD19, CD20, CD24 (B-lineage) CD1a, CD2, CD5, CD8, anti-TCR (T-lineage) CD15 (myeloid) |

A3.4 AML diagnosis and subclassification

Because cytogenetics requires some days to be adequately reported, the first diagnostic step is still based on morpho-cytochemical and immunophenotypic evaluation as per FAB/EGIL/WHO specifications, however keeping the lower BM blast cell threshold of the WHO ($\geq 20\%$). This should allow the identification of the clinical entities described below. Once cytogenetic/molecular biology study results are known, these data can be integrated into the new WHO classification. Thus, eventually, trial database will contain two strictly related AML classifications, which could be used or compared as needed. Very difficult cases can be provisionally labeled and will be reclassified after the central review process.

- **FAB M0 AML / WHO synonym: AML minimally differentiated.** This AML subtype displays undifferentiated morphology, negative SBB/MPO staining by light microscopy, and positivity $\geq 20\%$ for myeloid antigens CD13 and/or CD33 and/or CD117 and/or anti-MPO (positivity threshold is usually 10% and can be as low as 3% for cytofluorimetric MPO evaluation). Other myeloid/lymphoid antigens can be variously expressed but generally without meeting the criteria set for biphenotypic acute leukemia (see *Table A3.2*). This is a difficult diagnostic subtype and it cannot be recognized without SBB/MPO (negative) staining on light microscopy.
- **FAB M1 AML / WHO synonym: AML without maturation.** When 90% or more of nonerythroid BM cells are SBB/MPO positive blasts ($\geq 3\%$) with less than 10% maturation beyond blast stage. Immunophenotype is usually confirmatory. Lymphoid antigens may be co-expressed below the threshold indicating biphenotypic leukemia.
- **FAB M2 AML / WHO synonym: AML with maturation.** When $\geq 20\%$ -89% of nonerythroid BM cells are SBB/MPO positive blasts ($\geq 3\%$), with monocytic cells $< 20\%$ (see below for their identification) and with $\geq 10\%$ maturation beyond myeloblasts. A marked maturation pattern towards promyelocytes may be present. Immunophenotype is confirmatory. Cases co-expressing CD19 and/or CD56 and/or TdT often carry t(8;21). Lymphoid antigens may be co-expressed below the threshold indicating biphenotypic leukemia.
- **FAB M4 AML / WHO synonym: AMML.** When, in either BM or PB, $\geq 20\%$ but less than 80% of nonerythroid cells are blasts of monocytic lineage at variable maturation

stage. Other blasts are of myeloid type. The identification of the monocytic component lies in cytochemical reactions (ANBE/ANAE) and immunophenotypic study revealing positive results for monocytic antigens CD11b and/or CD14 (plus CD4/11c/36/64). Because in as many as 10% of monocytic leukemias the immunophenotype study fails to demonstrate CD11b/CD14 expression, ANBE/ANAE staining is always required (2). If morphological criteria are met with both immunophenotype and cytochemistry negative, the diagnosis is accepted. Lymphoid antigens may be co-expressed below the threshold indicating biphenotypic leukemia.

- **FAB M4eo AML / WHO synonym: AMML with eosinophils.** As above, with significant eosinophil cell present in BM. Eosinophils usually amount to 5% or more and display atypical basophilic granules in addition to specific highly eosinophilic granules. This entity is fairly often associated with inv(16).
- **FAB M5a/b AML / WHO synonym: Acute monoblastic/monocytic leukemia.** When $\geq 80\%$ of blast cells are of monocytic cell lineage, as shown by ANBE/ANAE (with NaF inhibition) and the immunophenotype study CD11b and/or CD14 plus CD4/11c/36/64. (2). The morphology of monocytes/promonocytes must be differentiated from that of monoblasts (M5a vs. M5b). Lymphoid antigens may be co-expressed below the threshold indicating biphenotypic leukemia.
- **FAB M6 (erythroleukemia) / WHO synonym: erythroleukemia, pure erythroid leukemia.** When (variously abnormal) erythroblasts constitute $\geq 50\%$ of all BM cells, and 20% or more of nonerythroid cells are blasts. If less than 20% of nonerythroid cells are blasts, the diagnosis is of MDS (RAEB). This subset is a mixed erythromyeloid leukemia and is the only recognized by the FAB. According to WHO, cases with $>80\%$ BM erythroid blast cells are classified as pure erythroleukemia. Erythroid antigens for immunophenotypic analysis are glycophorin A (CD238) and hemoglobin A. Anti-MPO and myeloid antigens are negative in erythroblasts. Concurrent myeloblasts generally lack CD34 and DR expression. In pure erythroleukemia, more immature cells may react to CD36 (not specific).
- **FAB M7 (megakaryocytic leukaemia) / WHO synonym: acute megakaryoblastic leukemia.** When a $\geq 20\%$ leukemic BM infiltrate, unreactive to MPO, is demonstrated as being of megakaryocytic nature on electron microscopy (platelet peroxidase) or by immunophenotypic tests (2 Bennett). Useful immune markers are CD41 and CD61 (and CD42). Myeloid markers and CD36 can be positive.
- **WHO Acute basophilic leukemia.** Very rare, with prominent basophilic differentiation in the blast cells. These are myeloid-type cells that are positively stained by toluidine blue. Immunophenotype is poorly specific except perhaps for CD9.
- **WHO acute panmyelosis with myelofibrosis.** Very rare, and best recognized through BM core biopsy showing consistent dysplasia with aberrant erythroblasts, scattered blasts, and increased atypical megakaryocytes/blasts, with fibrosis. Needs to be differentiated from megakaryoblastic AML.
- **WHO Myeloid sarcoma.** A tumoral mass of AML cells. Morphology and immunophenotype cover the entire range of myeloid and monocytic AML.
- **WHO Undifferentiated acute leukemia.** Mainly characterized by the lack of lineage specific markers MPO, cCD79a, cCD22, cCD3. Few other antigens expressed, associated with stem cells/immature cells: DR, CD34, CD38, TdT, CD7.
- **WHO Bilineal acute leukemia.** When two separate leukemic cell populations are found, i.e. myeloid and T or B lymphoid. Unclear relationship to biphenotypic leukemia.
- **WHO Biphenotypic acute leukemia.** When myeloid cell lineage markers are coexpressed with B or T, or B and T lymphoid lineage markers. Quantitative criteria were established to distinguish true biphenotypic leukaemia from lymphoid antigen positive AML. Biphenotypic leukemia is defined by an EGIL score >2 for myeloid antigens and >1 for lymphoid antigens (*Table A3.2*).

Table A3.2 EGIL score system for the diagnosis of biphenotypic leukemia (Cyt=intracytoplasmic expression; m=membrane expression; not indicated=means membrane expression; TdT=nuclear expression)

| Points | B-lineage | T-lineage | Myeloid |
|--------|------------------------------|---------------------|-------------------------|
| 2 | Cyt CD79a, Cyt IgM, Cyt CD22 | Cyt/m CD3, anti-TCR | MPO |
| 1 | CD19, CD20, CD10 | CD2, CD5, CD8, CD10 | CD117, CD13, CD33, CD65 |
| 0.5 | TdT, CD24 | TdT, CD7, CD1a | CD14, CD15, CD64 |

- **WHO AML with multilineage dysplasia.** When, besides AML cells, concurrent dysplastic changes are observed in $\geq 50\%$ of 2 or more other cell lineages. This can occur de novo or following an established diagnosis of MDS. Diagnostic hallmarks are dysgranulopoiesis, dyserythropoiesis and dysmegakaryocytopoiesis. Immunophenotype is that of AML cells.
- **High risk MDS.** According to WHO, RAEB-T with 20-30% BM blasts no longer exists. Cases with MDS eligible to AML-like therapy (i.e. HR MDS) have 5%-9% BM blasts (RAEB-1) or 10%-19% BM blasts (RAEB-2).

A3.5 How to differentiate AML vs. ALL/other

A joint morphological, cytochemical and immunological evaluation is necessary to distinguish AML from ALL and other neoplasms. Immunophenotype plays a crucial role, as lymphoid cells are always positive for one intracytoplasmic antigen (T lineage: cCD3; B lineage: cCD79a, cCD22) whereas most if not all AML (see above description of subtypes) react to intracytoplasmic MPO (cMPO). Other useful myeloid markers with a broad range of reactivity are CD117 (sometimes negative in AML, sometimes positive in T-ALL) CD15, CD13 and CD33 (fairly often positive in ALL).

A3.5 Other studies

The immunophenotypic and functional analysis of multidrug resistance mechanisms (MDR) expressed by AML cells can have significant prognostic and therapeutic relevance.

- The immunophenotypic detection of MDR molecules is possible for MDR1/PgP (MRK16, JSB-1 antibodies), LRP (LRP-56), MRP (MRPm6 antibody) and BCRP (BXP-34 antibody).
- The functional evaluation of drug cell efflux (with inhibition by cyclosporin A) is possible by the cytofluorimetric DiOC₂ or rhodamine-123 efflux/retention test.

Detailed methodological informations on these tests are available at the Coordinating Institution (contact A. Salvi, phone: no. +35 269491; e-mail: labpaolobelli@ospedaliriuniti.bergamo.it)

A3.6 Final diagnostic system

AML diagnostic subsets as detailed in *section A3.4* are eventually reconsidered in the light of prior clinical history and additional diagnostic tests (cytogenetics, molecular biology) and entered onto final WHO classification system:

1. AML with recurrent genetic abnormalities
 - AML with t(8;21)/AML1-ETO
 - AML with inv(16) or t(16;16)/CBFB-MYH11
 - AML with 11q23/MLL abnormalities
2. AML with multilineage dysplasia
 - Following MDS/chronic myeloproliferative disease
 - Without antecedent MDS
3. AML/MDS, therapy-related
 - Alkylating agent
 - Topoisomerase II inhibitor
 - Other
4. AML not otherwise categorised
 - AML minimally differentiated
 - AML without maturation
 - AML with maturation

- AMML
- Acute monoblastic/monocytic leukemia
- Acute erythroid leukemia
- Acute megakaryoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis
- Myeloid sarcoma
- Acute undifferentiated leukemia
- Acute bilineal leukemia
- Acute biphenotypic leukemia

A3.7 Final trial database

The diagnostic information eventually available for each patient will be checked centrally, with supplemental tests when required, and will contain: AML diagnosis according to FAB/EGIL/WHO specifications, cytogenetic/molecular biology integrations and MDR profile. All these informations, together with the clinical data, concur to determine the patient risk class and therefore the correct application of the treatment protocol and the interpretation of results.

A3.8 References

1. Falini B et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N ENGL J MED 2005,352:254.
2. Tallman MS et al. Acute monocytic leukemia (French-American-British classification M5) does not have a worse prognosis than other subtypes of acute myeloid leukemia: a report from the Eastern Cooperative Oncology Group. J CLIN ONCOL 2004,22:1276.