Toward Integrated Genomic Diagnosis in Routine Diagnostic Pathology by the World Health Organization Classification of Acute Myeloid Leukemia

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Abstract

Genomic characterization of cancer has transformed understanding of the pathogenesis for all types of cancer in the last two decades, with the study of hematologic malignancies at the forefront of the genomics revolution. With continuous rapid advances, as we envision future possibilities in diagnosis, it is helpful to reflect upon how we reached this point. This review includes a historical perspective, and touches upon significant milestones since the microscope's invention in 1674 toward the current diagnosis of acute myeloid leukemia (AML), which is an example of an aggressive hematologic malignancy that now requires integration with genomics for diagnosis, prognosis and clinical management. The 2016/2017 World Health Classification (WHO) classification criteria precisely identify AML with recurrent chromosomal abnormalities and the two new, molecularly-defined subtypes, AML with mutated *NPM1 (*AML-*NPM1*^{mut}) and biallelic mutated *CEBPA* (AML-bi*CEBPA*^{mut}). Epidemiologic and hematologic features of the two new molecularly-defined WHO subtypes of AML are highlighted, including for familial AML-*NPM1*^{mut} and familial AML-bi*CEBPA*^{mut}. Genomic testing, in conjunction with cytomorphologic evaluation, flow cytometric immunophenotypic, and cytogenetic analysis, is now essential for accurate diagnosis of AML. In light of current guidelines for testing, risk stratification, and newer therapies, considerations for integrated genomic testing are discussed toward routine diagnosis by the WHO classification of AML.
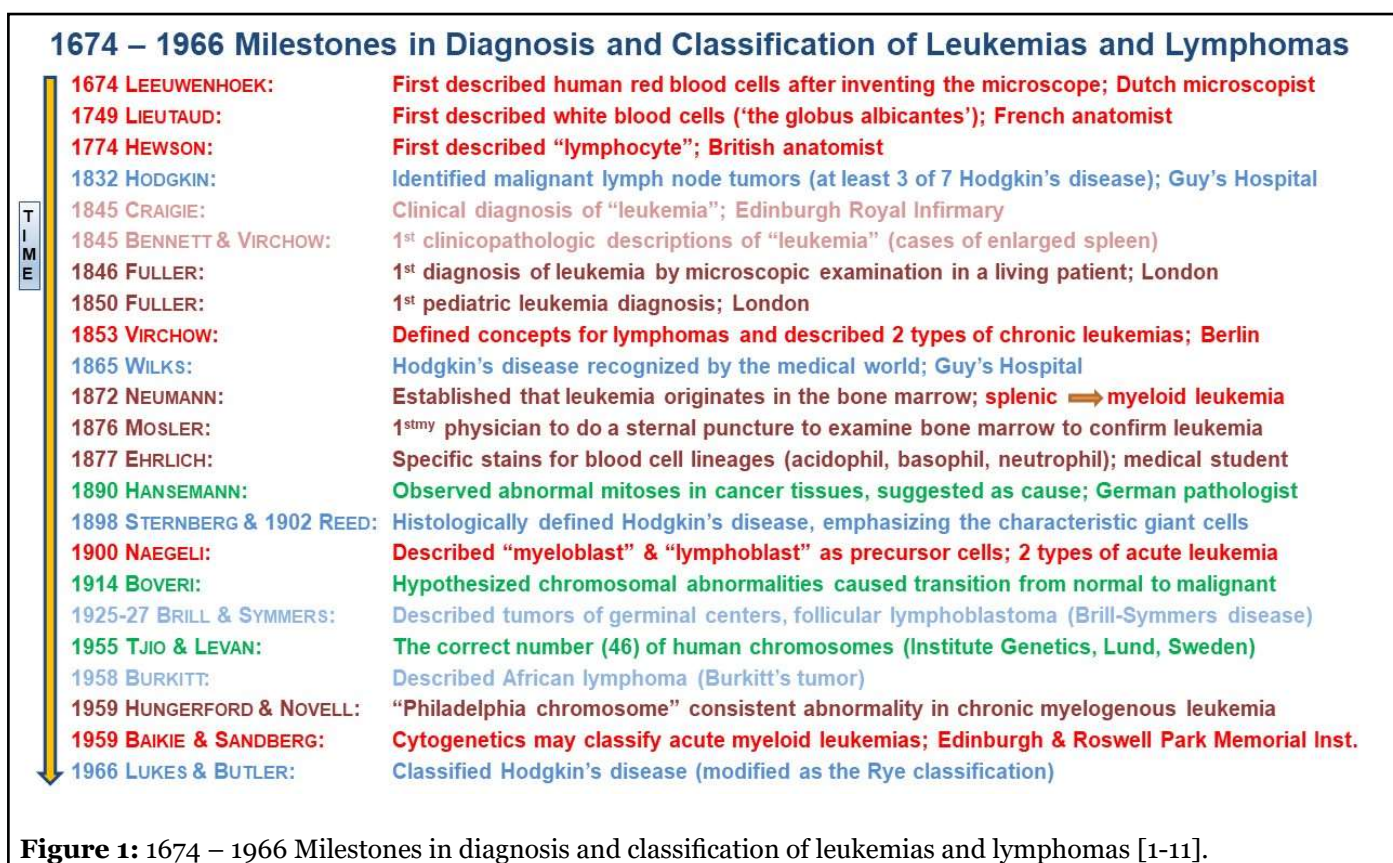
Abbreviations: AML: Acute Myeloid Leukemia; WHO: World Health Organization; AML-*NPM1*^{mut}: AML with mutated *NPM1*; AML-bi*CEBPA*^{mut}: AML with biallelic mutated *CEBPA*; FAB: French-American-British; NCI: National Cancer Institute; WF: Working Formulation; FCI: Flow Cytometric Immunophenotyping; EGIL: European Group for Immunological characterization of Leukemias; FLT3: FMS-like tyrosine kinase 3; ITD: Internal Tandem Duplication; TKD: Tyrosine Kinase Domain; NPM1: Nucleophosmin; CEBPA: CCAAT/Enhancer Binding Protein-alpha; NGS: Next Generation Sequencing; APL: Acute Promyelocytic Leukemia; MPAL: Mixed Phenotype Acute Leukemia; ALAL: Acute Leukemias of Ambiguous Lineage; AML-MRC: AML with Myelodysplasia-related Changes; AML-NOS: AML Not Otherwise Specified; NCCN: National Comprehensive Cancer Network; CAP: College of American Pathologists; ASH: American Society of Hematology

Introduction and Historical Perspective

Significant milestones and seminal discoveries during 1674-1966, by individuals who have made crucial contributions toward progress in the diagnosis of hematologic neoplasms as we understand today are depicted chronologically in Figure 1, with selected references [1-11]. It is notable that the path to progress in the understanding of disease and neoplasms initially took centuries for significant discoveries (17th-18th centuries), and subsequently, many decades (19th-20th centuries) for a breakthrough or a change from the prevailing norm. Further, that progress always required perseverance, dedication, innovation, and collaboration among individuals that were not necessarily recognized by the majority at the time, as exemplified by the respectful collaboration between John Hughes Bennett and Rudolph Virchow, as described by Piller [1].

Figure 1: 1674 – 1966 Milestones in diagnosis and classification of leukemias and lymphomas [1-11].

"Leukemia," lymphomas, and cancer genetics: first descriptions

As depicted in Figure 1, the first clear description for the thought of "leukemia" as the disease cause appears to have been in 1841 by David Craigie, a physician at Edinburgh Royal Infirmary, who observed a patient with thick blood and an enlarged spleen and who was puzzled by, and questioned, the cause of the clinical features in that patient [1,2]. Three years later, in 1844, Craigie attended to another patient with similar clinical features, and was convinced that the pathological cause was the same in both patients, and not pus and inflammation. John Hughes Bennett, a clinician, and pathologist at Edinburgh Royal Infirmary, who had received prior training with Alfred Donne, a French microscopist, performed an autopsy in Craigie's second patient and examined the deceased patient's blood under a microscope [1,2]. Bennett first reported in 1845 that the disease, which would now be recognized as chronic myeloid leukemia, was due to systemic involvement of blood (and not inflammation), along with Craigie's report of his first patient in the same journal. The second clinicopathologic report of leukemia was by Rudolph Virchow, a demonstrator of anatomy in Berlin, who, at age 24, described the unstained appearances of blood cells in the first report of a patient with chronic lymphatic leukemia. Subsequently, Virchow described the third case, also chronic leukemia with splenic enlargement, and recognized that there were two types of leukemias – splenic and lymphatic. While Bennett preferred the name "leucocythemia," Virchow coined the name "leukemia" for the disease, importantly, with both in agreement for the microscopic features in blood and the cause of the disease as systemic, instead of inflammation [1,2].

Five decades later, in 1900, the words "myeloblast" and "lymphoblast" were introduced by Naegeli, a Swiss hematologist, for precursor cells in the two types of acute leukemias. That nomenclature followed two crucial milestones: (1) first, in 1868, by Ernst Neumann, a Professor of Pathologic Anatomy at Konigsberg, who had stated that blood originated in the bone marrow, hematopoiesis is a continuous process, and that leukemias originate in the bone marrow, and (2) the seminal work of Paul Ehrlich, who, as a medical student in 1877, had described details of blood cells and developed stains to identify acidophil, basophil, and neutrophil granules in white blood cells [1].

Interestingly, malignant lymph node tumors (lymphomas), which represent the solid counterpart of hematologic lymphoid neoplasms, were first studied by post-mortem examination by Thomas Hodgkin at Guy's Hospital in London in 1832, a decade *before* Craigie observed his first patient with chronic leukemia in Edinburgh. Nevertheless, "Hodgkin disease" was first recognized by the medical world in 1865, two decades *after* Bennett and Virchow described leukemias due to Samuel Wilks, a curator at the same museum where Hodgkin had worked. Three decades later, at the turn of the 19th-20th century, Sternberg and Reed described the characteristic large nucleolated cells by histological examination in Hodgkin disease. Twenty-five years later, during 1925-

1927, Brill and Symmers described follicular tumors of germinal centers, and another 30 years later, in 1958, Dennis Burkitt described Burkitt lymphoma, which also arises from the germinal center [3,4].

In parallel with the milestones above in hematology in the second half of the 19th century, the field of the underlying genetics in cancer started with the observation by a German pathologist, Hansemann, who in 1890 first observed mitoses in tissues of malignant tumors. Two decades later, in 1914, Theodor Boveri hypothesized that chromosomal abnormalities caused the transition from benign to malignant. However, it was not until 1955 when Tjio and Levan at the Institute of Genetics in Lund ascertained the correct number of human chromosomes as forty-six [5]. Subsequently, in the late 1950s, the abnormally minute "Philadelphia chromosome" was identified as a causative abnormality in 7 patients with chronic myeloid leukemia in the absence of any observed chromosomal abnormalities in 10 AML patients [2,6].

Significantly, also in the late 1950s and 1960s, although not frequently noted, Baikie in Edinburgh, and Sandberg at Roswell Park Memorial Hospital in Buffalo, had described cytogenetic abnormalities in AML cases and suggested that cytogenetics could classify AML [7-9]. However, cytogenetics was not formally incorporated to classify AML until four decades later, when the 2001 WHO classification of tumors was introduced [12].

The morphologic and subsequent immunologic era for hematologic cancers

Ehrlich's groundbreaking work in 1877 initiated the morphological era for hematology, which has progressed for almost 1½ centuries. Major progress in the diagnosis and classification of leukemias was achieved by careful examination of peripheral blood and bone marrow aspirate smears, including cytochemical-stained smears, by the FAB Co-operative Group. As shown in Table 1, the FAB group classified acute leukemias during 1976-1985, and subsequently, chronic lymphoid and myeloid leukemias [13-16]. Figure 2 shows the AML subtypes by FAB classification, based on the differentiation of the leukemic cells and the extent of myeloid and monocytic maturation, as determined by microscopic evaluation of Wright-Giemsa-stained and cytochemical-stained smears [13,14].

The earlier classifications for lymphomas were proposed by multiple groups, as shown in Table 2 [10-23]. Before 1982, in addition to the three non-Hodgkin lymphoma classifications in Table 1, three additional classifications (Dorfman, WHO, and the British National Lymphoma Investigation) were also in use. The NCI-sponsored Working Formulation was developed in 1982 after evaluating those six classifications to classify lymphomas for clinical use, but the WF became most widely used for diagnostic pathology [21]. In 1994, the REAL classification was introduced by a group of expert hematopathologists

Figure 2: The French-American-British classification of acute myeloid leukemia [13,14]: M0: AML with minimal differentiation; M1: AML without maturation; M2: AML with maturation; M3: Acute promyelocytic leukemia; M4: Acute myelomonocytic leukemia; M5a: Acute monoblastic leukemia; M5b: Acute monocytic leukemia; M6: Acute erythroid leukemia; M7: Acute megakaryoblastic leukemia.

Lymphomas	Leukemias
1956 Rappaport classification – based on histologic pattern and cytologic features	1976 FAB Group classification of acute leukemias, updated 1985 - Based on careful cytologic examination of peripheral blood and bone marrow aspirate smears, including with cytochemical stains - Acute leukemia diagnosis required 30% blasts with ≥ 3% blasts positive for myeloperoxidase or the presence of Auer rods for a diagnosis of AML
1966 Lukes & Butler – classified Hodgkin disease, modified as the Rye classification	
1974 Lukes & Collins - based on immunological cell-of-origin (B or T-lymphoid) and cytology of cells (small and large cleaved and non-cleaved)	
1974 Kiel classification, updated 1988 - based on cytologic features to assign grade	
1982 National Cancer Institute Working Formulation for clinical usage	1989 FAB classification chronic (mature) lymphoid leukemias
1994 Revised European American-Lymphoma (REAL) classification	1994 FAB classification chronic myeloid leukemias
	1995 EGIL Immunological classification of acute leukemias
2001 World Health Organization (WHO) classification of tumours of the haematopoietic and lymphoid tissues	
Abbreviations: FAB: French-American-British Co-operative Group; AML: Acute Myeloid Leukemia; EGIL: European Group for the Immunological characterization of Leukemias	

Table 1: Earlier Diagnostic Classifications for Lymphomas and Leukemias [10-23].

Acute myeloid leukemia with recurrent genetic abnormalities
Acute myeloid leukemia with balanced translocations/inversions
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
Acute promyelocytic leukemia with *PML-RARA*
AML with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*
AML with t(6;9)(p23;q34.1); *DEK-NUP214*
AML with inv(3)(q21.3q26.2) or t(3;3)(q21;q26.2); *GATA2, MECOM*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); *RBM15-MKL1*
Provisional entity: AML with BCR-ABL1
Acute myeloid leukemia with gene mutations
AML with mutated *NPM1*
AML with biallelic mutation of *CEBPA*
Provisional entity: AML with mutated RUNX1
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia

Acute erythroid leukemia	
Acute megakaryoblastic leukemia	
Acute basophilic leukemia	
Acute panmyelosis with myelofibrosis	
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	
Transient abnormal myelopoiesis associated with Down syndrome	
Myeloid leukemia associated with Down syndrome	
Abbreviation: AML, acute myeloid leukemia	

Table 2: The 2016/2017 World Health Organization classification of acute myeloid leukemia and related precursor neoplasms (Adapted from Arber et al [41] and [42]).

who described clinicopathologic entities that could be precisely diagnosed, including with immunohistochemistry or flow cytometric immunophenotyping [22]. FCI was used in clinical laboratories at least since the 1980s for lineage determination (myeloid, B- or T-lymphoid) in acute leukemias. Rare bilineal or biphenotypic leukemias were recognized by cytomorphology or FCI, leading to the immunological classification of acute leukemias by the EGIL in 1995 [23].

The incorporation of cytogenetics and molecular genetics for diagnosis in acute myeloid leukemia

As noted earlier, as early as the late 1950s, cytogenetics was suggested to classify AML possibly [7-9]. As previously described, the diagnostic paradigm shifted only in 2001 when, based on the principles of the REAL classification, the WHO classification for hematopoietic and lymphoid tumors was introduced, including cytogenetics incorporated for acute leukemia classification [12,24]. That WHO volume represented the 3rd edition, which had included 10 volumes published by the International Agency for Research on Cancer during 2000-2006. The 4th edition (2006-2018) included 12 volumes and 2 revised editions, which had become necessary due to significant genomic advances in tumors of the central nervous system and hematolymphoid tissues. Before 2001, the WHO Classification of Tumors had included the 1st edition (1967-1981) containing a list of accepted terms with short histologic descriptions, and the 2nd edition (1982-2002), which included histologic and immunohistochemical features and one image for each histologic type [25].

Most importantly, the WHO 2001 classification represented a collaboration of pathologists, oncologists, and geneticists worldwide to standardize criteria for the definition and classification of cancer types and standardized nomenclature to ensure progress in clinical cancer care [12].

In the 1990s, large studies had established the role of cytogenetics in establishing prognosis in AML. The Medical Research Council Trial established 3 cytogenetic risk groups: t(8;21), t(15;17), inv(16) as favorable, -5 or -7,

del(5q), abn(3q) or complex karyotype as unfavorable, and normal as intermediate risk, with 5-year survival at 65%, 14% and 41%, respectively [26]. In 1999, a landmark study differentiated acute leukemias as myeloid or lymphoid by applying gene expression microarrays [27]. In 2004, Dutch investigators described 16 classes of AML, including chromosomal abnormalities, *CEBPA* and *FLT3* mutations, *EVI1* overexpression, and groups with normal cytogenetics in 285 cases, using gene expression profiling, FAB criteria, cytogenetics and clinical outcome [28].

Since normal cytogenetics comprised 45-50% of all AML, molecular genetic abnormalities were investigated for risk stratification in a normal karyotype. That motivation led to studies that established the prognostic significance of abnormalities in the *FLT3* and *NPM1* genes. The inactive FLT3 receptor is monomeric and is normally present on bone marrow CD34+ stem cells and immature hematopoietic progenitors (myeloid, monocytic, and B-lymphoid) [29]. The FLT3 receptor dimerizes in its active form, with phosphorylation activating downstream pathways for cell proliferation [29]. Mutations in the *FLT3* gene, located on chromosome 13q12, are most well-known to occur as internal tandem duplication in the juxtamembrane domain (*FLT3*^ITD) and as point mutations in the tyrosine kinase domain (*FLT3*^TKD), and these mutations lead to constitutive activation of the FLT3 receptor leading to uncontrolled cell proliferation [29]. In 2001, in a study of 854 AML, the presence or absence of *FLT3*^ITD mutations in the intermediate-risk cytogenetics group led to increased or decreased relapsed disease, respectively [30]. In 2002, another study of 224 AML showed the worst survival if *FLT3*^ITD-positive, intermediate if *FLT3*^TKD-positive, and best with absent *FLT3* mutations [31]. In 2005, Falini et al. identified insertion mutations in *NPM1* that led to abnormal cytoplasmic localization of the nucleolar protein in AML [32]. Subsequently, the prognostic role of *FLT3* and *NPM1* mutations in AML was ascertained, with the favorable effect of *NPM1* mutations present only if *FLT3*^ITD mutations were absent [33].

In 2001, Pabst et al. described mutations in AML in the *CEBPA* gene, which encodes for the transcription factor CEBPA, crucial for granulocytic differentiation [34].

Interestingly, germline *CEBPA* mutations underlying familial AML were identified in 2004 [35], several years after anticipation was observed in one pedigree with the same type of familial AML [36].

After the Human Genome Project was completed in 2003, the role of mutations in AML pathogenesis was intensely investigated, as reviewed earlier [37]. The Cancer Genome Atlas study of 200 *de novo* AML revealed gene mutations with intricate co-operation patterns and mutual exclusion between and within eight categories of biologically functional genes [38]. Mutations in *NPM1* co-occurred with mutations in *FLT3* or *DNMT3A* [38], similar to prior genomic profiling [39], while gene fusions

were mutually exclusive of mutations in *NPM1, RUNX1, TP53* and *CEBPA* [38]. Importantly, *NPM1* mutations were not detected in age-related clonal hematopoiesis, even with deep sequencing [37]. Subsequently, two types of short-read sequencers enabled analysis of the cancer genome by massively parallel sequencing, termed NGS, in clinical laboratories.

The World Health Organization classification of AML WHO[2001]-WHO[2016/2017]

The evolution of the diagnostic criteria for AML was previously described, with a schematic presented in Figure 3 [12-14,24,40-42].
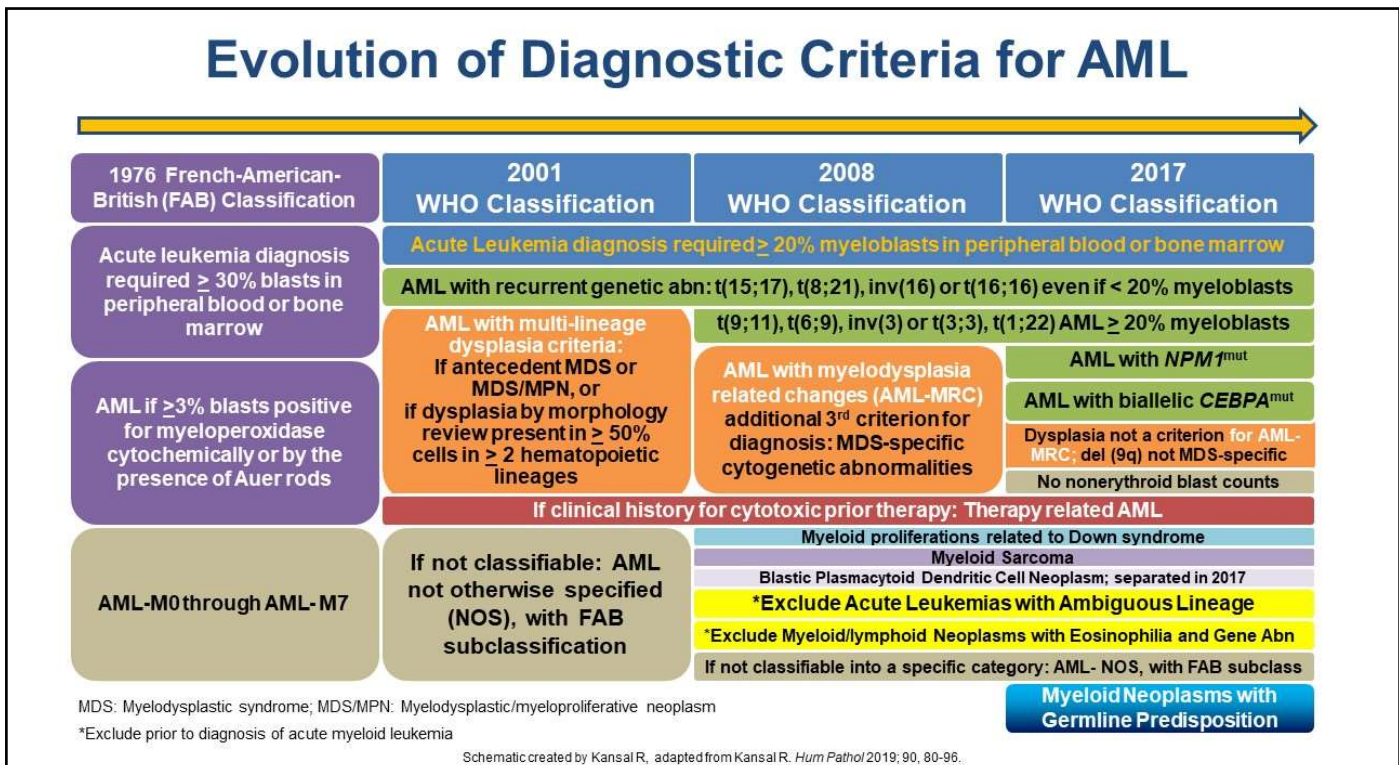
Figure 3: The evolution of diagnostic criteria for acute myeloid leukemia, from the French-American-British (FAB) Group classification to the 2016/2017 World Health Organization (WHO) classification. AML diagnosis by WHO[2001] required ≥ 20% myeloblasts (in the absence of AML-specific chromosomal abnormalities), instead of ≥ 30% by FAB criteria. Essentially, 3 specific categories of AML were described by 2001 WHO: (1) AML with recurrent genetic abnormalities (AML-RGA), (2) AML with multilineage dysplasia, (3) Therapy-related AML. The 4th category, AML, not otherwise specified (AML-NOS), was created for those AML cases that could not be classified into any of the 3 specific categories. By WHO[2008], (1) two major categories, AML-RGA and AML with myelodysplasia (MDS)-related changes (AML-MRC), were refined with additional inclusive cytogenetic criteria, (2) additional categories were recognized that needed to be excluded prior to rendering a diagnosis of AML, including acute leukemias with ambiguous lineage, myeloid/lymphoid neoplasms with eosinophilia and gene abnormalities, and blastic plasmacytoid dendritic cell neoplasm. The category of AML-NOS was retained similar to WHO[2001]. By WHO[2016/2017], AML with mutations in *NPM1* and biallelic *CEBPA* were included as 2 new subtypes. A major change in the WHO[2016] was that multi-lineage dysplasia alone by morphology (microscopic examination) was no longer a criterion for AML-MRC, the diagnosis of which required either MDS-specific cytogenetic abnormalities and/or a prior history of MDS or MDS/myeloproliferative neoplasm. A new category was introduced for germline predisposition in myeloid neoplasms, to encourage recognition of familial myelodysplastic syndromes and AML.

Abbreviations: AML: Acute Myeloid Leukemia; FAB: French-American-British Group; WHO: World Health Organization; MDS: Myelodysplastic Syndrome; MPN: Myeloproliferative Neoplasm

4A

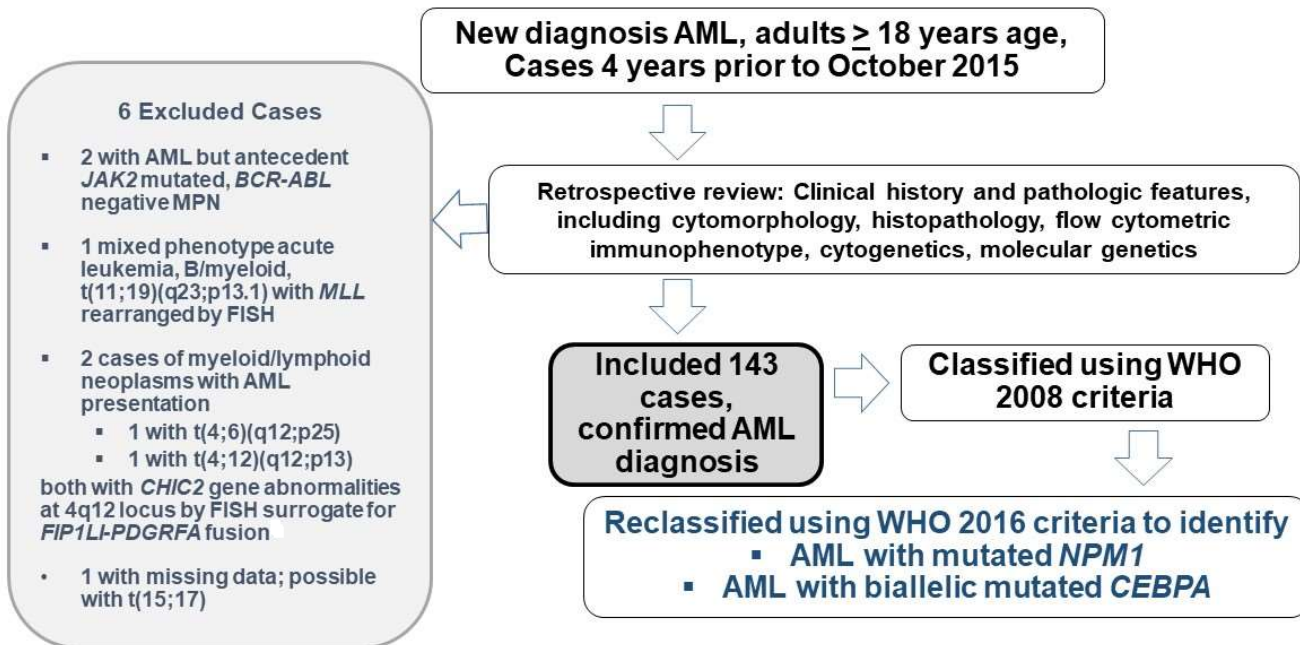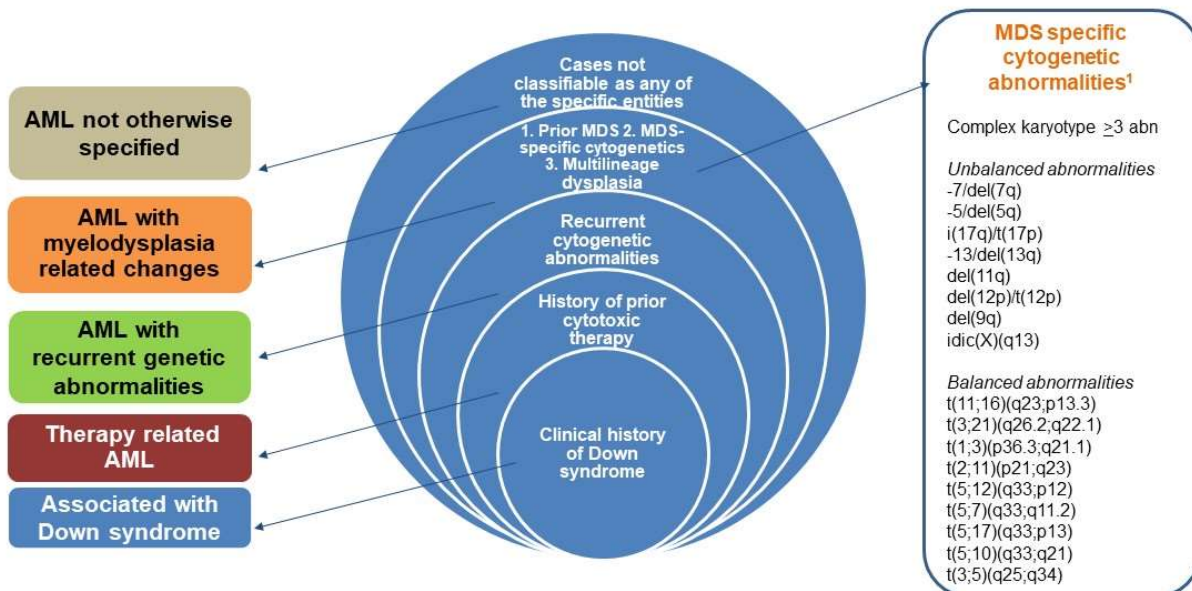## Study Design, Data Review and Excluded Cases

New diagnosis AML, adults ≥ 18 years age, Cases 4 years prior to October 2015

6 Excluded Cases

- 2 with AML but antecedent *JAK2* mutated, *BCR-ABL* negative MPN
- 1 mixed phenotype acute leukemia, B/myeloid, t(11;19)(q23;p13.1) with *MLL* rearranged by FISH
- 2 cases of myeloid/lymphoid neoplasms with AML presentation
  - 1 with t(4;6)(q12;p25)
  - 1 with t(4;12)(q12;p13)
both with *CHIC2* gene abnormalities at 4q12 locus by FISH surrogate for *FIP1LI-PDGRFA* fusion
  - 1 with missing data; possible with t(15;17)

Retrospective review: Clinical history and pathologic features, including cytomorphology, histopathology, flow cytometric immunophenotype, cytogenetics, molecular genetics

Included 143 cases, confirmed AML diagnosis

Classified using WHO 2008 criteria

Reclassified using WHO 2016 criteria to identify
- AML with mutated *NPM1*
- AML with biallelic mutated *CEBPA*

Figure Adapted by Kansal R. from Kansal R. *Hum Pathol* 2019; 90: 80-96.

4B

Methods: 143 AML Classified by WHO 2008

AML not otherwise specified

AML with myelodysplasia related changes

AML with recurrent genetic abnormalities

Therapy related AML

Associated with Down syndrome

Cases not classifiable as any of the specific entities

1. Prior MDS 2. MDS-specific cytogenetics 3. Multilineage dysplasia

Recurrent cytogenetic abnormalities

History of prior cytotoxic therapy

Clinical history of Down syndrome

MDS specific cytogenetic abnormalities[1]

Complex karyotype ≥3 abn

Unbalanced abnormalities
-7/del(7q)
-5/del(5q)
i(17q)/t(17p)
-13/del(13q)
del(11q)
del(12p)/t(12p)
del(9q)
idic(X)(q13)

Balanced abnormalities
t(11;16)(q23;p13.3)
t(3;21)(q26.2;q22.1)
t(1;3)(p36.3;q21.1)
t(2;11)(p21;q23)
t(5;12)(q33;p12)
t(5;7)(q33;q11.2)
t(5;17)(q33;p13)
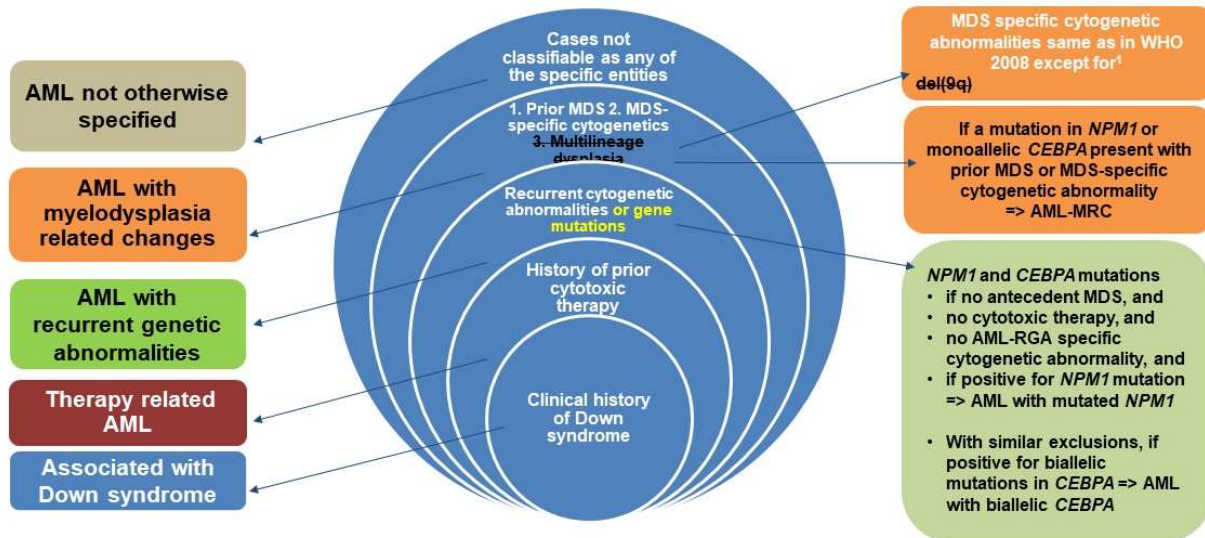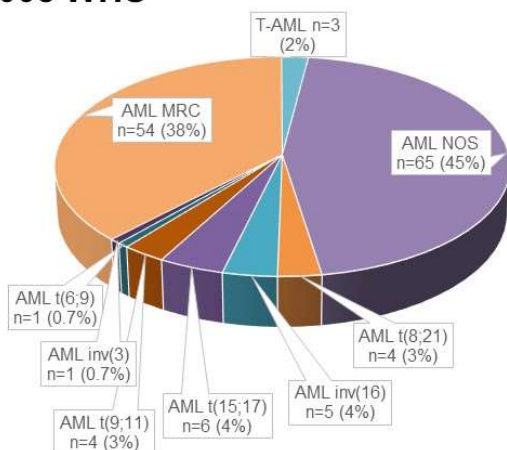t(5;10)(q33;q21)
t(3;5)(q25;q34)

1. Arber et al. AML with MDS related changes. In Swerdlow et al. [Eds.]. IARC: Lyon 2008.

Figure Adapted by Kansal R. from Kansal R. *Hum Pathol* 2019; 90: 80-96.

4C

Methods:143 AML Reclassified by WHO 2016/2017

1. Arber et al. AML with MDS related changes. In Swerdlow et al. [Eds.]. IARC: Lyon 2017.

Figure Adapted by Kansal R. from Kansal R. *Hum Pathol* 2019; 90: 80-96.

4D

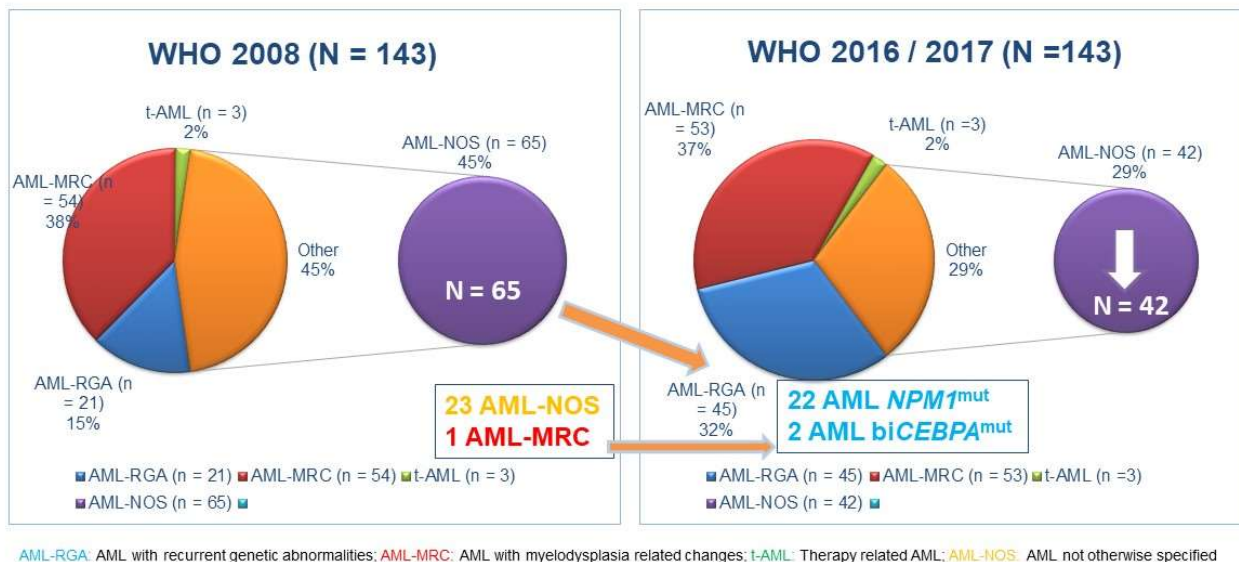# Distribution of 143 AML by WHO²⁰⁰⁸ and WHO²⁰¹⁶ᐟ²⁰¹⁷

Figure Adapted by Kansal R. from Kansal R. *Hum Pathol* 2019; 90: 80-96.

Figure 4: Composite 4A-4E, all figures adapted from *Hum Pathol* 2019; 90:80-96. 4A: Study design, data review and cases. The excluded cases highlight that careful consideration of all integrated findings is required for correct diagnostic classification. **4B**: Schematic for sequential steps to classify 143 cases by WHO[2008]. **4C**: Schematic for sequential steps to reclassify 143 cases by WHO[2016/2017]. **4D**: Distribution of 143 AML by WHO[2008] and WHO[2016/2017]. The numbers of AML cases with recurrent chromosomal abnormalities and for therapy-related AML remained unchanged in WHO[2008] and WHO[2016/2017]. **4E**: 24 (23 AML-NOS and 1 AML-MRC) WHO[2008]-classified cases were reclassified by WHO[2016/2017] as 24 AML with gene mutations (22 AML-*NPM1*[mut] and 2 AML biallelic *CEBPA*[mut]), reducing the AML-NOS cases from 65(45%) to 42(29%), and achieving the goal of classifying AML-NOS cases into specific AML subtypes.

Integrated Genomics for Acute Myeloid Leukemia Diagnosis and Classification

Most significantly, in 2020, we understand AML to be an aggressive clonal hematologic malignancy characterized by marked heterogeneity in genetic and clinical features, with this "single" disease comprised of numerous subtypes, with different prognosis and treatment options. Precise classification of AML, necessary for risk stratification and therapy, is best achieved by the WHO[2016/2017] classification [41,42], which requires not only microscopic skills, but also availability and collaboration for multiple modalities of tests, including clinical history, laboratory hematology, FCI, cytogenetics, and molecular genetics.
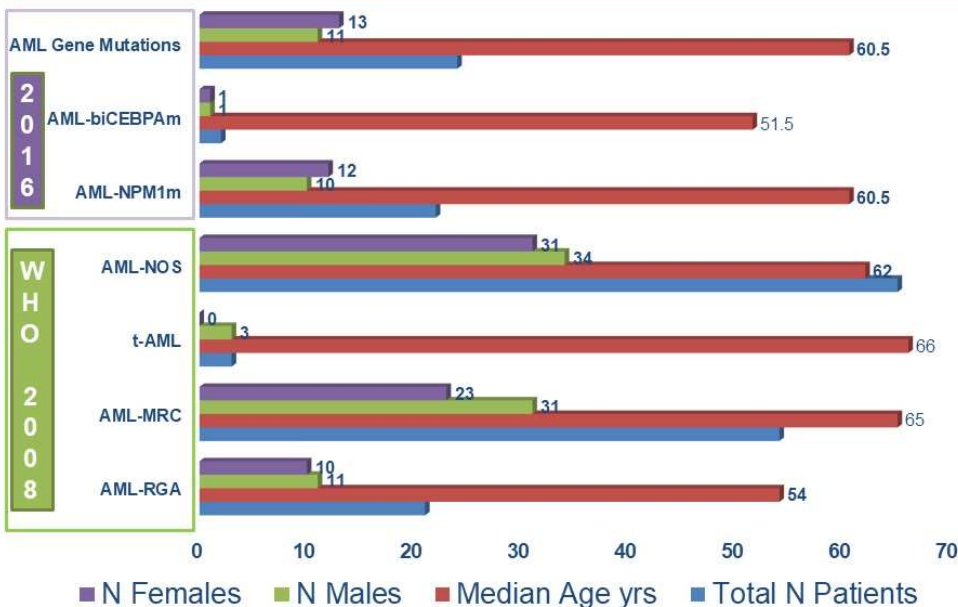
Table 2 shows the WHO[2016/2017] AML classification, including seven subtypes based on chromosomal translocations/inversion, AML-*NPM1*[mut], and AML-bi*CEBPA*[mut] based on gene mutations, and two provisional subtypes [41,42].

As reported in 2019, careful application of the WHO diagnostic criteria leads to the precise classification of the genetically-defined AML subtypes [24]. Here, features previously reported in text/tables [24] are highlighted in the following Figures: Figure 4A-4C, Study design, methods, and excluded cases; Figure 4D, Distribution of all cases by WHO[2008] and WHO[2016/2017]; Figure 4E, WHO[2008]-classified cases reclassified by WHO[2016/2017]; Figure 5A, Median age, numbers of males and females in all AML categories. Additionally, the median age for females with AML-*NPM1*[mut] was lower (49 versus 62 years) than in males with AML-*NPM1*[mut] [24]; Figure 5B, Hematologic findings in all categories; Figure 5C, Cytogenetics findings, and survival outcome in all categories; Figure 6, Smoking status in all molecularly-confirmed AML-*NPM1*[mut]; all three non-smokers with familial AML-*NPM1*[mut] had a history of female relatives with leukemia [24]; also, one young AML-NOS patient with features suggestive of AML-*NPM1*[mut] and with history of familial leukemia, was a non-smoker [24];

5A

5B

5C

Figure 5. Composite 5A-5C, all figures adapted from *Hum Pathol* 2019; 90:80-96. 5A: Patient demographics for 143 AML showing median age, numbers of males and females for all AML categories. Notably, median age (60.5 years) for AML with gene mutations in *NPM1* and *CEBPA* was close to the median age (62 years) of AML-NOS patients, while median age was lower (58 years) for AML with recurrent chromosomal abnormalities. Males dominated in all major WHO[2008] categories, but females dominated in the WHO[2016/2017]-reclassified AML subtypes with gene mutations in *NPM1* and biallelic *CEBPA*.

5B: Overall abnormal hematologic values for all patients, showing (a) Leucocytosis was present in a greater percentage of AML *NPM1*mut cases than in AML with recurrent genetic abnormalities (AML-RGA), AML with myelodysplasia-related changes (AML-MRC), and AML-NOS. (b) AML-*NPM1*mut was characterized by anemia, leucocytosis, and thrombocytopenia. As previously noted [24], by WHO criteria, all AML-*NPM1*mut were *de novo* AML, explaining possibly why thrombocytosis was observed in prior FAB-classified cohorts of AML with *NPM1* mutations, which had included secondary AML.
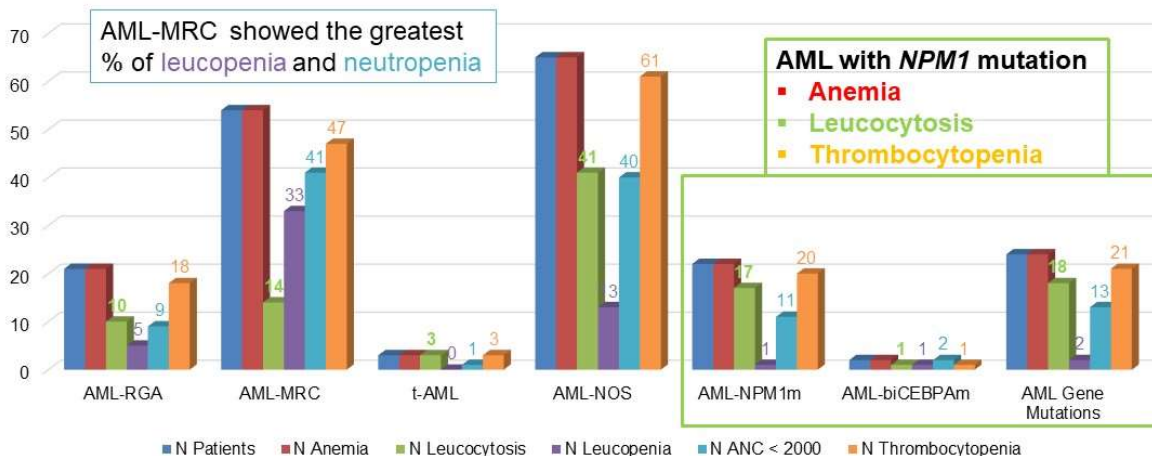
5C: Cytogenetics and overall survival in all 143 AML patients. Cytogenetics findings were as expected in all AML subtypes. Notably, patients with complex cytogenetics in the study cohort comprised a percentage virtually identical to that in a previous large study (Thiede et al. Blood 2006;107:4011-20). Further, alive patients exceeded dead patients among AML-RGA and AML-*NPM1*mut, whereas the reverse was true among AML-MRC, therapy-related AML and AML-NOS patients.
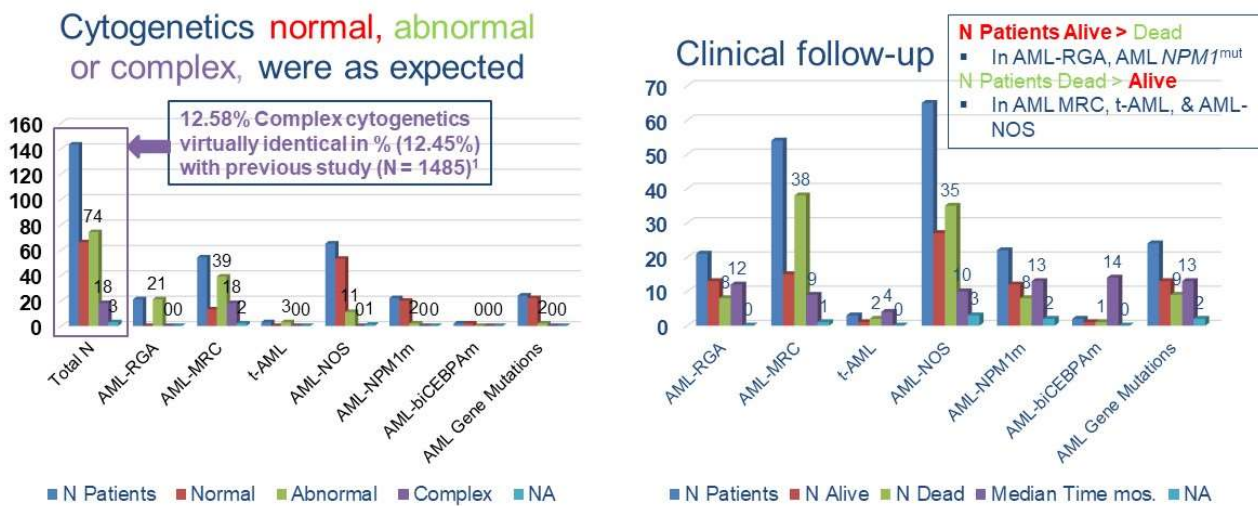
Figure 7, Hematologic features and normal cytogenetics in two AML-bi*CEBPA*mut patients, including a 74-year-old with FAB-M2 subtype with Auer rods and familial leukemia consistent with AML-bi*CEBPA*mut [24,35,43,44].

Table 3 is adapted from a previous review to show demographics of prior reported familial AML-*CEBPA*mut patients (20 males, 19 females, ages 1.75-62 years) from 14 families; 83% (15/18) patients showed normal cytogenetics
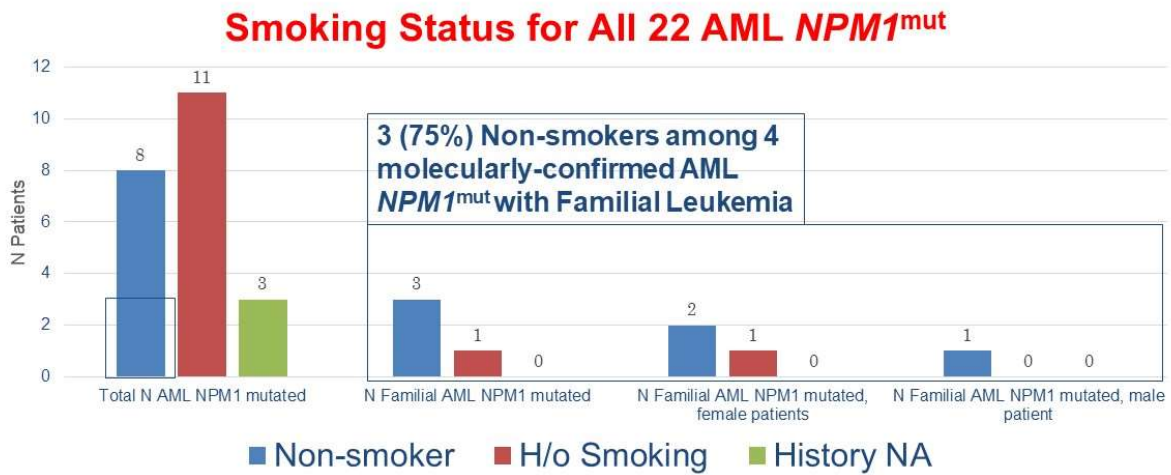
Figure 6: Smoking status in all AML-*NPM1*mut showing 75% (3/4) non-smokers among the 4 familial AML-*NPM1*mut. In contrast, only 26% (5/19) of the non-familial (sporadic) AML-*NPM1*mut were non-smokers, figure adapted from *Hum Pathol* 2019; 90:80-96.

Figure 7: Hematologic features and cytogenetics in 2 AML with biallelic *CEBPA*mut, figure adapted from *Hum Pathol* 2019; 90:80-96.

Reports for Families with Acute Myeloid Leukemia with mutated CEBPA	Patient Gender, Age in years at onset of Acute Myeloid Leukemia	Cytogenetics
Smith et al, 2004	Male, 10	Not done
	Male, 30; female, 18	Both Normal
	Male, 2	
Sellick et al, 2005, De Lord et al, 1997	Male, 34	NA
	Male, 25	del(6)(q21)
	2 Males, 24 and 4	Normal
Pabst et al, 2008, pedigree A	Female, 46	Monosomy 7
	Female, 40	Normal
Pabst et al, 2008, pedigree B	Male, 42	NA
	Female, 27	Normal
Renneville et al, 2009	Female, 23; male, 5	Both Normal
Nanri et al, 2010	2 Males, 39, 26	NA
Taskesen et al, 2011	Female, 25	NA
	Female & male, NA	NA
Taskesen et al, 2011, Stelljes et al, 2011	2 Females, 28, 2	Both Normal
Xiao et al, 2011	Male, 36	del(9)(q11q34)
Debeljak et al, 2013	2 Females, 1.75 (21 months), 15	Both Normal
Tawana et al, 2015	2 Females, 32 and 3	NA
	Female, 18	NA; failed
Yan et al, 2016	Male, 33	del(9)(q13q22)
Pathak et al, 2016	4 Males, 36, 41, 58, 62; 1 female, 53	NA for all 5
	3 Females, 11, 20, 22 y	Normal for all
	2 Males, 2.8 (34 months), 6 y	NA for both
Ram et al, 2017	Female, 36	Normal
Kansal, 2019	Male, 74	Normal
Abbreviation: NA, Not available		

Table 3: Characteristics of previous familial acute myeloid leukemia with mutated *CEBPA* patients compared with that of study case, adapted from publication [44].

[44]. Table 4 highlights that in the cohort by Green et al., the percentage of AML-bi*CEBPA*[mut] patients was highest in the youngest (15-29 years) age group [45].

Characteristics	Biallelic (double) CEBPA[mut]	
	No.	%
No. of patients	59	4%
Age in Years		
15-29	21	36
30-39	12	20
40-49	15	25
50-59	9	15
≥60	2	3
Median Age	35	
Age Range	16-67	
Sex		
Female	30	51
Male	29	49
Type of AML		
De novo	58	98
Secondary	1	2
Abbreviation: AML: Acute Myeloid Leukemia		

Table 4: Characteristics of AML patients with biallelic mutated *CEBPA* from the Green et al publication [45].

Epidemiologic studies are required to determine if there is a real increase in AML with gene mutations in *NPM1* and *CEBPA* in young patients, especially women and if yes, why? Why are women more affected by AML with genetic mutations in *NPM1* and *CEBPA*? Further, studies are necessary for familial AML, including AML-*NPM1*[mut], which comprises the most frequent genetic AML subtype to date in European cohorts [24,46], including for molecular epidemiology, which could potentially identify AML subtypes (familial or sporadic) that may be preventable in the future by lifestyle changes [47], including smoking, which increases the risk of AML [48], or by continued advances in precision gene editing [49].

For routine integrated genomics diagnosis, the WHO classification is meant to be applied worldwide, including in countries with limited resources or advanced molecular genetic techniques. Even in the developed world, laboratory hematologic tests with microscopic evaluation of peripheral blood and bone marrow smears are crucial for a prompt diagnosis of AML. Indeed, a specific diagnosis of APL may be preliminarily rendered with blood smear examination even before a bone marrow biopsy and rapid testing for *PML-RARA* are performed, particularly if combined with rapid myeloperoxidase cytochemical staining that typically shows intense diffuse positivity in APL even in the hypogranular variant, and if present with disseminated intravascular coagulation. The distinction of an APL from a non-APL--AML is the most urgent priority of the pathologist in communication with the clinician, for the correct life-saving therapy. Morphologic smear review is also invaluable to diagnose a possible AML with monocytic or myelomonocytic differentiation, particularly when a neoplasm might not be clinically suspected. Even with minimal monocytosis, a careful smear examination must be performed for abnormal, possibly neoplastic monocytic cells, as illustrated in the WHO books [12,40,42], with bone marrow biopsy performed if needed to rule-out or rule-in an AML, and as described for one AML-*NPM1*[mut] patient [24]. The identification of even rare but unequivocal Auer rods by morphologic review, although that may require time, confirms the diagnosis of a myeloid neoplasm.

FCI using a panel of markers that are required to assign lineage in acute leukemias correctly, is crucial to diagnose AML, including to definitively exclude MPALs, which were first classified as ALALs by WHO[2008], as depicted in Figure 3. ALALs also include acute undifferentiated leukemia, which is extremely rare and must only be considered after all other leukemia subtypes (including blastic plasmacytoid dendritic cell neoplasm and leukemias of other unusual lineages) and non-hematopoietic neoplasms are definitively excluded [42]. Immunohistochemical positivity of the leukemic blasts for myeloperoxidase, lysozyme, or CD117 may be useful to identify myeloid lineage. The reader is referred to a recent expert review by Porwit and Bene for recommendations (and pitfalls) for lineage determination in acute leukemias and diagnosis of MPALs by the EGIL and WHO[2001]-WHO[2016/2017] classifications [50]. The myeloid antigens, CD13, CD33, and CD117, included by the EGIL and WHO[2001] but not by WHO[2008], were myeloid lineage-specific for MPALs by WHO[2016/2017]. Also, while the EGIL classification included CD15 and not CD11c, the WHO[2001]-WHO[2016/2017] included CD11c and not CD15 as myeloid lineage-specific antigens [12,40,42,50]. Importantly, FCI also provides the patient-specific leukemia-associated immunophenotype that serves as a signature for subsequent disease detection, including the expression of CD33 on leukemic cells for anti-CD33 therapy.

Cytogenetics analysis is essential, with molecular analysis for genetic mutations. The presence of the t(8;21), t(15;17), t(16;16) or inv(16) abnormalities is diagnostic of AML even if blasts are less than 20%, and even if a MPAL is suggested

by FCI [12,40-42,50]. MPALs may also show a complex karyotype, which alone does not indicate a diagnosis of AML-MRC [50]. Additionally, MPALs may harbor gene mutations similar to those present in AML [50], further necessitating diagnostic distinction between MPAL and AML. Finally, in communication with the clinician for clinical history and laboratory scientists, the pathologist can best integrate findings from all necessary testing modalities to correctly classify AML, which guides the clinician for risk stratification and therapy.

Figure 8 shows the current NCCN guidelines for risk stratification [51,52]. Table 5 shows the CAP-ASH 2017 guidelines and NCCN guidelines for mutation testing in AML [51-53].

Moreover, AML treatment now includes drugs targeted against *FLT3* and *IDH* mutations, anti-CD33 antibody therapy, and therapies specific for AML-MRC, therapy-related AML, and AML in the elderly, as shown in Table 6, with several additional drugs, including in combinations, in clinical trials [54,55].

All of the above advances now increasingly necessitate comprehensive, integrated diagnostics, including genomics

for AML diagnosis, prognosis, and therapy. Considerations to implement such testing for AML include (1) first, correct diagnostic classification of AML with combined testing modalities and multidisciplinary teams as described above, with the pathologist best able to integrate all findings, (2) cost of testing, which is likely to be much less than treatment costs [56], and (3) turnaround time, which can take much longer by NGS than single-gene PCR assays, especially for *FLT3* and *IDH1/IDH2* mutations. Apart from institution-specific assays [57], commercial assays to allow clinical NGS results in possibly two days from sample collection will soon be available for AML [58]. (4) Several commercially available NGS assays include bulk RNA sequencing for detecting gene fusions [58,59]. (5) Clonal hematopoiesis may co-exist with myeloid neoplasms, including AML, with mutant clone detection dependent upon genomic methods, leading to challenges for interpretation of the significance of genetic mutations identified by NGS [37,60-62]. (6) Technically, in addition to sequencing depth and coverage, variability arising from the entire multi-step NGS process, including nucleic acid extraction, target enrichment, and library preparation, bioinformatics analysis, data analysis, and reporting for genomic aberrations must be considered, including for multi-institutional research, given the variability in NGS
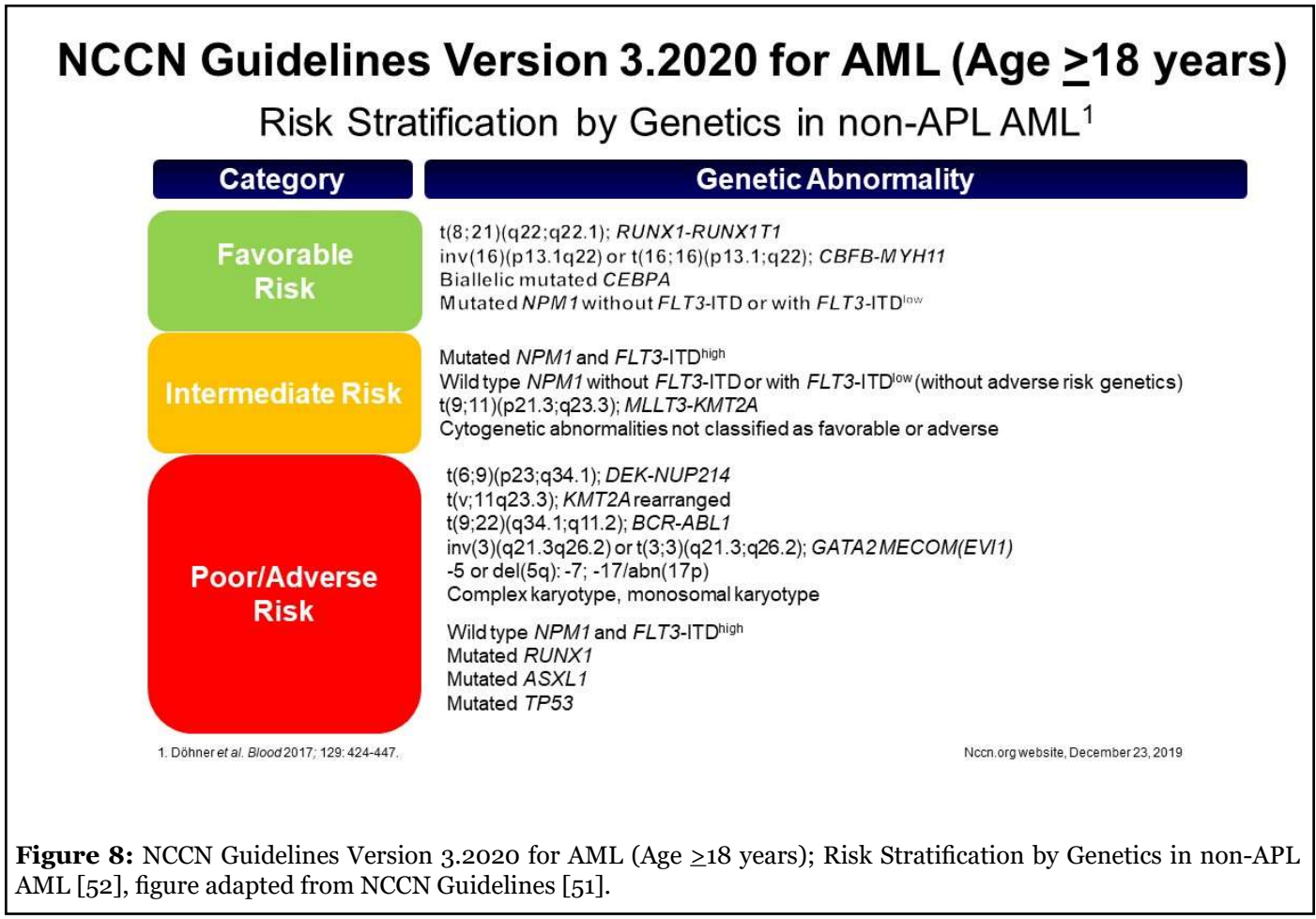
Figure 8: NCCN Guidelines Version 3.2020 for AML (Age ≥18 years); Risk Stratification by Genetics in non-APL AML [52], figure adapted from NCCN Guidelines [51].

2017 CAP-ASH guidelines [53]

In pediatric and adult patients with confirmed or suspected AML of any type
[1]*Strong recommendation*: $FLT3^{ITD}$ testing
[1]*Strong recommendation*: Mutational testing for *NPM1, CEBPA*, and *RUNX1* for AML other than confirmed CBF-AML, APL, or AML with myelodysplasia-related cytogenetic abnormalities
[2]Recommendation: Mutational testing including, but not limited to, *IDH1, IDH2, TET2, WT1, DNMT3A, TP53*

[1]*Strong recommendation*: *KIT* mutation testing in adult patients with confirmed CBF-AML
[3]*Expert consensus opinion*: *KIT* mutation testing in pediatric patients with confirmed CBF-AML

[1]*Strong recommendation*: In suspected APL, ensure rapid testing for *PML-RARA* is performed; ensure appropriate coagulation studies to evaluate for disseminated intravascular coagulation
[3]*Expert consensus opinion*: The pathologist may request cytochemical stains to assist in the diagnosis and classification of AML

[2]*Recommendation:* For molecular or genetic studies, may use cryopreserved cells or nucleic acid, formalin-fixed, non-decalcified paraffin-embedded (FFPE) tissue, or unstained marrow aspirate or peripheral blood smears obtained and prepared from peripheral blood, bone marrow aspirate or other involved tissues in which the use of such material has been validated. Such specimens must be properly identified and stored under appropriate conditions in a laboratory that is in compliance with regulatory and/or accreditation requirements.

[1]*Strong recommendation*: For extramedullary disease without bone marrow or blood involvement (myeloid sarcoma), the pathologist should evaluate and process a tissue biopsy for morphologic, immunophenotypic, cytogenetic, and molecular genetic studies, as recommended for the bone marrow.

[1]*Strong recommendation*: The initial pathology report should include laboratory, morphologic, immunophenotypic, and, if performed, cytochemical data, on which the diagnosis is based, along with a list of any pending tests. The pathologist should issue addenda/amended reports when the results of additional tests become available.

[1]*Strong recommendation*: Ensure that all tests performed for classification, management, predicting prognosis, and disease monitoring are entered into the patient's medical records.
This information should include the sample source, adequacy, and collection information, as applicable.

[1]*Strong recommendation*: Treating physicians and pathologists should use the current WHO classification for diagnosis and classification

[1]*Strong recommendation*: To avoid duplicate procedures, associated patient discomfort, and additional costs, if after examination of a peripheral blood smear, it is determined that the patient will require immediate referral to another institution with expertise in the management of acute leukemia for treatment, the initial institution should, whenever possible, defer *non-emergent* invasive procedures, including bone marrow aspiration and biopsies, to the treatment center.

[1]*Strong recommendation*: Provide the treatment center with all laboratory results, pathology slides, flow cytometry data, cytogenetic information, and a list of pending tests, if a patient is referred, at the time of referral, and forward results of pending tests when available.

NCCN Guidelines v3.2020 [51,52]
Mutational testing for risk stratification: *FLT3, NPM1, CEBPA, CKIT, TP53, ASXL1,* and *RUNX1,* with *FLT3*[ITD], allelic ratio (mutant/normal <0.5 low; ≥0.5 high), as per 2017 European Leukemia Network guidelines [52]
Recommend testing for both *FLT3*[ITD] and *FLT3*[TKD]
Abbreviations: AML, acute myeloid leukemia; CBF-AML, core-binding factor AML (AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* or inv(16)(p13.1q22) /t(16;16)(p13.1;q22); *CBFB-MYH11*); APL, acute promyelocytic leukemia; WHO, World Health Organization [1]Supported by convincing (high) or adequate (intermediate) quality of evidence and clear benefit that outweighs any harms [53]. [2]Some limitations in quality of evidence (adequate [intermediate] or inadequate [low]), balance of benefits and harms, values, or costs, but panel concluded that there is sufficient evidence and/or benefit to inform a recommendation [53]. [3]Serious limitations in quality of evidence (inadequate [low] or insufficient), balance of benefits and harms, values or costs, but panel consensus was that a statement was necessary [53]. Quality of evidence: *Convincing*, high confidence that available evidence reflects true effect, and further research is very unlikely to change the confidence in the estimate of effect; *Adequate*, moderate confidence that available evidence reflects true effect, and further research is likely to have an important effect on the confidence in estimate of effect and may change the estimate; *Inadequate*, Little confidence that available evidence reflects true effect, and further research is very likely to have an important effect on the confidence in the estimate of effect and is likely to change the estimate [53]

Table 5. The College of American Pathologists-American Society for Hematology (CAP-ASH) guidelines and NCCN guidelines for molecular genetic testing in patients with acute myeloid leukemia, adapted from publications [51-53].

Drugs targeted to specific genetic mutation		Approved Date	U.S.A. Food and Drug Administration Approved Indication
Midostaurin	*FLT3* ITD, *FLT3* TKD	April 2017	New diagnosis of *FLT3*-mutated AML in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation
Gilteritinib	*FLT3* ITD, *FLT3* TKD	November 2018	Relapsed/refractory *FLT3*-mutated AML
Enasidenib	*IDH2*	August 2017	Relapsed/refractory *IDH2*-mutated AML
Ivosidenib	*IDH1*	July 2018	Relapsed/refractory *IDH1*-mutated AML
		May 2019	New diagnosis of *IDH1*-mutated AML in patients aged ≥ 75 years or with comorbidities precluding induction chemotherapy
Antibody-drug conjugate targeted against surface antigen			
Gemtuzumab ozogamycin	CD33	September 2017	New diagnosis of CD33-positive AML in adults; relapsed/refractory CD33-positive AML in adults and pediatric patients aged ≥ 2 years

Drugs approved for specific AML patients			
CPX-351	Liposomal cytarabine and daunorubicin in a fixed 5:1 molar ratio	August 2017	New diagnosis of AML with myelodysplasia-related changes or therapy-related AML
Venetoclax	BCL2 inhibitor	November 2018	New diagnosis of AML in patients aged ≥ 75 years or with comorbidities precluding induction chemotherapy
Glasdegib	Hedgehog pathway inhibitor	November 2018	New diagnosis of AML in patients aged ≥ 75 years or with comorbidities precluding induction chemotherapy
Abbreviation: AML: Acute Myeloid Leukemia			

Table 6: Drugs approved for acute myeloid leukemia therapy since 2017.

methods (including manual versus automated) used in different laboratories [63-68]. A recent study showed genomic sequencing results to be institution-specific, with inter-institution heterogeneity in sequencing methods [69]. (7) Particularly for the AML classification, a technical challenge pertains to the detection of mutations in *CEBPA* [70], a GC-rich single-exon gene, and *FLT3*[ITD] mutations by short-read sequencers, as per the experience of many laboratories. Since NGS results for both targets are method-dependent, both genes are often preferentially analyzed by separate PCR-based assays. (8) Differences in NGS assays alone, including customized for academic institutions and commercially available (off-the-shelf or customized), add to the variability in sensitivity and specificity that might vary for different gene targets. Necessarily, if mutations in the genes necessary for precise diagnostic classification might be variably detected inter-institutionally, then ultimately, that would affect the collective diagnoses rendered universally, leading to inadequate or inaccurate assessment of the overall "true" AML subtypes. Similar to the standardized nomenclature required by the WHO classification, standardization of genomic testing for clinical diagnosis, albeit challenging, would be a major step forward towards the very challenging task of integrating genomics in AML for routine clinical care worldwide.

In conclusion, due to progressively rapid advances in technology and genomics, the last two decades have shown exponential progress for depth in our understanding of AML pathogenesis. The WHO[2016/2017] classification of AML represents a significant milestone for the care of patients with AML and has paved the way for future continued collaboration and innovation toward further progress for patient care.

Conflict of Interest

None.

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