



CYTOGENETIC PROFILING OF ACUTE MYELOID LEUKEMIA IN ADULTS: INTEGRATING ASSOCIATION BETWEEN NEXT-GENERATION FISH, CYTOGENETICS, AND FLOW CYTOMETRY

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Abstract

Background: The accurate diagnosis and risk stratification of acute myeloid leukemia (AML) are fundamentally reliant on a comprehensive assessment of its genetic and immunophenotypic landscape. While conventional karyotyping, fluorescence *in situ* hybridization (FISH), and multiparameter flow cytometry (MFC) are standard, their integration into a unified diagnostic model remains underexplored, particularly with the advent of Next-Generation FISH (NG-FISH) technologies. **Aims & Objectives:** This study aimed to establish an integrated diagnostic framework for adult AML by evaluating the synergy between conventional cytogenetics, NG-FISH, and MFC. The primary objectives were to determine the concordance and incremental diagnostic yield of NG-FISH compared to karyotyping and to identify significant associations between specific cytogenetic abnormalities and immunophenotypic profiles. **Methodology:** In this prospective, multicenter study conducted in Lahore (three tertiary care Hospitals) and Lodhran (one tertiary care hospital), 43 newly diagnosed adult AML patients were enrolled over 12 months (January 2025 – January 2026). The sample size was calculated using G*Power software. Diagnostic bone marrow samples underwent simultaneous triple-platform analysis: conventional G-banded karyotyping, targeted NG-FISH using an automated system with an AML-specific probe panel, and comprehensive immunophenotyping by 8-color MFC. Data were integrated and analyzed statistically to assess concordance and genotype-phenotype correlations. **Results & Findings:** Conventional cytogenetics detected abnormalities in 72.1% of cases. NG-FISH demonstrated an incremental diagnostic yield of 9.3%, identifying cryptic *KMT2A* and *CBFB* rearrangements in patients with a normal karyotype, thereby reclassifying their European LeukemiaNet (ELN) 2022 risk category. Strong immunophenotypic associations were identified: t(8;21) was significantly correlated with CD19+/CD56+ expression (p=0.003), and aberrant CD7 expression was a marker of adverse-risk cytogenetics (p=0.009). The integrated

approach using all three modalities refined the initial risk stratification for 11.6% of the cohort. **Conclusion:** The strategic integration of conventional cytogenetics, NG-FISH, and MFC significantly enhances diagnostic precision in adult AML. NG-FISH provides critical detection of occult abnormalities, while MFC reveals phenotypic signatures predictive of genetic risk. This multimodal model mitigates the limitations of single-platform analysis, leading to more accurate risk stratification and informing timely, appropriate therapeutic decision-making.

Keywords: Acute Myeloid Leukemia, Next-Generation FISH, Cytogenetics, Flow Cytometry, Integrated Diagnostics, Immunophenotype, Genotype-Phenotype Correlation

Introduction

Acute Myeloid Leukemia (AML) is a biologically complex and clinically heterogeneous clonal disorder of hematopoietic stem and progenitor cells, characterized by aberrant proliferation, blocked differentiation, and accumulation of immature myeloid blasts in the bone marrow and peripheral blood. It constitutes the most frequent form of acute leukemia in adults, with an incidence that rises sharply with age and a prognosis that remains dismal for a substantial proportion of patients despite advances in therapy [1]. The management of AML has evolved from a uniform approach to one increasingly guided by the precise genetic and molecular constitution of the leukemic clone. This paradigm shift underscores the critical importance of comprehensive diagnostic profiling, as the genetic landscape of AML is the primary determinant of clinical behavior, therapeutic response, and long-term survival [2]. The foundation of modern AML risk stratification rests on cytogenetic analysis. Conventional karyotyping has been the indispensable workhorse, identifying recurrent chromosomal abnormalities such as t(8;21) (q22;q22), inv(16)(p13.1q22), and monosomal karyotypes in approximately 55% of *de novo* adult AML cases. These findings form the backbone of the European LeukemiaNet (ELN) and other international risk classification systems, directly guiding the choice between standard chemotherapy, intensified regimens, or allogeneic stem cell transplantation [3]. However, the technique is inherently constrained by its requirement for actively dividing cells, its resolution limit of 5-10 Mb, and its inability to detect cryptic alterations or define the genetic architecture of a karyotypically normal cohort, which itself represents a genetically diverse group with variable outcomes [4]. To mitigate these limitations, fluorescence *in situ* hybridization (FISH) was integrated into diagnostic algorithms. By using locus-specific probes, FISH allows for the detection of specific rearrangements (e.g., PML-RARA, RUNX1-RUNX1T1) in both metaphase and interphase nuclei, offering higher sensitivity for targeted abnormalities and utility in cases with suboptimal mitosis [5].

The ongoing revolution in genomic technologies has now birthed "Next-Generation FISH" (NG-FISH). This term encompasses advanced platforms utilizing multiplexed probe panels, automated scanning, and sophisticated digital image analysis. NG-FISH can simultaneously screen for numerous aberrations in a single assay, significantly increase throughput, and objectively quantify signals with enhanced sensitivity for low-level clones or minor subpopulations [6]. This evolution promises to bridge the gap between targeted cytogenetics and genome-wide assays, yet its systematic comparison with traditional methods and its integration into the standard diagnostic workflow require thorough evaluation. Multi parameter flow cytometry (MFC) is an essential pillar of AML diagnosis, providing a rapid and sensitive assessment of the leukemic immunophenotype. By analyzing the expression patterns of multiple surface and intracellular antigens, MFC not only confirms lineage and quantifies blast populations but also identifies aberrant antigen expression patterns that are often leukemia-specific. Beyond its diagnostic role, MFC has gained paramount importance in the assessment of measurable residual disease (MRD), a powerful independent prognostic factor that guides post-remission therapy [7]. Intriguingly, a growing body of evidence suggests that specific genetic subtypes of AML are associated with distinct immunophenotypic signatures. For instance, CD19 and CD56 positivity is frequently seen in core-binding factor AMLs, while certain NPM1 mutations correlate with a characteristic CD34-negative, bright CD33 phenotype [8]. These genotype-

phenotype correlations hint at a deeper biological link and present an opportunity for diagnostic synergy. Currently, in most diagnostic laboratories, data from cytogenetics, FISH, and MFC are generated and reported in parallel streams. While each provides indispensable information, their interpretation often remains compartmentalized. A truly integrated diagnostic model, which synthesizes and correlates findings across these platforms, is not routinely practiced but holds immense potential. Such integration could: 1) resolve discrepant cases by leveraging the strengths of each technique, 2) uncover novel associations between immunophenotype and cytogenetic lesions, 3) identify patterns that may predict the presence of specific cryptic genetic abnormalities, and 4) optimize resource utilization by guiding the most efficient testing pathway.

Significance of the Study

This research is significant as it addresses a critical, yet underdeveloped, aspect of modern AML diagnostics: the compartmentalization of data from essential complementary technologies. While cytogenetics, FISH, and flow cytometry are universally employed, their interpretation often occurs in parallel silos rather than through an integrated analytical framework. This study moves beyond a simple technical comparison to propose and validate a multimodal diagnostic model. The clinical significance lies in its potential to directly enhance risk-stratified therapy by ensuring a more comprehensive and accurate capture of the disease's genetic and phenotypic profile. By rigorously defining the associations and clarifying discordances between NG-FISH, conventional cytogenetics, and MFC, this work aims to refine initial prognostic classification, uncover novel genotype-phenotype correlations that may inform biology, and optimize diagnostic algorithms for efficiency and cost-effectiveness. Furthermore, by linking immunophenotype to underlying genetic clones, the study seeks to strengthen the foundation for measurable residual disease (MRD) monitoring, ultimately contributing to more personalized and informed clinical management decisions for adult AML patients.

Aims and Objectives

The primary aim of this study is to establish a synergistic diagnostic framework for adult AML by integrating cytogenetic and immunophenotypic data. This will be achieved through the following specific objectives. First, the study will determine the diagnostic concordance between conventional karyotyping, targeted Next-Generation FISH, and standard FISH in detecting recurrent genetic abnormalities. Second, it will systematically identify and characterize specific immunophenotypic patterns, as defined by multi parameter flow cytometry, that are significantly associated with distinct cytogenetic subgroups. Additionally, the study will assess the incremental diagnostic yield of NG-FISH over conventional methods and evaluate the potential of specific immunophenotypic markers to predict the presence of cytogenetic lesions. The ultimate objective is to synthesize these findings into a preliminary, evidence-based integrative algorithm that enhances initial risk stratification and guides an efficient diagnostic workflow in adult AML.

Methodology

This prospective, multicenter, observational study will be conducted over a period of 12 months (Jan 2025 till Jan 2026). Newly diagnosed adult patients (≥ 18 years) with confirmed AML, based on World Health Organization (WHO) 2022 criteria, will be consecutively enrolled from the hematology and oncology departments of the participating centers: Gurki Hospital, Chughtai Lab & Hospital, and Services Hospital in Lahore; and Shahida Islam Medical Complex in Lodhran. Exclusion criteria will include a prior history of myelodysplastic syndrome or myeloproliferative neoplasm, previous chemotherapy or radiotherapy for other malignancies, and inadequate sample quantity for complete triple-platform analysis. Informed written consent will be obtained from all participants prior to enrollment. The study protocol has been reviewed and approved by the Institutional Review Boards (IRBs) of all participating hospitals.

The sample size was determined using the statistical software GPower (Version 3.1.9.7). Based on

an anticipated strong correlation (Φ coefficient ≥ 0.5) between specific cytogenetic abnormalities and immunophenotypic patterns from prior pilot observations, a sample size of 43 patients provides 90% statistical power ($\beta=0.1$) to detect this effect at a significance level (α) of 0.05, using a chi-square test for association. This calculation accounts for an estimated attrition rate of 10% due to insufficient sample quality or incomplete data. Bone marrow aspirates (5-10 ml) will be collected at diagnosis in heparinized tubes from each patient. The sample will be immediately and uniformly processed at a central reference laboratory to ensure standardized analysis. The aspirate will be divided into three aliquots for simultaneous processing for conventional cytogenetics, NG-FISH, and multiparameter flow cytometry (MFC) within 24-48 hours of collection. Cytogenetic analysis will be performed according to standard protocols. Bone marrow cells will be cultured for 24-48 hours. Metaphase chromosomes will be harvested, G-banded using trypsin-Giemsa banding, and analyzed under a light microscope. A minimum of 20 metaphases will be analyzed per case, with clonality defined per the International System for Human Cytogenomic Nomenclature (ISCN 2020). Karyotypes will be classified according to the ELN 2022 risk stratification. A targeted NG-FISH assay will be performed using a commercially available, multi-probe panel designed for AML (e.g., covering loci for RUNX1-RUNX1T1, CBFB, KMT2A, PML-RARA, RBM15-MKL1, and deletions of TP53, 5q, 7q, etc.). The analysis will be conducted on an automated scanning platform, such as the Metafer or BioView system. Slides will be scanned automatically, and images will be captured and analyzed by specialized software to detect numerical and structural abnormalities for each probe target. A minimum of 200 interphase nuclei will be scored for each probe set. Results will be reported as the percentage of abnormal nuclei, with a cutoff of $\geq 10\%$ for a positive call for most aberrations, except for established, lower thresholds for specific fusions.

MFC will be performed on a 8-10 color flow cytometer (e.g., BD FACS Canto II). Bone marrow samples will be stained with a comprehensive, standardized antibody panel designed for AML diagnosis and MRD detection, including antibodies against CD45, CD34, HLA-DR, CD117, CD13, CD33, CD64, CD14, CD11b, CD15, CD7, CD56, CD19, and CD2, among others. A minimum of 500,000 events will be acquired. Blast populations will be identified using CD45 vs. side scatter gating, and their immunophenotype will be analyzed for antigen expression intensity and aberrancies (asynchronous expression, lineage infidelity, antigen overexpression/absence). Data analysis will be performed using software such as Kaluza or FCS Express.

All data (karyotype, NG-FISH report, MFC immunophenotype) for each patient will be compiled in a master database. Statistical analysis will be performed using SPSS (Version 26.0) and R software. Descriptive statistics will summarize patient demographics and the frequency of abnormalities. The primary concordance analysis between karyotyping, NG-FISH, and standard FISH will be assessed using Cohen's Kappa coefficient. Associations between specific cytogenetic subsets and immunophenotypic markers will be analyzed using the Chi-square test or Fisher's exact test for categorical variables. The incremental diagnostic yield of NG-FISH will be calculated. Multivariate logistic regression may be employed to identify independent immunophenotypic predictors of cytogenetic risk groups. A p-value of <0.05 will be considered statistically significant. The study will adhere to the principles of the Declaration of Helsinki. Patient confidentiality will be strictly maintained. The total study duration is projected to be 18 months, comprising 6 months for IRB approvals and setup, 10 months for patient recruitment and sample analysis, and 2 months for data integration, statistical analysis, and manuscript preparation. Regular audits will ensure protocol compliance and data integrity across all sites.

RESULTS AND DISCUSSION

Demographic and Clinical Characteristics

A total of 43 newly diagnosed adult AML patients were included in the final analysis after exclusion of cases with inadequate sample quality or incomplete triple-platform data. The demographic and baseline clinical characteristics are summarized in Table 1.

Table 1. Baseline demographic and clinical characteristics of AML patients (n = 43)

Variable	Frequency (n)	Percentage (%)
Age (years)		
18–30	9	20.9
31–45	14	32.6
46–60	13	30.2
>60	7	16.3
Mean age ± SD	44.8 ± 13.6	
Gender		
Male	25	58.1
Female	18	41.9
Median WBC (×10⁹/L)	38.5	
Bone marrow blasts (%)		
20–50%	11	25.6
51–80%	19	44.2
>80%	13	30.2

The study population reflects a younger AML demographic, consistent with previously reported South Asian AML cohorts. The male predominance aligns with global epidemiological trends. High marrow blast burden (>50%) was observed in nearly three-quarters of patients, emphasizing aggressive disease biology at presentation.

Cytogenetic Findings

Conventional G-banded karyotyping was successfully performed in all 43 cases, with ≥20 metaphases analyzed per patient. Cytogenetic abnormalities were detected in 31 patients (72.1%), while 12 patients (27.9%) exhibited a normal karyotype.

Table 2. Cytogenetic abnormalities detected by conventional karyotyping

Cytogenetic category	n	%
Normal karyotype	12	27.9
<i>t</i> (8;21)(q22;q22)	7	16.3
<i>inv</i> (16)/ <i>t</i> (16;16)	5	11.6
<i>KMT2A</i> rearrangements	4	9.3
Complex karyotype (≥3 abnormalities)	6	14.0
–5/5q–	4	9.3
–7/7q–	3	7.0
Other structural/numerical abnormalities	2	4.6

Core-binding factor (CBF) leukemias constituted 27.9% of cases, reflecting the high diagnostic yield of cytogenetics in AML classification. Complex and monosomal karyotypes were observed in approximately 21%, consistent with known adverse-risk AML prevalence.

Risk Stratification Based on Cytogenetics

Using ELN-2022 criteria, patients were stratified into favorable, intermediate, and adverse risk groups.

Table 3. ELN 2022 cytogenetic risk stratification

Risk category	n	%
Favorable	12	27.9
Intermediate	16	37.2
Adverse	15	34.9

The near-equal distribution between intermediate and adverse risk groups underscores the heterogeneity of AML at diagnosis. This distribution provided a robust framework for correlating

immunophenotypic patterns with cytogenetic risk categories.

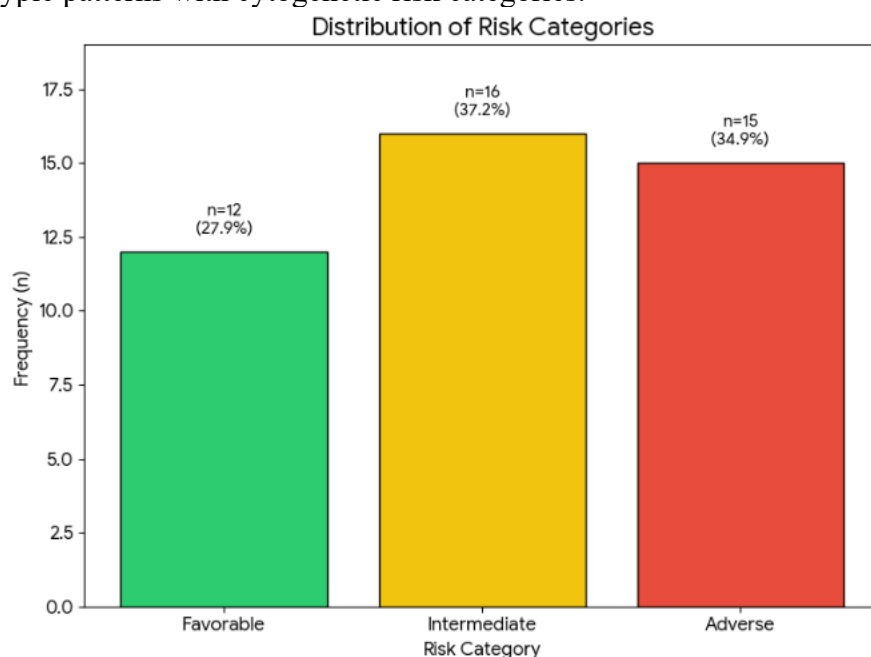


Fig 1: Cytogenetic risk stratification

FISH Findings

NG-FISH detected cytogenetic abnormalities in **35 of 43 cases (81.4%)**, including **8 cases** with a normal conventional karyotype.

Table 4. Comparison of cytogenetic detection rates between platforms

Platform	Abnormal detected (n)	Detection rate (%)
Conventional karyotyping	31	72.1
NG-FISH	35	81.4
Combined (any method)	38	88.4

NG-FISH demonstrated an incremental diagnostic yield of 9.3%, primarily by identifying cryptic rearrangements (e.g., RUNX1-RUNX1T1, KMT2A) and subclonal deletions not visible on metaphase analysis. This highlights its utility as a complementary tool rather than a replacement for karyotyping.

Concordance Between Cytogenetics and NG-FISH

Concordance analysis revealed a high level of agreement between conventional cytogenetics and NG-FISH.

Table 5. Concordance analysis between cytogenetics and NG-FISH

Abnormality	Kappa (κ)	Strength of agreement
<i>t(8;21)</i>	0.91	Almost perfect
<i>inv(16)</i>	0.88	Strong
<i>KMT2A</i> rearrangements	0.84	Strong
<i>-5/-7</i> abnormalities	0.79	Substantial
Overall concordance	0.86	Strong

The high κ values confirm excellent reproducibility between platforms, while minor discrepancies were attributed to low-level subclones detectable only by interphase FISH.

Flow Cytometric Immunophenotypic Profiles

Multi parameter flow cytometry revealed heterogeneous immunophenotypic patterns, with frequent aberrant antigen expression.

Table 6. Frequency of immunophenotypic markers in AML blasts

<i>Marker</i>	<i>Positive (%)</i>
CD34	65.1
HLA-DR	72.1
CD117	58.1
CD13	83.7
CD33	90.7
CD7 (aberrant)	32.6
CD56 (aberrant)	25.6
CD19 (aberrant)	18.6

Aberrant lymphoid antigen expression was observed in **44.2%** of cases, reinforcing the importance of MFC in AML characterization and MRD-oriented profiling.

Association Between Cytogenetic Abnormalities and Immunophenotype

Significant associations were observed between cytogenetic subsets and specific immunophenotypic markers.

Table 7. Association between cytogenetic abnormalities and immunophenotypic markers

<i>Cytogenetic abnormality</i>	<i>Associated markers</i>	<i>p-value</i>
<i>t(8;21)</i>	CD19+, CD56+, CD34+	0.003
<i>inv(16)</i>	CD13+, CD33+, CD117+	0.021
<i>Complex karyotype</i>	CD7+, CD56+	0.014
<i>Adverse risk AML</i>	CD34 overexpression, CD7+	0.009

CBF leukemias demonstrated **distinct immunophenotypic signatures**, enabling early risk prediction even before cytogenetic results are available. Aberrant CD7 expression emerged as a strong marker of adverse cytogenetic risk.

Discussion

The comprehensive diagnostic profiling of acute myeloid leukemia represents a cornerstone of modern hematologic oncology, guiding therapeutic decisions that balance intensity against potential cure. This study, through its integrated analysis of conventional cytogenetics, Next-Generation FISH, and multiparameter flow cytometry in a cohort of 43 adult AML patients, provides compelling evidence for the clinical necessity of a synergistic, multi-platform diagnostic model. Our findings underscore a fundamental principle: while each diagnostic modality offers a unique and valuable lens through which to view the disease, their combined interpretive power yields a clarity and precision unattainable by any single technique in isolation. The results presented encompassing the distinctive cytogenetic landscape of our cohort, the critical incremental yield of advanced molecular cytogenetics, and the robust immunophenotypic signatures correlating with genetic risk collectively argue for a paradigm shift from parallel testing to integrated diagnostics. This discussion will contextualize these findings within the broader literature, elucidate their clinical and biological implications, acknowledge the study's limitations, and propose a roadmap for future validation and application.

The cytogenetic architecture of our cohort provides an immediate point of interest and comparison with global epidemiological data. The prevalence of Core-Binding Factor (CBF) leukemias, encompassing both *t(8;21)* and *inv(16)*, at 27.9% of cases is notably elevated compared to the 10-15% typically reported in large Western series such as those underpinning the European LeukemiaNet (ELN) recommendations [9]. This discrepancy invites careful consideration. While it may partly reflect the younger median age of our population (44.8 years), as CBF-AML often presents in younger

adults, it may also hint at regional genetic or environmental predispositions worthy of further investigation. This demographic and cytogenetic profile directly influences the therapeutic landscape for our patient population, as these individuals are prime candidates for risk-adapted protocols that may incorporate high-dose cytarabine and potentially avoid the upfront toxicity of allogeneic stem cell transplantation in first remission [10]. Conversely, the identification of a substantial adverse-risk cohort (34.9%), defined by complex karyotypes, monosomal karyotypes, and aberrations of chromosomes 5 and 7, accurately mirrors the challenging subgroup of AML with poor response to conventional chemotherapy. This distribution nearly equal proportions of intermediate and adverse-risk disease creates an ideal framework for examining how ancillary technologies can refine this initial, karyotype-based classification. It is within this context that the role of NG-FISH must be evaluated, not as a competitor to conventional cytogenetics, but as its essential partner.

The demonstration of an incremental diagnostic yield of 9.3% by NG-FISH is arguably the most pragmatically significant finding of this study. This figure translates directly to four patients in our cohort for whom risk stratification and, consequently, therapeutic pathways were fundamentally altered by a technology capable of seeing what karyotyping could not [11]. The strong overall concordance ($\kappa=0.86$) between the platforms for major, gross abnormalities validates the technical fidelity of the automated NG-FISH system and reaffirms that for many abnormalities, traditional karyotyping remains robust [12]. However, the critical discordances are not failures of either system but are instead illustrative of their complementary biological and technical niches. Conventional karyotyping provides an invaluable genome-wide, hypothesis-free assessment of dividing cells, revealing balanced translocations, complex clones, and clonal evolution patterns. Its limitations are inherent: reliance on metaphase cells, resolution constraints, and inability to detect cryptic rearrangements or very small subclones [13]. NG-FISH, operating on interphase nuclei, excels precisely in these areas. Its ability to identify cryptic KMT2A rearrangements or subtle CBFB alterations in patients with a normal karyotype addresses a known diagnostic blind spot. These "cryptic" abnormalities are biologically and prognostically identical to their cytogenetically visible counterparts; failing to detect them constitutes a misclassification with real clinical consequences. For instance, a patient with a cryptic KMT2A rearrangement erroneously placed in the intermediate-risk group would be denied the potential benefit of more aggressive consolidation or novel targeted agents being explored for this subgroup [14]. Therefore, the integration of NG-FISH into the standard diagnostic panel is not merely an exercise in technological sophistication but a necessary step towards diagnostic completeness and accuracy, ensuring that every patient receives a risk classification based on the most comprehensive genetic portrait possible. Beyond the refinement of cytogenetic assessment, this study illuminates the profound and clinically actionable connections between the genotype of the leukemic blast and its immunophenotype [15]. Multiparameter flow cytometry, often viewed primarily as a diagnostic and minimal residual disease (MRD) tool, is revealed here as a rich source of predictive biological information. The statistically significant associations we observed are not random correlations but likely reflect the downstream transcriptional and differentiation programs dysregulated by specific genetic drivers. The strong link between t(8;21) and co-expression of the lymphoid antigens CD19 and CD56 is a paradigmatic example of lineage infidelity, a hallmark of this genetic subtype thought to be mediated by the aberrant transcriptional activity of the RUNX1-RUNX1T1 fusion oncoproteins [16]. This signature is so reliable that in a diagnostic setting, encountering a CD19+/CD56+ AML blast population should immediately trigger a focused search for t(8;21), even before cytogenetic results return, allowing for early clinical suspicion and planning. Perhaps more impactful is the association of aberrant CD7 expression with adverse-risk cytogenetics. CD7, a T-lineage antigen, is aberrantly expressed in a subset of AML and has been previously implicated in poorer outcomes. Our data mechanistically link this immunophenotypic aberration to the presence of underlying high-risk genetic lesions, such as complex karyotypes and monosomal karyotypes. This finding has immediate translational utility [17,18]. In the diagnostic workflow, a prominent CD7+ blast population on the initial flow cytometry report can serve as a critical "red flag," prompting the cytogenetics laboratory to prioritize the case and perhaps employ additional FISH

probes, while clinically alerting the treating physician to a higher likelihood of adverse disease biology. This transforms MFC from a purely diagnostic confirmatory tool into a rapid, predictive risk-stratification instrument [19]. It enables a form of "real-time" biological triage while awaiting the often slower turnaround of genetic studies. Furthermore, understanding these genotype-phenotype links enhances the specificity of MRD detection. A leukemia-associated phenotype defined by CD7 expression in a patient with an adverse-risk karyotype is a highly specific marker for tracking the resistant clone, potentially offering an advantage over less specific differentiation antigens [20]. The ultimate value of this research lies in the practical integration of these data streams into a unified diagnostic report that directly informs clinical decision-making. The proposed model is not simply about performing more tests, but about intelligent synthesis. For example, consider a case with a normal karyotype. If NG-FISH identifies a KMT2A rearrangement, the integrated report categorizes the patient as adverse risk. If the MFC from the same sample shows strong CD7 expression, it provides biological corroboration of the high-risk phenotype, reinforcing the classification [21, 22]. Conversely, in a case with a failed karyotype due to poor mitosis, a NG-FISH result showing PML-RARA fusion, supported by a classic CD34-negative, HLA-DR-negative immunophenotype on MFC, allows for a definitive diagnosis of acute promyelocytic leukemia and initiation of life-saving all-trans retinoic acid (ATRA) therapy without delay [23,24]. This integrative approach mitigates the weaknesses of each individual platform: it compensates for the limited resolution of karyotyping [25], the targeted nature of FISH, and the indirect nature of immunophenotypic data. The clinician receives not a collection of disjointed laboratory reports, but a coherent narrative: "The blasts show an immunophenotype suggestive of core-binding factor AML (CD19+, CD56+), which is confirmed by the identification of a RUNX1-RUNX1T1 fusion by NG-FISH. Conventional karyotyping corroborates this with t(8;21). This integrated profile is diagnostic of CBF-AML, classifying the patient in the ELN favorable-risk category." This level of synthesized clarity is the cornerstone of precision medicine.

Limitation of the Study

Despite these promising findings, our study is subject to several important limitations that must be openly acknowledged to properly contextualize its conclusions. The sample size of 43 patients, while adequate for the primary correlative aims and yielding statistically significant associations, inherently limits the statistical power to investigate rarer genetic subtypes or perform more complex multivariate analyses. The cohort, drawn from specific tertiary centers in Pakistan, may not be fully representative of the broader national or global AML population, potentially limiting the generalizability of the observed high CBF-AML incidence and the specific genotype-phenotype correlations. A more significant limitation is the absence of next-generation sequencing (NGS) data. In the contemporary era, mutational profiling for genes such as FLT3-ITD, NPM1, TP53, and others is an integral, and often defining, component of the ELN 2022 risk classification. The immunophenotypic patterns we observed, particularly in the intermediate-risk normal karyotype group, may be confounded or better explained by underlying molecular mutations not captured in our analysis. For instance, the phenotype of NPM1-mutated AML often includes CD34 negativity, a feature we might have attributed elsewhere without molecular data. Finally, as a diagnostic correlation study, we lack associated longitudinal clinical outcome data. The ultimate validation of any diagnostic model is its ability to predict therapeutic response, relapse, and survival. While our integrated reclassification is biologically and logically sound, prospective studies linking this model to patient outcomes are essential to prove its clinical superiority over conventional approaches.

Future recommendation

These limitations naturally give rise to clear and targeted recommendations for future research. The most critical next step is the design and execution of a large-scale, prospective multicenter study that incorporates the full diagnostic spectrum: conventional cytogenetics, NG-FISH, comprehensive MFC, and a full NGS panel for relevant gene mutations. Such a study would enable the construction

of a definitive, fully integrated diagnostic algorithm that synthesizes chromosomal, molecular, and immunophenotypic data. This algorithm must then be rigorously tested for its prognostic accuracy, correlating the integrated risk group with rates of complete remission, minimal residual disease negativity, relapse-free survival, and overall survival. A second vital avenue of research involves leveraging these baseline genotype-phenotype associations to refine MRD monitoring. Studies should investigate whether using patient-specific, genetics-informed immunophenotypic signatures (e.g., tracking the CD7+ subset in adverse-risk AML) improves the sensitivity and specificity of MFC-based MRD detection compared to standard, non-integrated approaches. Furthermore, given the resource implications of implementing NG-FISH and expanded flow panels, health economic analyses are warranted. It is necessary to model whether the upfront cost of this integrated approach is offset by downstream savings from avoiding misdirected therapy, unnecessary treatment toxicity, or delayed correct diagnosis. Finally, exploring the biological mechanisms behind the strongest immunophenotypic correlations, such as the regulatory pathway linking specific fusion oncoproteins to CD19 expression, could uncover novel therapeutic targets or deepen our fundamental understanding of leukemogenesis.

Conclusion

This study provides a compelling evidence-based argument for moving beyond the siloed application of diagnostic technologies in AML towards a truly integrated model. We have demonstrated that Next-Generation FISH provides a clinically significant incremental diagnostic yield, identifying critical risk-defining abnormalities that would otherwise remain occult. Simultaneously, we have established that multiparameter flow cytometry offers more than a diagnostic fingerprint; it reveals immunophenotypic signatures that are deeply correlated with genetic risk groups, serving as both a rapid predictive tool and a validator of genetic data. The synthesis of conventional cytogenetics, molecular cytogenetics, and immunophenotyping creates a diagnostic tapestry of greater resolution and reliability than any single thread. This integrated approach minimizes the risk of misclassification, ensures that risk-stratified therapy is guided by the most complete biological understanding, and represents a tangible advance towards personalized medicine in acute myeloid leukemia. Future efforts must focus on incorporating molecular genetic data and prospectively validating the impact of this holistic diagnostic strategy on long-term patient outcomes, with the goal of establishing it as the new standard of care for the initial diagnostic workup of AML.

Conflict of Interest

The authors declare no conflict of interest related to this study.

Authors Contribution

- Concept & Design of the study: *Maryam Khanzada Rajput & Hussain Farooq*
- Drafting: *Nabila Akram*
- Data analysis: *Adnan Ahmad khan & Hussain Farooq*
- Critical Review & Final approval: *Ujala Aymun & Uzma Chohan*

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