



MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES



TRANSCRIPTOME BASED MOLECULAR SUBTYPING OF AML AND ELUCIDATION OF MOLECULAR RESPONSES UNDER DIFFERENT THERAPEUTIC AGENTS

NURDAN KELEŞOĞLU

Ph.D.THESIS

Department of Bioengineering

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Thesis Co-Supervisor

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ÖZET

TEZ BAŞLIĞI: AKUT MİYELOİD LÖSEMİNİN TRANSKRİPTOM BAZLI MOLEKÜLER ALT TİPLEMESİ VE FARKLI TERAPÖTİK AJANLAR ALTINDA MOLEKÜLER CEVAPLARIN AYDINLATILMASI

Akut miyeloid lösemi (AML), kanda ve kemik iliğinde biriken ve normal kan hücrelerine müdahale eden anormal hücrelerin hızlı büyümesiyle ortaya çıkan, bir tür habis ve heterojen hematopoetik sistem hastalığıdır. Bugüne kadar, AML'nin ilk olarak uygulanan tedavisi hala geleneksel kemoterapi rejimine dayanmaktadır. Bununla birlikte, AML'nin remisyon oranının tamamı, özellikle yaşlı hastalarda hala iyi bir durum sergilememekte ve tam remisyondan sonra nüks oranı hala yüksektir.. Son birkaç yıl içerisinde AML'nin anlaşılmasında ve tedavisinde büyük gelişmeler kaydedilmesine rağmen, AML hastalarının hayatta kalmalarını uzatacak çok az ilerleme kaydedilmiştir. Bunlara ek olarak, son on yıldaki gen ekspresyonu profillemeye çalışmaları AML hakkında önemli moleküler bulgular ortaya koymuştur, ancak yeterli tarama ve etkili tedavi stratejileri henüz uygun bir şekilde başırlanamamıştır. Bu çalışmada yetişkin AML hastalarının transkriptom verileri üzerinde bir meta-analiz yapılması ve genom ölçeğinde biyo-moleküler ağlara sahip ekspresyon profilleri tarafından RNA, protein ve metabolit seviyelerinde reporter genlerin belirlenmesi hedeflenmektedir. Bu yaklaşım, AML'deki halihazırda bilinen biyobelirteçleri, tümör baskılayıcıları ve onkogenleri, ayrıca yeni biyobelirteç adayları ve potansiyel terapötik hedefler olarak miRNA'ları, transkripsiyon faktörlerini, diğer ve metabolitleri ortaya çıkarmıştır. Prognostik biyobelirteçlere ek olarak, AML tedavisi için potansiyel terapötik adayları önceliklendirmek için ilaç yeniden konumlandırma yapılmıştır. Nortriptilin, doksepin ve estramustinin AML hücre dizileri üzerindeki inhibitör etkileri, bu ilaçların AML tedavisinde kullanıldığı ileri çalışmalar için umut verici çıktılar sağlamıştır. Bu çalışma prognostik biyobelirteçlerde ilacın yeniden konumlandırılması yoluyla yeni hastalık adaylarının tespit edilmesini ve hastalık teşhisinde iyileşmeyi sağlayacak gelecekteki çalışmalar için yeni bir sistem sağlar. Tekrarlayan mutasyona uğramış AML genleri ve yolakları arasındaki potansiyel önemli birçok ilişkinin tanınması, patogenezin genetik kurallarının anlaşılması için kapsamlı bir temel oluşturacaktır.

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ABSTRACT

THESIS TITLE: TRANSCRIPTOME BASED MOLECULAR SUBTYPING OF AML AND ELUCIDATION OF MOLECULAR RESPONSES UNDER DIFFERENT THERAPEUTIC AGENTS

Acute myeloid leukemia (AML) is a malignant and heterogeneous disease of the hematopoietic system characterized by rapid growth of abnormal cells that accumulate in the blood and bone marrow and interfere with blood cells. To date, first-line treatment of AML has been based mostly on conventional chemotherapy. However, the rate of complete remission of AML is still not optimistic, especially in elderly patients, and the relapse rate after complete remission is still high. Despite significant improvements in the understanding and treatment of AML in recent years, little progress has been made in AML patient outcomes and survival. In addition, gene expression profiling studies have provided molecular insights into AML over the past decade, but adequate screening and effective treatment strategies have yet to be developed. In the current study, we aim to perform a meta-analysis of transcriptome data of adult AML patients and identify reporter biomolecules at the RNA, protein, and metabolite levels by integrating gene expression profiles with genome-level biomolecular networks. This approach revealed already known biomarkers, tumor suppressors and oncogenes in AML, as well as miRNAs, transcription factors, others and metabolites as novel biomarker candidates and potential therapeutic targets. In addition to prognostic biomarkers, we performed drug repositioning to prioritize potential therapeutic candidates for the treatment of AML. As a result of these analyzes, we proposed repositioned drugs including desipramine, doxepin, estramustine, hydrochlorothiazide, nortriptyline, and risedronate for the drug treatment of AML. The inhibitory effects of the nortriptyline, doxepin and estramustine on AML cell lines gave us promising output for further studies using these drugs in the treatment of AML. This study provides a framework for future studies that will improve disease diagnostics and enable the identification of new drug candidates by repositioning drugs on prognostic biomarkers. The recognition of many potentially important relationships between recurrent mutant AML genes and pathways provides a comprehensive foundation for understanding the genetic rules of pathogenesis.

February, 2023

Nurdan KELEŞOĞLU

CLAIM OF ORIGINALITY

THESIS TITLE: TRANSCRIPTOME BASED MOLECULAR SUBTYPING OF AML AND ELUCIDATION OF MOLECULAR RESPONSES UNDER DIFFERENT THERAPEUTIC AGENTS

Acute myeloid leukemia (AML) is a heterogeneous group of blood cell neoplasms characterized by the accumulation of immature blast cells in the bone marrow and the suppression of normal blood cell production. Despite recent developments in treatment and improvements in certain subtypes of AML, the disease is still notoriously difficult to cure. Although quite a few patients achieve remission after induction therapy, relapses are common, and the 5-year overall survival rate is still quite poor. Intensive basic and translational research has shed much light on the pathobiology and genetic variability of AML in recent decades. This research has led to the discovery of several promising new therapies for AML. Large-scale genomic studies have greatly improved our understanding of the molecular landscape of AML, including the impact of numerous recurrent mutations that often have prognostic and, in some cases, therapeutic value. The main goal of this dissertation was to identify prognostic biomarkers, drug-responsive molecules, and modules that play essential roles in AML and to reposition drugs for the treatment of the disease using a systems biomedicine approach. The dissertation consists of three main parts and each part contains novelties in many respects, such as: (i) We have applied for the first time three biomolecular networks at the whole genome level to the study of AML to identify novel prognostic biomarkers and potential therapeutic targets by considering the interconnected nature of signaling, regulatory and metabolic processes within a cell. (ii) We have proposed novel prognostic networks for AML by applying differential co-expression analysis for the first time. Using comparative and integrative analysis, we constructed a co-expression network linking genes involved in drug response and unresponsive genes. Data extracted and analyzed included patients with AML who were refractory, relapsed, or had never been treated. Patients who have not responded to previous therapy and/or untreated patients may benefit from learning about potentially treatment-relevant genes and their co-expressed network by identifying changes in co-expression patterns of genes in patients. iii) The currently used therapeutic options may fail in some patients and they are or become resistant

to drugs. Therefore, an innovative approach that can evaluate AML at different biological levels and propose drug candidates is needed. Integration of expression profiles at transcript levels from AML patients allowed deciphering the major aberrations that may occur as a consequence of the disease. The proposed systems biology and drug repositioning methods allowed us to prioritize currently approved drugs for the treatment of prolactinoma. Six drugs, including desipramine, doxepin, estramustine, hydrochlorothiazide, nortriptyline, and risedronate were proposed as potential therapeutics for AML. Experiments on cell lines demonstrated a significant decline in cell viability, the initiation of cell death, and changes in the protein expressions of Akt.

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Prof. Dr. Kazım Yalçın ARGA

Nurdan KELEŞOĞLU

SYMBOLS

α Alpha

β Beta

μ Micro



ABBREVIATIONS

AML	Acute myeloid leukemia
ALL	Acute Lymphocytic Leukemia
APL	Acute promyelocytic leukemia
AR	Allelic ratio
ATRA	All-trans retinoic acid
BIND	The Biomolecular Interaction Network Database
CALGB	Cancer and Leukemia Group B
CBFs	Core-binding factors
CEBPA	CCAAT/enhancement-binding protein alpha
ChIP	Chromatin immunoprecipitation
CK-AML	Complex karyotype Acute Myeloid Leukemia
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CN-AML	Cytogenetically normal Acute Myeloid Leukemia
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEGs	Differentially expressed genes
DLBCL	Diffuse Large B-cell Lymphoma
ELN	European LeukemiaNet
ESCs	Embryonic stem cells
FAB	French-American-British
FLT3	FMS-related tyrosine kinase 3
GEO	Gene Expression Omnibus
GO	Gene Ontology
H3K4	Histone H3 on lysine residue 4
HCT	Hematopoietic cell transplantation
HDAC	Histone deacetylase
HiDAC	High-dose Ara-C
HMR	Human Metabolic Reaction
HPID	The Human Protein Interaction Database
HRAS	Harvey RAS

HSCT	Hematopoietic stem cell transplantation
HSPs	Heat shock proteins
HTRIdb	Human Transcriptional Regulation Interactions database
IC	Induction chemotherapy
IDAC	Intermediate dose cytarabine
IDH	Isocitrate dehydrogenases
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRAS	Kirsten RAS
LDH	Lactate dehydrogenase
LIMMA	Linear Models for Microarray Data
MBRole	Metabolites Biological Role
MINT	Molecular Interaction database
miRNA	Micro RNA
MLL	Mixed lineage leukemia
MRD	Minimal residual disease
NGS	Next-generation sequencing
NOS	Not otherwise specified
NPM1	Nucleophosmin 1
NRAS	Neuroblastoma RAS
PANTHER	Protein Analysis Through Evolutionary Relationship
PCC	Pearson's correlation coefficients
PGDBs	Pathway or Genome DataBases
PPIN	PPI networks
PPIs	Protein-protein interactions
R/R	relapsed/refractory
RMA	Robust Multi-Array Average
RTK	Receptor tyrosine kinase
TCGA	The Cancer Genome Atlas
TFs	Transcription factors
TKI	Tyrosine kinase inhibitor
TRNs	Transcriptional regulatory networks

VPA	Valproic acid
WHO	World Health Organization
Y2H	Yeast two-hybrid



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1. INTRODUCTION

Systems biology is one of the most exciting topics that gained popularity in the field of biomedicine at the beginning of the century. Although the number of definitions for the term "systems biology" is currently close to the number of practitioners, there is general agreement that systems biology is an interdisciplinary discipline that uses principles, insights, and techniques from biology, computer science, medicine, physics, chemistry, and engineering to connect the dots between these fields. Ideker et al. (Ideker et al. 2001) defined systems biology as the study of biological systems through the systematic application of perturbations (biological, genetic, or chemical), the measurement of the responses of genes, proteins, and pathways, the integration of these data, and the development of mathematical models that describe the structure of the system and its response to individual perturbations. In this sense, the computational tools provided by bioinformatics are also essential. This whole process required constant interaction with experimental biologists. Systems biology is thus an approach to model-based, hypothesis-driven science accompanied by experimental testing of these ideas.

1.1. System Biomedicine: Networks Based Approach for Understanding The Mechanism And Biomarker Discovery

The systemic approach examines the underlying interactions between molecules to better understand biological processes rather than focusing on the specific features. These methods have been slow to develop in part due to a lack of big data. The omics revolution has made it possible to generate such datasets, and the networks constructed from them have become standard research in molecular systems biology. In what is often referred to as network or systems medicine, these systemic concepts are applied to the study of human disease. Evidence and insights from the application of systems medicine are leading to a paradigm shift in therapeutic target discovery and new drug development, giving rise to new areas of study such as network pharmacology. Biological systems can be studied from the perspective of a network model, which represents them as dynamic graphs whose structure is determined

by the interdependence of many components (nodes) and their associated interactions (edges).

The expression of genes in a cell is controlled by a gene regulatory network, which is a complex set of highly interconnected activities. Many pairings of proteins and genes exhibit this type of network, in which one protein or gene controls the amount of another protein or gene in the cell or the activity of the second protein or gene. The proteins in the network regulate the production, activity, and degradation of other proteins, which in turn regulate the distribution of materials and cellular resources. The term "network" can refer to either a permanent or a constantly changing set of connections. The structure and data of a network can evolve over time and space, raising the possibility that the network has both a geographic and temporal dimension. Gene regulatory networks cover a wide range of systems that deal with different facets of the intricate interplay between genes and the proteins they produce in a cell (Schlitt and Brazma 2007). They can be considered in the context of networks of gene expression, gene co-expression, and gene interaction, as well as networks of protein interaction, signal transduction, transcription, and more. They are designated according to the level of abstraction used to describe them and their unique physical, chemical, and functional properties. The components responsible for gene expression and signal transduction exhibit significant duplication. Proteins not only regulate gene expression but are also the end result of gene transcription. Metabolic reactions are facilitated by proteins. The availability of metabolites is a critical requirement for most regulatory processes.

1.1.1. Molecular Networks

1.1.1.1. Transcription Regulatory Networks

One of the most fundamental biological regulatory mechanisms of gene expression is transcriptional regulation. Transcription factors (TFs) are proteins that can recognize and bind to specific sequence elements in DNA located in the control regions of genes. Many transcription factors can regulate a single gene, and many genes can be regulated by a single transcription factor. Transcriptional regulatory networks (TRNs) describe the connections

between TF proteins and the genes they control by binding to specific DNA motifs. TRNs characterize the gene expression patterns that determine the expression of cell type-specific proteins during development and determine ultimate fate and responsiveness to environmental changes (He and Tan 2016). Since the production of molecular data is no longer a constraint, systems biology must now address the modeling and representation of biological networks. The description of TRNs should allow us to learn about their role and predict their actions. In addition, it should contribute to the search for novel pharmacological targets, oncogenes, and candidates for cellular reprogramming. One subset of interest is the transcription network, which is formed exclusively by genes encoding transcription factors (TF genes). Gene networks consisting of TF genes and their targets (target genes) form the backbone of the entire gene network. A directed graph can be used to represent the TF gene network in mammalian cells. The vertices of this graph represent the TF genes, and the edges represent the causal connections between the genes. Each edge represents a combination of gene expression and trans-regulatory events (Panditrao et al. 2022).

The methods currently used to develop computer models for TRNs can be divided into three groups, depending on the type of data used for the analysis. The first methods are those that use only gene expression profiles as data input. The term "reverse engineering" is used to describe these methods. In particular, they start with the regulatory output (e.g., expression level). Several methods in this category have been developed using different computational frameworks (Lefebvre et al. 2012). Some examples of these techniques are linear regression, statistical correlation, and Bayesian networks. The second techniques include those that combine gene expression information with ChIP-X data. The combination of high-throughput methods such as sequencing or microarrays (hereafter referred to as ChIP-Seq/Chip) with chromatin immunoprecipitation (ChIP) provides information about the occupancy of a particular TF throughout the genome (Qin et al. 2011). The amount of this information has increased exponentially in recent years. Although ChIP-X is beneficial, it is difficult to infer regulatory relationships from data from this approach alone. This is because ChIP-X can only detect binding events, and binding events are necessary but not sufficient for functional regulatory interactions. The third techniques include gene expression data,

transcription factor data, and information obtained by analysis of chromatin interactions with ChIP-X. The methods presented here take a direct approach to the subproblem of TRN modeling that requires identification of enhancer-promoter interactions (He and Tan 2016).

1.1.1.2. Protein Interaction Networks

Proteins play important roles in living systems as catalysts, structural elements, signal transducers, and molecular mechanisms. Protein-protein interactions (PPIs) play a critical role in coordinating cellular processes. They serve as building blocks in numerous transcriptional regulatory networks and cellular signal transduction pathways. Large-scale proteome-wide studies of proteins interacting in the coordinated network of metabolic, signaling, and regulatory pathways in a cell have become standard since the accessibility of comprehensive and analyzed genome sequences from different organisms has moved the research approach away from studying individual proteins in an organism. Systems biologists are increasingly interested in studying PPIs. Protein folding, protein assembly, and PPI rely on non-covalent contacts between side chains of residues. All types of PPIs and compounds are initiated by these contacts. PPIs can be divided into a variety of subgroups based on their unique structural and functional properties.

PPIs are also critical for understanding protein function in cells. By observing how a protein interacts with a protein whose function is known, one can draw conclusions about the protein's potential functionality. The molecular mechanisms of cellular processes can now be better visualized thanks to extensive research on PPIs, which has accelerated the modeling of functional pathways. Determining the biochemistry of a cell is greatly facilitated by a better understanding of the interactions between proteins in a given proteome. Several methods exist to determine the outcome of a protein interaction with a known functional target.

The discovery of PPI data is useful in the search for therapeutic targets. Proteins with a higher number of contacts (hubs) have been found in a variety of contexts, such as enzyme families, transcription factor networks, and inherently disordered protein networks. PPIs, on the other hand, are involved in more diverse processes and expand the scope of regulation required.

To understand their function in the cell, it is necessary to identify the multiple interactions that occur and the consequences of these interactions. Two-hybrid systems, mass spectrometry, phage display, and protein chip technology are just a few of the current high-throughput experimental approaches used to reliably improve PPI data. Extensive PPI networks have been established through the use of these experimental approaches. While validation in the laboratory is essential, the volume of PPI data poses a significant problem. Understanding the role of as yet unidentified proteins increasingly depends on the computational study of PPI networks. Protein-protein interaction (PPI) is currently one of the most important areas of investigation in modern systems biology.

There are three broad categories of techniques for detecting protein-protein interactions: *in vitro*, *in vivo*, and *in silico*. One method is performed in a controlled environment outside of a living being, as is the case with *in vitro* techniques. Tandem affinity purification, affinity chromatography, co-immunoprecipitation, protein arrays, protein fragment complementation, phage display, X-ray crystallography, and nuclear magnetic resonance spectroscopy are all *in vitro* approaches used to detect PPIs. *In vivo* methods use a whole living organism to perform an experiment or test. Yeast two-hybrid (Y2H) and yeast synthetic lethality (Y3H) are *in vivo* techniques used for PPI identification (Brückner et al. 2009). *In silico* methods are fully computerized using digital simulation. Sequence-based approaches, structure-based approaches, chromosome proximity, gene fusion, *in silico* 2 hybrid, mirror tree, phylogenetic tree, and gene expression-based approaches are all types of *in silico* methods used for PPI discovery.

The constant production of a huge amount of experimental PPI data necessitated the development of machine-readable biological databases to store and analyze this information. The Biomolecular Interaction Network Database (BIND) is based on a flexible specification system that allows a detailed description of the experimental procedure that led to the data from PPI and often includes direct links to the concluding evidence in the literature (Bader et al. 2003). In addition, experimentally determined binary protein-protein interactions can be found in the interacting proteins database (DIP) (Xenarios 2002). Another important source of data is the database STRING, which contains both empirically determined and

anticipated physical and functional interactions with confidence values (von Mering 2004). Another source of experimentally obtained data PPI is the Molecular Interaction database (MINT), which obtains this information from the scientific literature and also contains the strength of evidence for each interaction (Zanzoni et al. 2002). Data on protein and genetic interactions among thirteen species are stored in BioGRID (Oughtred et al. 2021). HitPredict is a database of reliable protein-protein interactions that allows us to view all interactions for a given protein and obtain confidence scores for each (A. Patil et al. 2011). The Human Protein Interaction Database (HPID) was created to provide precomputed data on human protein interactions using already available structural and experimental information (K. Han et al. 2004). Protein interaction data can be stored, viewed and analyzed using IntAct, an open-source database and toolbox (Hermjakob et al. 2004). The protein interaction network can be explored in the context of the GO annotations of the interacting proteins, and both textual and graphical representations of the interactions are available through the online interface. APID is an interactive bioinformatics web tool developed to enable the exploration and analysis of current knowledge on protein-protein interactions integrated and unified in a common and comparative platform (Prieto and de Las Rivas 2006).

PPI networks (PPIN) are described as heterogeneous networks whose edges represent protein interactions. As a first step in the computational study of PPIN, a diagram of the structure of the network is presented (Ryan and Matthews 2005). A mathematical graph consisting of vertices and edges represents the basic version of a model. In such a graph, a protein serves as a node and the other proteins that physically interact with it serve as nodes that are physically close to each other and connected by an edge. The results of a network analysis are highly variable. For example, proteins that are close to each other in a network diagram often have similar functions. Densely connected subgraphs in the network are likely to form protein complexes as a unit in some biological processes, apart from their utility. Therefore, looking at the proteins with which a protein interacts and the protein complexes in which it resides can provide insight into the protein's function. The topological information from PPIN alone can be used to predict potential new contacts, which is an interesting and practical development. The network is visualized using various analysis techniques, such as random

layout algorithm, circular layout algorithm, hierarchical layout algorithm, and so on. A large amount of data is produced every year, and it is difficult for researchers to keep up with all this data and make sense of it. Over the years, many tools have been developed to visualize PPI networks, as visualizing this high-throughput data is particularly valuable for user interpretation. Cytoscape is a tool for visualizing molecular interaction networks and linking them to gene expression profiles (Shannon et al. 2003). Large databases of gene expression data, protein-protein, protein-DNA, and genetic linkages are becoming increasingly available for humans and model organisms, and this tool works best when used in conjunction with these resources. Several methods for representing networks are available in Cytoscape. Cytoscape can be customized with a number of useful plug-ins. Several clustering methods have been used, including MCODE, ClusterONE, and ClusterViz. The cytoHubba plugin can also use topological techniques such as degrees to locate important nodes and subnetworks (e.g., hub proteins and protein modules in the PPI network). Most importantly, the NetworkAnalyzer plugin can be used to calculate a wide range of network features.

1.1.1.3. Metabolic Networks

Metabolites can be either small molecules such as glucose and amino acids or large molecules such as polysaccharides and glycans, which are critical for cell growth, survival, and proliferation. With the exception of a few processes in the cell, enzymes usually serve as catalysts for transformation. An important aspect in biochemistry is a metabolic pathway, which is a series of sequential or closely related biochemical events that carry out a particular metabolic activity. A metabolic network provides a more comprehensive and accurate picture of cellular metabolism than its individual pathways.

Reconstructions of metabolic networks provide a useful framework for pooling information from different types of genomic and proteomic studies (Palsson 2002). A metabolic network reconstruction is a manually controlled, computational framework that allows the description of gene-protein reaction interactions. This framework is constructed in part using annotated genomes as well as data on biochemical pathways, genetic traits, and cell phenotypes (Chavali et al. 2012). These metabolic reconstructions have proven useful in a number of

studies to support the formation of new biological hypotheses and insights (Aydin et al. 2018; Kelesoglu et al. 2022). Metabolic networks have gained popularity in the last three decades. Many studies using current genomic data have been conducted to reconstruct and model metabolic networks. Typically, genomic sequence data and molecular physiology are used as the starting point for this reconstruction. Processing and understanding all this high-throughput data can be very challenging. Due to the development of bioinformatics technologies, many biological metabolic networks from a variety of species can now be reconstructed and analyzed. Information can also be found by exploring online databases. As a result, it is likely that effective and user-friendly tools will be created to accomplish this task. The process of reconstructing a metabolic network requires several processes such as creating a model, building a detailed model, describing the model quantitatively, filling gaps, and simulation and visualization. The first two phases are the processes of searching for functional annotations of genes, which can be achieved by extracting and matching biochemical reaction data from databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Covert et al. 2004; Ogata et al. 1999). The metabolic model is built by converting the functional annotations into the corresponding biological reactions.

Among the many metabolic network databases are those that focus on a single organism, such as EcoCyc (for *Escherichia coli*) (Peter D. Karp et al. 2014), HinCyc (for *Haemophilus influenzae*) (P D Karp et al. 1996), and PseudoCyc (for *Pseudomonas aeruginosa*) (Romero and Karp 2003), and those covering a variety of organisms, even across entire kingdoms of life, including KEGG, Biocyc, and MetaCyc. To facilitate the transition from gene catalogs to pathway catalogs, KEGG was one of the first pathway databases to be introduced (Ogata et al. 1999). This bioinformatics database stores data such as proteins, genes, pathways, and reactions. It is also a useful bioinformatics tool for making connections between genetic material and ecological systems. Karp et al. created BioCyc as a collection of 161 Pathway or Genome DataBases (PGDBs) that provide structured data on genomes and cellular networks and facilitate extensive computational study and use of the data (Peter D Karp et al. 2005). The popular MetaCyc database, created by Karp et al, contains details on the enzymes and metabolic pathways of about 158 different organisms (Peter D. Karp et al.

2014). In addition, metabolic information can be found in the MetaCyc online database. The substrates of compounds, products, and enzymes in their metabolic pathways are documented here. These databases have the metabolic pathway as the primary unit of description. Such pathways and their links are documented in the databases in the form of data sets and images.

Another set of databases, including BRENDA, ENZYME, and the combined IntEnz project, are dedicated to a single reaction. BRENDA is a protein function database that is constantly updated and reviewed by extracting data directly from the literature (Schomburg et al. 2004). This database contains a huge amount of enzymatic and metabolic information. Sheu et al. conducted a study using the database ENZYME to make predictions about enzyme interaction sites (Bairoch 1999; Sheu et al. 2005). As enzymes, proteins catalyze biological reactions and are therefore essential for life. Enzyme-related information such as reaction specificity, functional parameters, substrates, products, and inhibitors are all included. More importantly, IntEnz is a relational database that combines enzyme information from each of these resources (Fleischmann et al. 2004). Each metabolic database has its own set of data retrieval tools that allow the user to search for specific information and navigate the results found.

These methods depend on a detailed description of the dynamics of the network, but in many cases these data are not available. The goal of large-scale metabolite screening studies is to obtain cell-wide metabolite concentration measurements. Although there are significant gaps that need to be filled by direct experimental studies and reviews, metabolic network maps may be the most comprehensive of all biological networks.

1.1.2. Multi-Omic Studies for Biomarker Discovery

Taking a closer look at the molecular side of things, we now have omics at many different levels, such as genomics, transcriptomics, proteomics, metabolomics, metagenomics, epigenomics, epitranscriptomics, and so on. If we consider the possibility that many of these omics will have a temporal and/or spatial dimension, the complexity increases even further. The hope is that by combining multiple types of omics, a more complete molecular profile of the disease or individual patient can be obtained. It is anticipated that this molecular profile

will serve as a step toward the pursuit of several major goals, including computational diagnosis/prognosis, identification of disease subtypes, diagnosis of complex biological patterns associated with disease, understanding of regulatory processes involved in disease development, and prediction of response to drug treatment.

Biomarkers are biological entities or characteristics that can be used to determine whether tissues or individuals are healthy or diseased. Nowadays, biomarkers are usually molecular producers such as genes, proteins, metabolites, glycans, and other molecules that can be used for disease diagnosis, prognosis, and development of therapies. This has led to omics-based approaches, in conjunction with computational and bioinformatics methods, providing new opportunities to accelerate the identification of biomarkers, which are then used to improve the diagnosis and therapy of many diseases, especially malignancies (Arga 2019; Aydin et al. 2020; Caliskan et al. 2021; Kelesoglu et al. 2022). Biomarkers have been discovered at many different molecular levels, including genetic (Novelli et al. 2008), mRNA, protein/peptide, epigenetic (J. W. Martens et al. 2009), microRNA (miRNA) (Ruan et al. 2009), glycans (Brooks 2009; Kori et al. 2021), and metabolites (Donatti et al. 2020).

For biomarkers to be used in clinical applications, they must possess the properties of specificity, stability, and consistency across a variety of testing platforms. We have limited understanding of disease etiology and poor understanding of the distinction between healthy and diseased processes, both of which contribute to the difficulties in identifying biomarkers in many diseases. Traditional studies comparing only one protein metabolite or cell from cellular disease models or tissues with control and disease samples may prove difficult. For biomarkers to be used in clinical applications, they must have the properties of specificity, stability, and consistency across a variety of testing platforms. We have limited understanding of the etiology of disease and poor understanding of the distinction between healthy and diseased processes, both of which contribute to the difficulties in identifying biomarkers in many diseases. Traditional studies that compare only one protein metabolite or cell from cellular disease models or tissues to control and disease samples can prove to be a difficult approach to hypothesis-driven biomarker development. Tissue-specific factors are required for optimal gene expression and tissue-specific signaling, making this strategy

insufficient. For this reason, there are not many biomarkers used in the clinic (Perlis 2011). The identification of a broadly generalizable biomarker is complicated by the fact that patients with complex diseases usually need to be subdivided into sub-phenotypes based on genetic traits to be considered. The development of high-throughput omics technologies and advances in computational biology have enabled scientists to generate, analyze, and interpret a wide range of information that can be used to identify biomarkers at a scale that was previously unattainable.

1.2. Acute Myeloid Leukemia

AML is characterized by the accumulation of various somatically acquired genetic abnormalities in hematopoietic stem cells that ultimately lead to impaired cell proliferation and differentiation (Papaemmanuil et al. 2016). Although advances in genomics have developed a wide range of assays that can identify many molecular abnormalities underlying the biological heterogeneity of AML, these significant biological developments have had only a modest impact on the development of therapeutics for AML patients.

AML is a disease with a highly variable prognosis and a high mortality rate despite extensive research to identify predictive biomarkers. The 5-year overall survival rate is less than 50%, and in the elderly, only 20% survive two years after diagnosis (Kantarjian et al. 2021). Adults with AML have nonrandom chromosomal abnormalities in their leukemic blasts. These abnormalities are associated with rearrangements of key segments of proto-oncogenes that produce an abnormal fusion protein. This protein is usually a transcription factor, or a protein involved in intracellular signaling pathways for cell growth and differentiation. This situation, in turn, increases the risk that the cell will undergo malignant transformation (Grove and Vassiliou 2014; S. C. Meyer and Levine 2014).

Once diagnosed, prognosis depends largely on cytogenetic and molecular abnormalities, currently considered the most important prognostic markers. In AML, there are three cytogenetic risk categories: favorable, intermediate, and poor. The t(8;21), t(15;17), and inv(16) abnormalities belong to the favorable risk group, as do those with normal

cytogenetics and NPM1 mutation in the absence of FLT3- ITD or biallelic CEBPA mutation (Delaunay 2003; Nguyen 2002). Poor-risk karyotypes include inv(3), t(3;3), t(6;9), 5, 5q, 7, 7q, or complex karyotype abnormalities, patients with normal cytogenetics and an FLT3-ITD mutation. Patients have a low disease-free period and overall survival rate of 5 to 15 percent and a significant treatment resistance rate during induction chemotherapy, which increases the risk of relapse. Approximately 45 percent of adult AML patients have a normal karyotype and are at intermediate risk (Papaemmanuil et al. 2016). Following chemotherapy and allogeneic hematopoietic cell transplantation (HCT), AML patients often suffer from late effects due to side effects such as cardiotoxicity, infertility, secondary tumors, central nervous system dysfunction, and overall decreased quality of life (S. C. Meyer and Levine 2014). There is a significant unmet need for novel treatments that are more effective, less toxic, and better target key molecular pathways involved in the development of AML leukemia.

Next-generation sequencing (NGS) and other high-throughput technologies have produced significant genetic and genomic data. Drug discovery for oncology has been revolutionized by using molecular biomarkers for AML, which have helped shift the focus of research from potential targets to clinically relevant driver alterations or molecular features. On the other hand, there is an urgent need for screening and treatment alternatives that are cost-effective and easily quantified. Prognostic and predictive biomarkers made possible by advances in molecular biology could help inform treatment decisions for AML patients. Clinical trials focusing on the individual response of each patient, rather than the general population, are needed in precision cancer medicine to find the right drug at the right dose for a specific person with a specific genetic event or molecular trait.

1.2.1. Subtypes and Classifications of AML

The clinical manifestations, morphological characteristics, and immunophenotypes of AML are quite diverse. The AML classification is revised to highlight recent advances in our understanding and treatment of the illness. Significantly, AML with identifying genetic anomalies can be distinguished from AML defined by differentiation. AML has been

subclassified based on clinical, morphologic, and genetic aspects in the 4th edition of the World Health Organization (WHO) classification, which was developed in 2016 and published in book form in 2017 (Arber et al. 2016; Khoury et al. 2022). Originating from the morphologic French-American-British (FAB) classification, the WHO AML classification has since developed to describe disease groups based on cytogenetic abnormalities, mutational profile, and patient history (previous MDS, MDS/MPN, MPN, or cytotoxic therapy). AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, and AML, not otherwise specified (NOS) were involved as subgroups. But, with the new update on 2022 the group “AML with myelodysplasia-related changes” is recategorized as new class of AML with myelodysplasia-related cytogenetic abnormalities, AML with myelodysplasia-related gene mutations, and AML with mutated TP53. The confusing category of AML NOS were removed, under the term “defined by differentiation” were formerly listed (Table 1.1).

Table 1.1 World Health Organization classification of AML

World Health Organization classification of AML
Acute myeloid leukaemia with defining genetic abnormalities Acute promyelocytic leukaemia with PML::RARA fusion Acute myeloid leukaemia with RUNX1::RUNX1T1 fusion Acute myeloid leukaemia with CBFB::MYH11 fusion Acute myeloid leukaemia with DEK::NUP214 fusion Acute myeloid leukaemia with RBM15::MRTFA fusion Acute myeloid leukaemia with BCR::ABL1 fusion Acute myeloid leukaemia with KMT2A rearrangement Acute myeloid leukaemia with MECOM rearrangement Acute myeloid leukaemia with NUP98 rearrangement Acute myeloid leukaemia with NPM1 mutation Acute myeloid leukaemia with CEBPA mutation Acute myeloid leukemia with myelodysplasia-related cytogenetic abnormalities a complex karyotype (≥ 3 unrelated clonal chromosomal abnormalities) del(5q)/t(5q)/add(5q) – 7/del(7q) + 8, del(12p)/t(12p)/add(12p) i(17q) – 17/add(17p) or del(17p) del(20q) idic(X)(q13)

AML with myelodysplasia-related gene mutations

ASXL1
BCOR
EZH2
SF3B1
SRSF2
STAG2
U2AF1
ZRSR2

AML with mutated TP53**Acute myeloid leukaemia, defined by differentiation**

Acute myeloid leukaemia with minimal differentiation
Acute myeloid leukaemia without maturation
Acute myeloid leukaemia with maturation
Acute basophilic leukaemia
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia

1.3. Molecular Biomarkers In AML

The study of genetic alterations in AML raises several issues from a clinical perspective. According to the current WHO classification, patients with AML must first be screened for genetic abnormalities (Campo et al. 2011). Certain genetic abnormalities and molecular prognostic indicators are becoming increasingly important since they can be used for risk stratification. It is essential to tailor treatment for different AML subtypes based on identifying specific genetic abnormalities, which may lead to better outcomes. Genes with mutations associated with AML pathogenesis or that may have prognostic significance are discussed in this context (Table 1.2).

Table 1.2 Summary of molecular biomarkers of AML

	<i>Biomarker</i>	<i>Clinical feature</i>
<i>Cytogenetic</i>	t(8;21)	AML1-ETO oncofusion protein, favorable response
	inv(16)	CBF-MYH11 oncofusion protein, favorable response
	t(15;17)	PML-RARA oncofusion protein, hallmark of APL, favorable response

	der(11q23)	MLL mutations, poor prognosis, treated according to high-risk protocols
<i>Genetic</i>	FLT3 (FLT3-ITD/FLT3-TKD)	Most frequent mutation, poor prognosis, important role in proliferation, survival, and differentiation of hematopoietic progenitor cells, activated signaling
	RAS	Regulating mechanism of proliferation, differentiation, and apoptosis, variable prognosis, activated signaling
	IDH1/2	Poor prognosis, DNA methylation related
	DNMT3A	Adverse prognosis, decrease in overall survival, DNA methylation related
	NPM1	Higher survival and remission rates, DNA repair and cell cycle mechanism
	RUNX1	Adverse prognosis, Myeloid Transcription Factor
	CEBPA	Improved prognosis, longer overall survival and remission rate, transcription factor in hematopoiesis, Myeloid Transcription Factor
	KIT	Unfavorable outcome, role in survival, proliferation, differentiation, and functional activation of progenitor cells, Kinase Signalling Pathway
	TP53	Poor prognosis, Tumor Suppressor
<i>Epigenetic</i>	Aberrant DNA Methylation	AML subtypes classification, predictor of clinical outcome
	Non-coding RNA: miRNAs	AML subtypes classification, predictor of clinical and therapeutical outcome
<i>Proteomic</i>	LDH	Poor prognosis
	nm23-H2	Predictor of clinical outcome
	CXCL12/CXCR4	Poor prognosis
	BTG1	Good prognosis
	Heat Shock Proteins (HSPs)	Poor prognosis

1.3.1. Cytogenetic Biomarkers

Chromosomal rearrangements are detected in approximately 50-60 percent of newly diagnosed AML patients (J. H. A. Martens and Stunnenberg 2010). Rearrangements are further divided into three groups: AML with balanced translocations/inversions, AML with various cytogenetic abnormalities (such as deletions, monosomies, and trisomies), and AML with a complex karyotype (with at least three acquired chromosomal aberrations). In AML, there are three prognostic cytogenetic categories: favorable, intermediate, and poor-risk groups.

1.3.1.1. t(8;21)(q22;q22) (AML1/RUNX1-ETO/RUNX1T1, CBF)

The AML1-ETO fusion protein is the product of a reversible translocation t(8;21)(q22;q22) between chromosomes 8 and 21. Heterodimeric transcription factors referred to as core-binding factors (CBFs) or the RUNX family of proteins, consist of two subunits: the α -subunit (CBF α ; encoded by three different genes: Runx1/Runx2/Runx3) and β -subunit (CBF β ; encoded by another gene: CBF β) (Chiaretti et al. 2014). While ETO acts as a transcriptional repressor, AML1 is a critical transcription factor for hematopoietic differentiation (Al-Harbi et al. 2020). AML1-ETO recognizes AML1 consensus binding sites and forms heterodimers with CBF β , which are transcriptional repressors. There is a strong correlation between this type of translocation and AML M2 FAB subtype (AML with maturation), but it is rare in AML M1 or M4 subtypes. This chromosomal abnormality is associated with favorable outcomes, high remission rates, and longer median survival. However, concurrent mutations in the c-Kit gene are an independent unfavorable prognostic indicator for this disease. t(8;21) patients respond well to standard treatment (anthracyclines and doxorubicin). Complete remissions were achieved in these patients with the 7+3 regimen (cytarabine). Cytarabine should be administered for at least three or four rounds of intensive post-remission therapy in adults to maximize the benefit of chemotherapy (Lagunas-Rangel et al. 2017).

1.3.1.2. Inv(16)(p13.1q22)/t(16;16)(p13.1;q22) (CBF β -MYH11, CBF)

Inversion of the 16th chromosome (p13;q22) results in the development of the CBF-MYH11 oncogene. The chimeric protein functions as a transcriptional and epigenetic complex repressor and cooperates with AML1 to block transcription of the tumor suppressor genes PTEN, Bcl-2, CEBPA, ARF, and PSGL-1 (Y. Li et al. 2013; Ponnusamy et al. 2015; Zhuang et al. 2013). The first 165 residues of CBF β are fused to the C-terminal coiled-coil region of MYH11. In progenitor cells, the CBF-MYH11 fusion gene forms at least eight different transcripts. At Runx binding sites on DNA, RUNX1 (CBF α) and CBF β form a complex in progenitor cells that regulate gene expression. The CBF-MYH11 and RUNX1-ETO fusion proteins compete for heterodimerization and/or DNA binding at Runx binding sites, thereby impairing the regular function of the RUNX1 (CBF α)/CBF β complex (Chiaretti et al. 2014). Since CBFs are essential regulators of several stages of hematopoiesis during hematopoietic ontogeny, abnormally fused products hinder their function and prevent hematopoietic differentiation. The prognosis for patients with inv(16)/t(16;16) who respond well to conventional chemotherapy (the 7+3 regimen of cytarabine and anthracyclines) and achieve complete remissions is favorable. 3-4 cycles of histone deacetylase (HDAC) are also effective as in t(8;21) patients. The increased sensitivity of leukemic cells to cytarabine and apoptosis may be due to the incorporation of cytarabine into genomic DNA, leading to a better outcome with repeated treatment (Lagunas-Rangel et al. 2017; Mrózek 2008).

1.3.1.3. t(15;17)(q24;q21)(PML-RARA)

10-15% of AML cases are caused by acute promyelocytic leukemia (APL). The t(15;17)(q24;q21) encoding a fusion protein called PML-RARA is found in approximately 98% of cases of APL, which is considered a biomarker for APL (Liquori et al. 2020). The PML-RARA rearrangement in patients with APL indicates a favorable response to retinoic acid treatment (de Braekeleer et al. 2014). More remarkably low-risk patients with APL using arsenic trioxide and all-trans retinoic acid (ATRA) can achieve a 97 percent 2-year event-free survival rate with 100 percent complete remission (Kayser et al. 2018). ATRA is approved for treating adult and pediatric leukemias or as a component of remission induction

therapy. Retinoic acids (ATRA or 9-cis-retinoic acid, CTRA) have been shown to activate the RAR of the nuclear hormone receptor superfamily, which is essential for hematopoietic cell differentiation. DNA at sites responsive to retinoic acid interacts with the heterodimer RARRXR formed by RAR and RXR, a retinoid X receptor response element (RARE) (Nagpal et al. 1993). The transcriptional activator complex formed by RAR and RXR is required for promyelocytic differentiation. An important effect on apoptosis is exerted by PML-RARA, which impairs the wild-type function of PML and regulates its expression by negative dominance. ATRA induces dissociation of PML-RARA co-repressors, which are then destroyed by the proteasome in a manner that activates transcription. ATRA is required for immature leukemic promyelocytes to differentiate into mature granulocytes, bypassing the co-repressor activity of PML-RARA (Chiaretti et al. 2014; Prada-Arismendy et al. 2017).

1.3.1.4. 11q23 (MLL Mutations)

The mixed lineage leukemia (MLL) gene was discovered due to chromosomal rearrangements at 11q23 that have been associated with pediatric, adult, and therapy-related leukemias. MLL causes at least 10% of acute leukemias. MLL is a histone methyltransferase that plays an essential role in controlling transcription, embryonic development, and hematopoiesis. MLL binds to one of more than 50 recognized partner genes in patients, resulting in an MLL fusion protein that acts as an oncogene (Krivtsov and Armstrong 2007). The fusion partner, MLL-ENL, acts as an effector unit that promotes prolonged transactivation (Yokoyama et al. 2010). As a member of the trithorax family, it is involved in the methylation of histone H3 on lysine residue 4 (H3K4), which is linked to gene expression (Krivtsov and Armstrong 2007). MLL is ubiquitously expressed in hematopoietic cells and regulates homeobox (HOX) genes that control hematopoiesis. The fusion protein activates HOX genes such as HoxA9 and HoxA10, which are frequently upregulated in leukemia (Chen et al. 2019). Although MLL has domains that can bind directly to DNA, recent research suggests that this interaction may be more indirect. 11q23 rearrangements have an intermediate to poor prognosis, depending on other genetic abnormalities (Meyer et

al., 2009). The 11q23 rearrangement with the partner t(9;11)(p22;q23) has a better prognosis. Patients with this rearrangement respond to conventional treatment as intermediate .

1.3.1.5. Complex Karyotypes, Trisomies, And Monosomal Karyotypes

It is estimated that 10-15 percent of all AML cases are caused by complex karyotypes, characterized by three or more acquired chromosomal abnormalities (Meyer and Levine, 2014). A chromosomal loss or gain is characteristic of these types of AML, and they are commonly accompanied by TP53 mutations, which negatively influence overall survival (Lindsley et al. 2015). Trisomies are usually found in a complex karyotype but may also be detected alone or in association with other structural rearrangements. The chromosomal aberration trisomy 8 has been identified in many karyotypic contexts, including t(8;21), inv(16), and t(9;11) mutations, but also as a single chromosomal aberration. Patients with trisomy 8 or with a complex karyotype that includes trisomy 8 are expected to have poor treatment outcomes. However, patients with trisomy 8 associated with t(8;21), inv(16), or t(9;11) are expected to respond better to standard treatment (Mrózek 2008). Trisomies associated with de novo AML occur in decreasing order of frequency: +8, +22, +13, +21, and +11.

Autosomal monosomies can also be found in complex karyotypes, with chromosomes 5 and 7 being the most frequently observed single monosomies in AML patients (Mrózek 2008). Compared to del(7q) and del(5q), loss of whole chromosomes (-7 and -5) has been shown to result in shorter overall survival (5q). A monosomal karyotype is defined as two or more autosomal monosomies or single monosomy accompanied by other structural abnormalities. It has been shown to be a more robust indicator of poor prognosis compared to a complex karyotype (Breems et al. 2005).

1.3.2. Genetic Biomarkers

Many cases of AML (40-50% of adults and 25% of pediatric AML) are considered cytogenetically normal (CN-AML). They exhibit gene alterations and unregulated gene expression (Walker and Marcucci 2012). AML patients with normal karyotype had a 5-year

survival rate of 24% to 42%. CN-AML patients have some gene mutations such as Nucleophosmin 1 (NPM1), FMS-related tyrosine kinase 3 (FLT3), CCAAT/enhancement-binding protein alpha (CEBPA), Neuroblastoma RAS viral oncogene homolog (NRAS) gene, and RUNX1 gene. However, these mutations can also be found as secondary abnormalities in AML with an aberrant karyotype (Prada-Arismendy et al. 2017).

1.3.2.1. Activated Signaling (FLT3-ITD, FLT3-TKD, RAS)

FLT3 is a member of the receptor tyrosine kinase (RTK) family involved in the proliferation and differentiation of hematopoietic progenitor cells. FLT3 mutations are found in approximately 30% of all AML patients. There are two groups of activation mutations in FLT3; FLT3-ITD and FLT-TKD. FLT3-ITD is an in-frame mutation that results in an elongated juxta-membrane domain by duplicating short sequences ranging from 3 to over 400 base pairs in length. The receptor is constantly active, triggering intracellular signaling pathways that lead to cell proliferation (Daver et al. 2019a). FLT3-ITD mutations are found in AML with a frequency of 25%. The prognostic significance of the mutation is determined by allelic burden. Allogeneic stem cell transplantation was found to be beneficial only in AML patients with a high allelic ratio (AR) FLT3-ITD. Individuals with high AR FLT3-ITD had lower complete remission rates, poorer survival, and relapse (Linch et al. 2014).

FLT3-TKD are mainly point mutations, deletions, or insertions in the TK domain and are less frequent than FLT3-ITD mutations, with a frequency of 7% (Daver et al. 2019a). FLT3-TKD mutations are mainly point mutations in codon D835 or deletions of codon I836 in the activation loop of FLT3 (Ahn and Kim 2022). They activate RTK by causing constant tyrosine phosphorylation, which is considered a gain-of-function mutation. Mutations in the activation loop have been observed in several receptor tyrosine kinases, e.g., KIT at site D816.

In combination with regular chemotherapy, tyrosine kinase inhibitors may be able to target FLT3 mutations. Three FLT3 subgroups, FLT3-TKD, FLT3-ITD low, and FLT3-ITD high AR, showed significantly prolonged overall survival and event-free survival after a tyrosine kinase inhibitor (TKI) was added to conventional chemotherapy (Daver et al. 2019b).

Sunitinib, sorafenib, midostaurin, lestaurtinib, and ponatinib are examples of inhibitors that target FLT3 (Larrosa-Garcia and Baer 2017). AML cells may benefit from inhibiting numerous RTKs, including FLT3 and additional RTKs downstream of FLT3 and/or acting in parallel signaling pathways. Off-target activities, on the other hand, could potentially cause health problems. Second-generation FLT3 inhibitors, on the other hand, have fewer off-target effects and are more selective and effective than first-generation FLT3 inhibitors (Larrosa-Garcia and Baer 2017). Although second-generation FLT3 inhibitors are effective in AML cells, they do not act against targets downstream of FLT3 or in parallel signaling pathways. Mutations in the RAS gene have been found in 15-30% of AML patients and may play a role in the development of the disease. The RAS gene encodes membrane-associated proteins critical for cell growth, proliferation, apoptosis, migration, division, and differentiation. There are three types of RAS isoforms: Neuroblastoma RAS (NRAS), Kirsten RAS (KRAS), and Harvey RAS (HRAS). Approximately 8% to 13% of AML patients had KRAS mutations. KRAS codons 12, 13, and 61 are the most common sites for point mutations (Prada-Arismendy et al. 2017; Zaidi et al. 2008).

In adult AML, the NRAS gene is frequently mutated, making it the most prominent member of the RAS family. Mutations in the NRAS gene have been found in 11-30% of patients. AML patients with NRAS t(3;5) or KRAS inv(16) mutations had a higher than average frequency of RAS mutations. RAS mutations had little impact on prognosis in patients with normal karyotype, inv(16) and t(8;21). Increasing NRAS activity has been shown to improve survival in young patients receiving high doses of cytarabine. The other activating mechanisms are more critical than the NRAS mutations, while patients with KRAS mutations are more likely to relapse their disease. KRAS mutations were not predictive of survival in certain patients throughout treatment, and most patients who received chemotherapy had complete remission. RAS mutations serve as important prognostic markers in the pathogenesis of AML (Metzeler et al. 2008; Zaidi et al. 2008).

1.3.2.2. DNA Methylation Related (IDH1/2, DNMT3A)

Isocitrate dehydrogenases (IDH1 and 2) are enzymes in various metabolic and epigenetic cellular processes. Somatic mutations of IDH1 or IDH2 occur early in AML progression at rates of 4-9% and 8-19%, respectively, resulting in genomic DNA hypermethylation, abnormal gene expression, cell proliferation, and abnormal differentiation (Dohner and Dohner 2008). The oncometabolite 2-hydroxyglutarate is formed by amino acid substitutions in conserved residues. The amino acid changes causing the hotspot mutations are located in codon 132 of exon 4 of the IDH1 gene and codons 140 or 172 of exon 4 of the IDH2 gene (Marcucci et al. 2010).

IDH mutations in AML have a wide range of biological, clinical, and therapeutic consequences. Depending on the location of the mutation and other associated genomic abnormalities, IDH mutations have different implications for the prognosis of AML patients. Under typical intensive chemotherapy conditions, IDH mutations' impact on AML prognosis is still controversial. However, IDH1 mutations generally have a poor impact on prognosis, whereas IDH2 mutations, especially R172K IDH2 mutations, have a fairly good impact on prognosis. IDH1 and IDH2 mutations are associated with several patient- and disease-specific factors, including older age, diploid or other intermediate-risk cytogenetics (such as trisomy 8), and a stable platelet count at presentation. IDH1 and IDH2 mutations have a context-dependent effect with IDH mutations in association with NPM1 mutations (but not FLT3-ITD), appearing to lead to better treatment outcomes (Marcucci et al. 2010; Paschka et al. 2010).

Somatic mutations in DNMT3A are among the most severe and common epigenetic abnormalities in myeloid malignancies, affecting 20-22% of people with de novo AML. Approximately 30-37% of CN-AML patients have DNMT3A loss-of-function mutations. Almost all CN-AML patients carry at least one point mutation in a DNMT3A allele. In the methyltransferase domain, the most common missense mutation was identified as R882H. De novo methyltransferase activity is reduced by R882H, which inhibits wild-type expression of DNMT3A and leads to focal hypomethylation at specific CpGs throughout the genome of AML cells (Russler-Germain et al. 2014; Yan et al. 2011). DNMT3A mutations

are generally associated with intermediate-risk AML and with poor treatment outcomes. Patients with DNMT3A mutation who received decitabine responded better to therapy. However, patients with the wild-type DNMT3A gene had a lower rate of clinical remission and shorter overall survival. A positive response to decitabine treatment would be indicated by a high level of miR-29b (a miRNA directed against DNMT3A) (Wagner et al. 2010). Patients with DNMT3A and IDH1/IDH2 mutations respond well to the DNMT inhibitors, decitabine, and azacytidine (Pourrajab et al. 2020).

1.3.2.3. DNA Repair and Cell Cycle (NPM1)

NPM1 plays a role in ribosome biogenesis and transport, apoptosis, response to stress stimuli, and genome stability. DNA repair is also facilitated by NPM1. NPM1 mutations are the most common adult AML mutation (insertion and deletion) and account for approximately one-third of all adult AML cases. NPM1 mutations are found in approximately 50-60% of CN-AML patients that respond well to induction chemotherapy, and positive outcomes are observed in CN-AML subtypes without FLT3- ITD mutations. Researchers have discovered many variations of NPM1, all of which have similar biological effects. In most cases, these genetic abnormalities are associated with normal karyotypes and are mutually exclusive of RUNX1 and CEBPA. NPM1 mutations occur alongside other epigenetic modifiers such as DNMT3A, TET2, and IDH1/2 (Falini et al. 2020). AML with an NPM1 mutation often has a favorable prognosis and a good response to initial treatment (Patel et al. 2019). In addition, the allele ratio of the FLT3 allele influences prognosis. On the other hand, AML patients with mutant NPM1 and a high FLT3-ITD AR (> 0.5) together with wild-type NPM1 are considered intermediate-risk patients. The absence of NPM1 mutations in AML is a hallmark of complete remission. However, an unfavorable prognosis can be inferred from the persistent presence of the mutation during follow-up (Falini et al. 2020; Ivey et al. 2016).

1.3.2.4. Myeloid Transcription Factors (RUNX1, CEBPA)

Various forms of abnormalities, including translocations, mutations, and copy number variations, have been associated with the involvement of RUNX1 in leukemia. It is estimated

that up to 15% of patients with AML have somatic mutations. There are numerous hematopoietic genes controlled by RUNX1, including those encoding transcriptional activators (STAT3, MYB), surface receptors (TCRA, TCRB, M-CSF receptor, FLT3), growth factors (GM-CSF, MPO, IL3), and signaling molecules (CDKN1A, BLK, BCL2). In AML, mutations, amplifications, and translocations of the RUNX1 gene have formed three different types of acquired gene alterations. AML M0, myelodysplastic syndromes (MDS), AML after MDS, and therapy-related MDS and AML have all been associated with intragenic mutations (van der Kouwe and Staber 2019).

RUNX1 mutations are associated with chromosomal abnormalities such as monosomy 7 and trisomy 13. The RUNX1 mutation highlights the difficulty in developing class-defining biomarkers with clinical and prognostic significance. RUNX1-mutated AML has been associated with certain clinical features, such as older age (men were more likely to develop AML), immature morphology (tumors were less mature), and secondary AML arising from MDS. These clinical factors have long been known to negatively impact clinical outcomes (Gaidzik et al. 2016; van der Kouwe and Staber 2019).

CEBPA is expressed in the myeloid lineage and is located on chromosome 9q13.11 as a member of the transcription factors. It is essential for the proliferation and differentiation of myeloid progenitor cells into granulocytes or monocytes. Increased expression of CEBPA leads to granulocyte and neutrophil differentiation under certain conditions. CEBPA mutations can lead to early blockade of granulocyte maturation and are associated with a favorable prognosis in approximately 10% of AML patients, most of whom have an intermediate risk karyotype (Song et al. 2015). CEBPA mutations are found in 7-15% of CN-AML patients, with the majority having the FAB -M2 subtype. Patients with CEBPA double mutations had a better prognosis (especially at age 16-60) and a longer remission time (H.-Y. Li et al. 2015; Walker and Marcucci 2012).

1.3.2.5. Kinase Signaling Pathway (KIT)

On chromosome band 4q11-12 is the gene KIT, which encodes a transmembrane glycoprotein of the tyrosine kinase family. The link between the receptor KIT and stem cell

factor activates downstream signaling pathways critical for cell proliferation, differentiation, and survival (Lennartsson and Rönstrand 2012). Most commonly, KIT activating mutations (encoding a transmembrane glycoprotein) are found in CBF leukemias, which include AML with t(8;21) and AML with inv (16). Gain-of-function mutations in KIT were found in 2% of all AML and 33% of all CBF leukemias. Alterations in exon 17 and exon 8 of the KIT gene are the most common sites for these mutations (Paschka et al. 2006). CBF-AML with KIT mutations as intermediate risk. However, the co-occurrence of KIT mutations with inv(16) and t(8;21) has been associated with poor prognosis and a higher incidence of relapse. KIT inhibitors such as dasatinib and avapritinib have been studied as frontline therapy. Results show that KIT-mutated CBF-AML relapse rates can be reduced to levels comparable to CBF AML without KIT mutations (Gulley et al. 2010; H. Jin et al. 2021; Paschka et al. 2006).

1.3.2.6. Tumor Suppressor TP53

AML patients with a complex karyotype (CK-AML) have a TP53 mutation in more than 75% of cases; however, this is the case in only a small percentage of patients. In both CK-AML and therapy-related AML, the most important marker of poor prognosis is a TP53 mutation. Older patients with decreased complete remission, overall survival, event-free survival, and relapse-free survival are more likely to have TP53 mutations. Several aspects should be considered when developing targeted therapy for TP53 mutations, such as alterations affecting associated pathways and other therapeutic options, such as BCL2 inhibitors. In the latter case, TP53 activation may overcome resistance to BCL-2 inhibitors (Boddu et al. 2018; Kadia et al. 2016).

1.3.3. Epigenetic Biomarkers

1.3.3.1. Aberrant DNA methylation

In leukemia, DNA methylation is the most common epigenetic marker, but changes in histone modifications and noncoding RNAs also play critical roles (Claus et al. 2010; Tickenbrock

et al. 2011; Wallace and O'Connell 2017). AML patients can be classified into different categories based on methylation profiles associated with specific cytogenetic or molecular subsets. Several studies have also established the association between clinical prognosis and DNA methylation. Patients with a good prognosis and normal cytogenetic profiles have aberrant hypermethylation. Hypermethylation of CpGs promoter sites in nonclustered CpGs with different methylation profiles is associated with specific clusters. Genes involved in the Wnt/ β -Catenin signaling system, tumor suppressors, cell cycle control, development/differentiation, and apoptosis exhibited abnormal methylation (Alvarez et al. 2010).

It has been examined the genomes and epigenomes of two "high-risk" APL patients, who were distinguished from "lower-risk" APL patients by their resistance to the ATRA therapy, which is routinely used to treat APL. Despite the lack of significant genetic differences, high-risk APL was distinguished from lower-risk APL instances by a collection of different epigenetic signals. A 23-CpG signature that can differentiate between high- and low-risk APL cases was discovered using supervised clustering to identify CpG sites that were differentially methylated (Arteaga et al. 2015; Singh et al. 2018).

Compared with the study of genetic alterations in AML, epigenetic research in AML is still under development. DNA methylation patterns can predict AML patients' prognosis in terms of survival and response to therapy; these results need to be validated in different groups with different risk factors.

1.3.3.2. Non-coding RNA: miRNAs

Research into miRNA function in AML began with the search for disease-specific miRNA expression patterns. miRNAs have recently been proposed as potential biomarkers, as their altered expression may contribute to disease development and progression. Many cytogenetic subtypes of AML and certain mutations in CN-AML have distinct miRNA profiles. The association between miRNA expression profiles and prognosis is further evidence that miRNAs may play a role in this disease. Few miRNAs may have functional effects on AML, even when enriched in leukemia-associated genomic alterations. In this regard, some

miRNAs have been associated with carcinogenesis and cancer progression, and they may be valuable in predicting response to therapy (Buhagiar et al. 2020; Y. Liu et al. 2019).

In MLL-rearranged AML, miR-9 is an important oncomiR, promotes MLL fusion-mediated leukemogenesis, and might be a therapeutic target for the disease (P. Chen et al. 2013). Serum exosomal miRNA expression profiles in AML have been studied, and it was found that an increase in miR-125b expression was significantly associated with an increased risk of AML relapse and mortality. Therefore, researchers propose miR-125b as a new biomarker for intermediate-risk AML with a poor prognosis (Jiang et al. 2018). It is worth noting that either overexpression or knockout of miR-126 may enhance leukemogenesis because miR-126 regulates a variety of targets (Li et al. 2015). miR-124 has been proposed as a tumor suppressor because it restricts cell proliferation while promoting differentiation and apoptosis. Its underexpression is associated with favorable survival in AML patients and more common patients with t(15;17) (X. Chen et al. 2014).

Overexpression of the carcinogenic miRNA miR-155 has been associated with poor outcome of AML. miR-155 has been shown to be a potential tumor suppressor under certain circumstances. To reconcile these conflicting findings, a recent study further investigated the role of miR-155 in AML. The researchers observed that miR-155, when overexpressed at intermediate levels (5 to 10 times above control) in three mouse models of AML, exhibited oncogenic activity that increased proliferation and colony-forming ability (Narayan et al. 2017). miR-155, on the other hand, behaved as a tumor suppressor by inhibiting colony formation and proliferation at high levels (> 10-fold above control), indicating a dose-dependent effect of miR155 in these AML mouse models (Garzon et al. 2008). Studies of miR-155 in other cancers have shown similar increases in miR-155 expression, suggesting that the gene may have a primary oncogenic effect in human AML at intermediate miR-155 expression levels. In light of these studies, the impact of a miRNA in AML may depend on the underlying genetic defects causing the disease or cell type expression.

1.3.4. Proteomic Biomarkers

Current efforts to discover novel therapeutically useful biomarkers are driven by advances in high-throughput proteomic techniques, particularly mass spectrometry. Studies have identified numerous potential therapeutic targets and protein biomarkers for predicting AML recurrence and treatment efficacy based on proteomic profiles of AML patients.

Lactate dehydrogenase (LDH), an established biomarker for several malignancies, including AML, has been shown to be useful in determining patient prognosis. The prognostic value of LDH in AML patients receiving allogeneic hematopoietic stem cell transplantation and in AML patients undergoing bone marrow transplantation has been demonstrated in recent studies (Geva et al. 2019). Low levels of nm23-H2 or nucleoside diphosphate kinase B have previously been an excellent indicator of prognosis in AML (Wakimoto et al. 1998). The CXCL12/CXCR4 complex is used in AML to activate pro-survival signals and direct AML blasts to the bone marrow niche for protection. Increased CXCR4 expression and CXCL12 secretion at BM by bone marrow stromal cells in AML may be related to oxygen deprivation in the microenvironment (Abe-Suzuki et al. 2014; Fiegl et al. 2009). High CXCL12 levels are thought to help retain AML blasts in the bone marrow and reduce their resistance to chemotherapeutic treatment. CXCL12 has been shown to be higher in patients at moderate and severe risk of AML. A previous study found that AML patients with high CXCR4 expression have a poor prognosis (Du et al. 2019). A study comparing proteomes before and after treatment showed that patients with complete remission of AML had higher expression of BTG1 in their proteomes than patients who were not in remission (Cho et al. 2004). The role of heat shock proteins (HSPs) in apoptosis and resistance to therapy was demonstrated by Western blot analysis, which showed that patients with higher expression of HSPs had poorer rates of complete remission as well as poorer overall survival (Thomas et al. 2005). Despite advances in protein biomarkers for AML, there is still a limited body of evidence that is retrospective and, in most cases, unverified. Because large patient cohorts and representative AML samples are difficult to obtain, the disease is heterogeneous and complex. The number of protein biomarkers already discovered but clinically validated biomarkers are relatively small. To ensure that the proposed biomarkers are accurate, reliable,

interpretable, and suitable for clinical practice, verification studies need to be performed using various technologies and an independent patient cohort.

1.4. Treatment Strategies of AML

1.4.1. Current Treatment Methods Of AML

AML has a 5-year survival rate of less than 30%, making it one of the most lethal leukemias. As indicated, multiple chromosomal abnormalities and gene mutations commonly found in leukemic blasts are responsible for the poor outcome. This leads to a clinically heterogeneous collection of diseases with high relapse rates and resistance to therapy (Döhner et al. 2015). Despite advances in AML biology and new technologies since the 1970s, treatment techniques have not changed significantly. Research into the molecular landscape of AML and its impact on key signaling pathways such as apoptosis, proliferation, and differentiation has led to a new wave of therapies that increasingly use targeted agents, either as single agents or in combination with standard chemotherapeutic agents, to eliminate leukemic cells and improve treatment outcomes (Table 1.3) more efficiently. These revolutionary treatment approaches have ushered in a new era in treating AML by successfully expanding the therapeutic regimen.

Table 1.3 Current treatment methods in AML

Chemotherapy	Targeted therapy
Pyrimidine analogs: Cytarabine	FLT3 inhibitors: Type I inhibitors; Midostaurin, Gilterinitib, Sunitinib, Lestaurtinib, Crenolanib Type II inhibitors; Sorafenib, Quizartinib, Ponatinib
Anthracyclines: Daunorubicin, Doxorubicin, Idarubicin	IDH inhibitors: Ivosidenib, Enasidenib, Olutasidenib

Hypomethylating agents: Azacitidine, Decitabine	BCL-2 inhibitor: Venetoclax
	Hedgehog signaling pathway inhibitors: Glasdegib, Vismodegib, Sonidegib, Erismodegib
	HDAC inhibitor: Vorinostat

1.4.1.1. Frontline induction therapy

Intensive cytarabine-anthracycline-based induction chemotherapy (IC) remains the standard of care for remission in AML patients. Using 7+3 as the standard combination, cytarabine is dosed on days 1-7 and anthracycline on days 1-3. Higher anthracycline or cytarabine doses or the addition of a third drug during induction have recently been investigated in many randomized trials. Nevertheless, it is difficult to compare these trials because of substantial differences in crucial characteristics, including the number of induction sessions, doses in the control arm, and further therapy administered to patients who responded to treatment or who still had blasts after the first induction cycle (Kantarjian et al. 2021). Therefore, differences in the experimental or control groups of the study could account for some discrepancies in the results.

1.4.1.2. Post-remission (consolidation) therapy

Post-remission therapy builds on remission induction therapy and results in a modest but substantial increase in overall survival and establishment of complete remission. Although induction chemotherapy aims to reduce the number of AML cells in circulation, the primary goal of post-remission therapy is to prevent disease recurrence. More than 90% of AML patients relapse within a few weeks or months if they do not receive treatment after remission. Consolidation and maintenance therapy are the two main goals of therapy after remission.

Consolidation therapy consists of intensive chemotherapy administered immediately after recovery from induction therapy. When induction and consolidation are as close together as possible, the risk of disease recurrence is reduced (Kumar 2011).

1.4.1.3. HiDAC consolidation

Finding the right dose and schedule of cytarabine to achieve a long-lasting disease response while minimizing toxicity is the focus. The hematologic, neurologic, gastrointestinal, and cutaneous toxicity of cytarabine is of great concern. Studies have examined a variety of cytarabine dosing regimens for the post-remission period, and it has proven difficult to establish a uniform guideline. However, a typical regimen developed by the Cancer and Leukemia Group B (CALGB) uses 2-3 g/m² of cytarabine infused twice daily for six consecutive days, for a total dose of 12-18 g/m² per cycle. Early research has shown that high-dose Ara-C (HiDAC) provides better disease-free survival than cytarabine infusion over five days. Intermediate dose cytarabine (IDAC) has been shown to be effective in the treatment of AML when administered at a dose of 1 g/m² (Böhm et al. 2005; Kolla et al. 2019).

1.4.1.4. Allogeneic HSCT

One of the most important therapeutic options for AML patients is to weigh the potential benefits and risks of allogeneic hematopoietic stem cell transplantation (HSCT) in the first remission. Transplantation is better for preventing AML relapse and is associated with higher treatment-related morbidity and mortality. If the risk of relapse is low and survival is high enough in patients with favorable AML, HSCT can be postponed until after the second remission. Allogeneic HSCT from a sibling or a fully matched unrelated donor is usually considered a viable option for patients with intermediate- and unfavorable-risk AML who have achieved first remission (Magliano and Bacigalupo 2020). The current benefit/risk assessment based on the European LeukemiaNet (ELN) genetic classification needs to be reconsidered. Many factors must be considered when deciding whether a patient should

receive allogeneic HSCT, including the presence of minimal residual disease (MRD) and the presence of leukemia-associated aberrant immunophenotypes.

1.4.2. Targeted therapies in AML treatment

1.4.2.1. FLT3 inhibitors

TKI drugs have been developed targeting different sites of the ATP-binding site in the intracellular domain of FLT3-RTK as progress has been made in understanding the mechanism of FLT3 gene mutation (Kennedy and Smith 2020). Midostaurin, gilteritinib, sunitinib, lestaurtinib, and crenolanib are type 1 inhibitors that bind to the RTK ATP -binding site in the active conformation and in the inactive state. Sorafenib, quizartinib, and ponatinib are type 2 inhibitors that bind to the hydrophobic region adjacent to the ATP-binding domain and prevent receptor activation. For FLT3 gene-mutated AML patients with FLT3-ITD or FLT3-TKD, midostaurin has been recommended as first-line therapy (Shimada 2019). The use of midostaurin in combination with regular chemotherapy to treat newly diagnosed FLT3-mutated AML patients has also demonstrated its cost-effectiveness (Stone et al. 2017; Tremblay et al. 2020). Gilteritinib is a small molecule receptor TKI that can be taken orally and is used to treat AML with FLT3 mutations. Gilteritinib inhibits FLT3 signaling in cells carrying the FLT3-ITD, the FLT3-D835Y TKD mutation, and the FLT3-ITD-D835Y double mutant, causing apoptosis (Perl et al. 2019). Sunitinib is a small molecule FLT3 inhibitor with direct and indirect anti-tumor and anti-angiogenesis activities (O'Farrell et al. 2003). Lestaurtinib inhibits FLT3 tyrosine kinase and induces hematologic remission in AML patients with FLT3- ITD. However, many patients in clinical trials became resistant to lestaurtinib (Al-Jamal et al. 2015). TKD mutations as well as ITD mutations, can be effectively treated with crenolanib. In a phase II study of newly diagnosed FLT3-mutated AML patients, crenolanib was well tolerated in combination with 7+3 induction therapy (Haijiao Zhang et al. 2019). In patients with FLT3-ITD AML, quizartinib is a successful treatment. It reduces tumor cell viability by inhibiting FLT3. For patients with rapidly proliferating diseases and a poor prognosis, quizartinib could be considered a new standard of care (Cortes, Khaled, et al. 2019). As a single agent, sorafenib has shown modest efficacy

in FLT3+ AML. Sorafenib and sunitinib have a similar effect on AML. Sorafenib prolongs life when used with regular treatment in people younger than 60 years (Röllig et al. 2015). Standard induction chemotherapy does not affect survival in people over 60 (Serve et al. 2013). Continued treatment with sorafenib after allo-HSCT treatment improves overall and event-free survival. Ponatinib is indicated for patients with TKI-resistant chronic myeloid leukemia (CML) and is a promising agent in FLT3-ITD positive AML (Zirm et al. 2012).

1.4.2.2. BCL2 inhibitors

Nearly twenty years ago, BCL-2 was an attractive therapeutic target for a wide range of cancers, including AML. The BCL-2 inhibitor venetoclax showed excellent activity in AML clinical trials after showing promising results as a single treatment in lymphoid malignancies (Pollyea et al. 2019). Many studies have shown that combining venetoclax with decitabine, azacitidine, or low-dose cytarabine improves outcomes and increases survival rates in patients with AML (DiNardo et al. 2019). Since then, the FDA has granted accelerated approval for venetoclax in combination with chemotherapeutic regimens in AML patients who are 75 years or older or unsuitable for conventional chemotherapy.

1.4.2.3. IDH inhibitors

AML patients with IDH1 or IDH2 gene mutations that lead to abnormal maturation patterns in white blood cells are treated with IDH inhibitors (IDH-i). Ivosidenib and enasidenib block IDH1 and IDH2, respectively. Immature blast populations are reduced, and the frequency of mature myoblasts is increased by blocking leukemic cells with IDH-i. In patients with IDH1-mutated relapsed/refractory (R/R) AML, ivosidenib has been shown to be overall safe and effective, leading to FDA approval. Patients with a poor prognosis may benefit from ivosidenib. Despite developing resistance to these mutant IDH inhibitors, combination therapy may address this issue (Norsworthy et al. 2019; Sidaway 2018). The FDA has also approved the drug enasidenib for the treatment of R/R AML. This is the first mutant IDH2 inhibitor to demonstrate safety and efficacy in a phase 1/2 dose-escalation and expansion study. It was well tolerated by patients with AML and resulted in molecular remissions and

hematologic responses not achieved with previous treatments. Because enasidenib is a highly selective inhibitor acting on an early stable mutation, this treatment may work better when used in conjunction with other targeted therapies (Reed et al. 2019; Stein et al. 2019). Based on the results of preclinical studies, olutasidenib is a highly potent, orally bioavailable, and selective IDH1 inhibitor. Olutasidenib causes profound responses in some of those treated for AML, including eliminating IDH1 mutations (Watts et al. 2019). This drug may have significant therapeutic potential for the treatment of AML and MDS; however, further clinical trials are needed.

1.4.2.4. Hedgehog signaling pathway inhibitors

The hedgehog pathway inhibitor glasdegib was studied in combination with conventional chemotherapy in patients with AML or high-risk MDS. Low-dose cytarabine with glasdegib was approved in November 2018 to treat newly diagnosed AML patients over 75 years of age or ineligible for intensive chemotherapy (Cortes et al. 2018; Hoy 2019). Recently, similar SMO inhibitors such as vismodegib, sonidegib, and erismodegib in combination with chemotherapy such as azacytidine are being developed in clinical trials for patients with AML and MDS (Cortes, et al., 2019; Tibes et al., 2015).

1.4.2.5. Histone Deacetylase (HDAC) inhibitors

Vorinostat is approved as a histone deacetylase inhibitor for the treatment of cutaneous lymphoma. According to a phase II clinical trial, patients with AML or MDS may benefit from vorinostat in combination with idarubicin and cytarabine (Garcia-Manero et al. 2012). The overall response rates of this combination are incredibly high, especially in patients with diploid or FLT3- ITD. The impact on survival remains to be studied over a longer period.

1.5. Why Is Drug Repositioning Needed for AML Treatment?

The prognosis of AML patients has remained largely unchanged in recent decades, despite significant improvements in the development of targeted therapies and a growing

pharmacologic library. Only a small proportion of patients with AML have a long-term sustained response to current therapy. Despite the exploration of several potential drug candidates, combination chemotherapy remains the standard treatment. Survival rates of patients with AML have not improved due to the ineffectiveness of current treatments, and there is a need for novel, tailored therapies with different perspectives.

Finding new therapeutic indications for already approved drugs by repurposing or repositioning drugs is a promising strategy for finding effective candidates for the treatment of the disease. Biomarkers must accompany drug development for leukemia for narrowly defined patient subgroups to verify the response. The discovery of new mechanisms of action and the identification of new molecular targets are often associated with drug repositioning. Unfortunately, in AML, there are no precise biomarkers to identify responders for such simple and effective therapy, except for APL. With systems biology approaches, screening tools for diseases and pathways have revitalized the field. From this perspective, *in silico* drug repurposing could help improve the efficacy of personalized and targeted therapies in AML and open up new indications. It is advantageous to repurpose drugs that are already familiar with the full range of adverse effects and how they are delivered and distributed in the body (Table 1.4).

Table 1.4 Repurposed clinical drugs for AML

Class	Drug	Original Indication
Chemotherapeutic Agents		
<i>Antineoplastic</i>	Cladribine	B-cell disease hairy cell leukemia
	Clofarabine	Acute lymphocytic leukemia (ALL)
	Actinomycin D	Wilms Tumor, Rhabdomyosarcoma, Ewing Sarcoma, Trophoblastic Neoplasm, Testicular Carcinoma
	Melphalan	Multiple Myeloma, Retinoblastoma, Melanoma, Ovarian Cancer
	Hydroxyurea	Chronic myeloid leukemia (CML), Melanoma, Ovarian Cancer, Head and Neck Cancer
Non-Chemotherapeutic Agents		
Antimicrobial		
<i>Antibacterial</i>	Tigecycline	Intra-Abdominal infections, Skin infections

Antiprotozoal	Hydroxychloroquine	Lupus Erythematosus, Malaria, Rheumatoid arthritis
	Artemisinin	Malaria
	Quinacrine	Malaria
	Pyrimethamine	Toxoplasma Gondii infection, Malaria
Antiviral	Ribavirin	Hepatitis C
Anthelmintic	Niclosamide	Tapeworm infections
	Mebendazole	Intestinal Helminthiasis
	Clioquinol	Dermatologic disorders
	Ivermectin	Parasitic Roundworm infections
Neuropsychiatric	Valproic acid	Bipolar disorder, Epilepsy, Migraine Prophylaxis
	Bromocriptine	Parkinson's disease, Acromegaly, Hyperprolactinemia
	Tranlycypromine	Major depressive disorder
	Sertraline	Major depressive disorder, Post-traumatic stress disorder, Premenstrual dysphoric disorder
Metabolic	Metformin	Diabetes mellitus, type 2
	Pravastatin	Hypercholesterolemia, Cardiovascular Disease prevention
Antiarrhythmic	Digoxin	Heart Failure

1.5.1. Repositioning Drugs For AML

1.5.1.1. Chemotherapeutic Drugs

Cladribine is an adenosine analog resistant to adenosine deaminase and has been shown to be a relatively new cytotoxic agent. Hairy cell leukemia, a disorder of B lymphocytes, responds extremely well to this treatment, with efficacy of more than half. AML patients may benefit from the antileukemic properties of cladribine when used as a single agent (Sigal et al. 2010). Refractory AML patients can also be treated with cladribine, cytarabine, and granulocyte colony-stimulating factor (CLAG) (Ramsingh et al. 2014). Adding mitoxantrone to the CLAG regimen may also enhance the antileukemic effect. Clofarabine is a second-generation nucleoside analog in combination with fludarabine and cladribine. It has potent antileukemic activity because it can block ribonucleotide reductase and DNA polymerase, cause apoptosis, and increase the amount of cytarabine in cells. Clofarabine has been shown

to be effective in treating AML as salvage therapy or as a first line of treatment for patients unresponsive to intensive chemotherapy (Ghanem et al. 2013). Actinomycin D was the first antibiotic to show anticancer activity. Wilms tumor, rhabdomyosarcoma, Ewing sarcoma, trophoblastic neoplasia, testicular carcinoma, and ovarian cancer can be treated with Actinomycin D (Gaspar et al. 2015; Lawrie et al. 2016). It binds directly to DNA and prevents RNA synthesis by inhibiting RNA polymerase I, II, and III; however, it inhibits RNA polymerase I activity only at low concentrations. AML patients with NPM1 mutations have a 14-month CR with actinomycin D (Falini et al. 2005). The clinical effect in NPM1 AML has been demonstrated with actinomycin D causing acute mitochondrial stress, formation of ROS, recovery of PML nuclear bodies, and progression of cancer to senescence (Wu et al. 2021).

One potential treatment for AML is the alkylating drug melphalan, which is currently being studied for the treatment of multiple myeloma, retinoblastoma, melanoma, and ovarian cancer. Low doses of melphalan in elderly patients with R/R AML have produced positive results. Overall survival was prolonged in patients who achieved complete or partial remission with melphalan treatment (Kerr et al. 2000; Stratmann et al. 2019). Hydroxyurea has been studied in combination with cytarabine in AML (Burnett et al. 2007). It is used to treat CML, melanoma, ovarian cancer, and head and neck cancer (Madaan et al. 2012). Hydroxyurea improved outcomes, particularly in samples with high SAMHD1 enzyme levels, without significant adverse effects (Rudd et al. 2020). The use of hydroxyurea in combination with valproic acid has resulted in unique effects on DNA repair in preclinical studies (Leitch et al. 2016).

1.5.1.2. Non-Chemotherapeutic Drugs

1.5.1.2.1. Antimicrobials

Antibacterial

Tigecycline is an antibiotic that is effective against Gram-positive and Gram-negative bacteria (Pankey 2005). By inhibiting mitochondrial translation, tigecycline effectively kills

leukemic stem and progenitor cells. Tigecycline delayed disease progression at the maximum tolerated dose in xenograft mouse models, comparable to daunorubicin and bortezomib. Tigecycline is a promising prospective therapy because it kills AML cells and leaves healthy cells alive (Škrtić et al. 2011).

Antiprotozoal

Glioblastoma, pancreatic adenocarcinoma, sarcoma, multiple myeloma, melanoma, and AML are among the malignancies being studied in clinical trials with the antimalarials hydroxychloroquine and chloroquine (Verbaanderd et al. 2017). In AML cells, hydroxychloroquine caused cell death by suppressing autophagic degradation. In cytarabine-resistant leukemia cell lines, hydroxychloroquine-induced cell death was higher than that in cytarabine-sensitive cells (Y. Kim et al. 2015). Artemisinins have been shown to cause cell cycle arrest, activation of apoptosis via ROS -dependent and independent pathways, and lysosomal disruption in leukemia. Through the formation of ROS, activation of caspases, reduction of lysosomal integrity, and activation of p38 mitogen-activated protein kinase, artemisinins showed anti-leukemic effects *in vitro* and synergistic effect with chemotherapeutic agents in AML cells (Drenberg et al. 2016; J.-J. Lu et al. 2008).

Quinacrine was discovered after screening over a thousand chemicals from the LOPAC library. Both *in vitro* and *in vivo* experiments showed moderate toxicity in mononuclear cells and a synergistic effect with cytarabine or azacytidine, resulting in a reduction in the total number of circulating blast cells and increased survival in mice (A Eriksson et al. 2015; Anna Eriksson et al. 2017). Malaria and toxoplasmosis treatment Pyrimethamine was discovered in a high-throughput drug screen associated with ATRA. When used *in vitro* and *in vivo*, it can induce apoptosis and differentiation in both human and murine AML cell lines without harming normal cells (Sharma et al. 2016).

Antivirals

The antiviral guanosine analog ribavirin competes with the 5'-mRNA 7-methylguanosine (m7G) cap and inhibits the action of eukaryotic translation initiation factor 4E (eIF4E) (de Benedetti and Graff 2004). Ribavirin has been shown to negatively affect AML cells' ability to form colonies (Kentsis et al. 2004). eIF4E is overexpressed in the M4/M5 subtype of AML

(Sarit Assouline et al. 2009). Overexpressing individuals were enrolled in a clinical trial of ribavirin and low-dose cytarabine to treat R/R AML. Among the 21 patients who received a combination of treatments, there were two CR, one PR, and two blast responses (S. Assouline et al. 2015).

Anthelmintic

Niclosamide is used to treat tapeworm infections and acts by inhibiting oxidative phosphorylation and targeting mitochondria. Due to its potential to suppress NF- κ B, mTOR, Wnt/ β -catenin, and CREB signaling pathways, niclosamide has been considered a promising option for AML treatment (Balgı et al. 2009; Y. Jin et al. 2010; Ren et al. 2010). Preclinical tests have shown that it improves the survival of mice xenografted with AML cells when combined with regular treatment (Chae et al. 2018). Intestinal worm infection is often treated with mebendazole, which binds permanently to tubulin and prevents microtubule assembly. The chaperone mechanism of heat shock protein 70 is disrupted by mebendazole, leading to the degradation of c-MYB in AML cells. Mebendazole decreased MLL viability and colony-forming activity and prolonged survival of MLL-AF9 xenograft mice (Walf-Vorderwölbecke et al. 2018). Proteasome activity is blocked by the cyclin D2 inhibitor clioquinol, which is used specifically in malignant AML. Tumor volume and weight decreased without side effects in three separate xenograft models of AML mice. Clioquinol proved safe and effective in individuals with refractory AML (Schimmer et al. 2012). Ivermectin induces caspase-dependent apoptosis in AML cells and causes membrane hyperpolarization and production of ROS. Cytarabine and daunorubicin worked better when combined with ivermectin, proving that the drugs work well together (Sharmeen et al. 2010).

1.5.1.2.2. Neuropsychiatric

The epilepsy drug valproic acid (VPA) has been tested as an anti-cancer therapy. It inhibits the catalytic activity of class I HDACs and promotes proteasomal degradation of HDAC2, which causes hyperacetylation of histones H3 and H4 *in vitro* and *in vivo*. Murine PML-RARA and RUNX1-RUNX1T1-driven leukemias, AML cell lines, and primary blasts undergo differentiation and/or die due to this inhibitory effect on HDAC activity. Synergistic

anti-leukemic effects between VPA and cytarabine (AraC) were demonstrated in a retrovirus-mediated mouse model with MLL-AF9 leukemia, three leukemia cell lines (THP-1, K562, and HL-60), and seven primary human AML samples (Nan Liu et al. 2016). Anti-leukemic effect of cytarabine was enhanced by VPA, which induces cell death. Bromocriptine is a dopamine agonist used in the treatment of Parkinson's disease. It has shown selectivity for leukemic cells and synergistic effect when combined with conventional therapy to treat high-risk MDS and secondary AML (Lara-Castillo et al. 2016).

The antidepressant tranylcypromine acts as an epigenetic modulator by inhibiting LSD1. LSD1 is necessary for MLL-AF9 cells to initiate AML, and its inhibition or knockdown reduces the ability of AML leukemic stem cells to proliferate (Harris et al. 2012; Magliulo et al. 2018). The antidepressant sertraline has been shown to have anti-cancer properties in several malignancies, including liver, colorectal, and lymphomas. The study showed that sertraline has potent antiproliferative effects in both AML cell lines and primary cell lines and that it can kill cells through both the apoptosis and autophagy pathways (D. Xia et al. 2017). Sertraline and cytosine arabinoside are being tested in a phase I trial (NCT02891278) in adults with relapsed and resistant AML (Valli et al. 2020).

1.5.1.2.3. Metabolism

By inhibiting mTOR activation via the liver kinase B1 (LKB1)/5' AMPK/tuberous sclerosis protein, metformin, a drug for type 2 diabetes, inhibited AML cell growth while preserving normal hematopoiesis. When combined with sorafenib, metformin is effective against AML with FLT3-ITD (Fangfang Wang et al. 2015). To address these promising results, a phase I clinical trial (NCT01849276) was conducted to evaluate metformin in combination with cytarabine for treating R/R AML. Due to a lack of participants, the project was discontinued. By inhibiting HMG-CoA reductase and preventing HMG-CoA conversion to mevalonic acid, statins lower blood cholesterol levels in patients (Kornblau et al. 2007). In AML, overexpression of genes involved in the mevalonate pathway occurs. Co-administration of pravastatin doses blocked the adaptive cholesterol response in AML cells with idarubicin/high-dose cytarabine. Complete remission was achieved in 20 of the 37 patients

taking pravastatin without toxicity. In a phase II study, patients with relapsed AML responded effectively to idarubicin and cytarabine in combination with pravastatin (NCT00840177) (Advani et al. 2014).

1.5.1.2.4. Antiarrhythmic

Digoxin is a drug used to treat heart failure and abnormal heart rhythms. The proliferation of human malignant cell lines is inhibited by digitoxin, which induces apoptosis without destroying the proliferating normal cells. Digitoxin was found to successfully destroy human AML stem cells in an *in silico* study followed by an *in vitro* leukemic stem cell assay (Laverdière et al. 2018). A phase Ib/ II trial (NCT03113071) was initiated to evaluate the safety and efficacy of digoxin and decitabine in adults with AML and MDS. However, the study was discontinued due to a lack of patients (Valli et al. 2020).

1.5.2. Challenges For Repositioning

Drug repositioning is an efficient and potentially less toxic treatment alternative for diverse and heterogeneous groups of AML. Preclinical research supporting the efficacy and safety of a drug approved by the FDA should be considered during repositioning. Repositioning can only be successful if pharmaceutical companies, motivated researchers, and philanthropic organizations collaborate in novel ways. Researchers estimate that 75% of drugs can be repurposed, but only 6% of drugs studied in another therapeutic area can be repositioned (Neuberger et al. 2019). Phase I and II clinical trials of several drugs have been discontinued due to uncertainties about efficacy.

In the past, AML treatment relied exclusively on a few agents with antiproliferative properties that could be used in all cases of AML. The complex biological pathways and the broad spectrum of disease manifestations require specialized drugs targeting specific disease vulnerabilities (Valli et al. 2020). To date, there has been no "long-term win" in finding new uses for old drugs to treat AML. Clinical trials have been conducted to evaluate the effects of VPA, ribavirin, and HMG-CoA reductase inhibitors, with varying degrees of success. Because of slow recruitment in clinical trials for drugs such as metformin,

hydroxychloroquine, and cardiac glycosides, additional funding may be needed for a new trial to succeed. Overall, drug repurposing in AML does not inspire confidence. In AML therapy, it is clear that the experimental backbone and challenging clinical trials require advanced biomarkers to identify treatment-responsive patients (Stenvang et al. 2013). If the data generated are successful, they should solve the problem of regulatory approval methods and regulations by drug regulatory agencies.

1.6. Multi-omics studies in AML

According to the principles of systems biology, all living things are composed of interconnected and ever-changing networks of biological reactions. Disruption of one or more signaling cascades is associated with disease etiology, which can originate at the molecular level and lead to clinical disease manifestation via gain or loss of normal cascade function. Many cells employing various signaling pathways are involved in complicated diseases. This systems perspective on the disease or biological process is the consequence of integrating the systems of molecular pathways impacting these cell types. High-throughput multi-omics molecular analysis have revolutionized our understanding of diseases in clinical medicine. Nonetheless, the difficulty of retrieving useful information from massive amounts of "big omics data" remains a significant barrier to clinical application. Yet, in specific clinical circumstances, both diagnostic and therapeutic utility of large-scale sequencing or gene expression studies are pave the way for elucidating of the disease. Although a great number of AML-initiating mutations have been identified and characterized, which has led to several direct advances in the area, the processes by which the malignancy grows are still not completely understood. From that perspective exploring system-wide profiles is crucial to construct the novel strategies for the diagnosis, prognosis, treatment, and chemoresistance of AML.

Researchers could take an integrative approach, combining multiple types of data to gain a more complete picture of the molecular mechanisms underlying the disease. This approach would allow researchers to identify key genes, proteins, and metabolites involved in AML

and to uncover interactions and pathways that may not be apparent from any single type of data. By integrating multiple types of data, researchers could potentially identify novel targets for therapy and develop more effective treatments for AML. Analysis of the genomic and epigenomic landscape in AML led to the identification of genetic risk groups for AML, which in turn provided the release of the recommendations for risk stratification (Döhner et al, 2017). Molecular responses to therapeutics have been elucidated at the transcriptomic level, and gene expression signatures for responders and non-responders have been identified (Raponi et al, 2007; Raponi et al, 2008). More recently, mathematical modeling and machine learning approaches came to the forefront to predict survival outcomes of AML patients (Hoffmann et al, 2020; Karami et al, 2021). On the other hand, multi-omics techniques that reconcile and integrate data streams from different levels of the biological hierarchy, from genes to proteins to metabolites, offer promising potential for AML discoveries toward systems medicine. The researchers performed multi-omics clustering analysis to identify distinct subgroups of AML patients based on their molecular profiles. They identified three subgroups of patients with distinct molecular features and clinical outcomes. These subgroups were characterized by differential expression of genes involved in cell cycle regulation, DNA repair, and cell signaling pathways. The study also identified several dysregulated molecular pathways in the highest risk AML patients, including the JAK-STAT pathway. Furthermore, the study identified several potential prognostic biomarkers that may be useful for predicting the outcome of AML patients with the highest risk of relapse and mortality (Nguyen et al, 2021).

Another study utilized a multi-omics approach to identify potential druggable targets in AML cancer stem cells. The study identified several dysregulated pathways and validated the efficacy of targeting one of the potential druggable targets in vitro and in vivo. The findings from this study may lead to the development of new therapies for AML (Salavaty et al, 2022). A study utilizing a multi-omics approach to understand the molecular mechanisms of APL revealed the PML-RARA fusion protein's context-specific transcription factor activity, which regulates different sets of genes in different APL subtypes, indicating the importance of personalized treatments. By identifying thousands of binding sites, the study identified

key target genes involved in cell proliferation, differentiation, and apoptosis, which provides new insights into the molecular mechanisms of APL and may aid in the development of targeted therapies. These findings suggest that different APL subtypes may require different treatments, and personalized approaches may lead to more effective treatments (Villiers et al, 2022). Our study was designed to specifically interrogate the effect of changing mechanism of treatment response. We report here a systems medicine and multi-omics approach to integrate the AML transcriptome data and reporter biomolecules at the RNA, protein, and metabolite levels using genome-scale biological networks. This study represents the first attempt to perform an integrative multi-omics analysis, combining transcriptomics, proteomics, and metabolomics data, to gain a more comprehensive understanding of complex biological processes in responder and non-responder AML patients and their overall survival status. Moreover, novel drugs were repurposed based on the results of the analysis, providing new therapeutic opportunities for AML patients.

2. MATERIAL AND METHODS

2.1. Implications For Systems Medicine of Emerging Molecular Signatures Derived from Multi-Omics Developments in Diagnostic and Treatments For Acute Myeloid Leukemia

2.1.1. Transcriptome Datasets and Data Preprocessing

The design of the present multi-omics integrative analyses was directed at deciphering how patients display individual differences in response to treatment and progression of the disease. Datasets that are pertinent to these aims and those that include adult patient groups and at least 25 samples in each dataset were accepted as inclusion criteria.

To analyze gene expression profiles in AML, we screened datasets in Gene Expression Omnibus (GEO) (Barrett et al. 2012) and ArrayExpress (Athar et al. 2019). We used two independent transcriptome datasets from two consecutive studies, GSE5122 (Raponi et al. 2007) and GSE8970 (Raponi et al. 2008), in the GEO database.

The GSE8970 dataset contains both treatment response and overall patient survival data, whereas the GSE5122 dataset contains only treatment response data in addition to gene expression profiles. Therefore, both datasets were used in the comparative analysis of treatment response groups, i.e., responders and non-responders, to identify gene signatures associated with treatment response. On the other hand, the GSE8970 dataset was used to identify genes associated with overall survival. Considering patients with overall survival $\pm$ 10% of the median of the dataset, patients were divided into two groups, i.e., high survival and low survival, and analyzed comparatively.

The farnesyltransferase inhibitor, tipifarnib, was used to treat patients in both datasets. ‘Treatment response’ was defined as patients who had an objective response (complete remission, complete remission with incomplete platelet recovery, or partial remission) or a hematologic response (decrease of $>50\%$ of leukemic blast cells in the bone marrow). “Stable disease” was defined as no hematologic response but no disease progression (Raponi et al. 2007). For the purpose of the study, patients with stable disease were considered neither responders nor non-responders and were therefore not included in the analysis. GSE5122 comprises 58 patients, five of whom were removed according to our criteria for treatment response. GSE8970 consists of 34 patients, eight of whom were removed based on their response status. After preprocessing of the data in the GSE5122 and GSE8970 datasets, the male-to-female ratio was 26:27 and 18:8, respectively, and the mean age was 60 and 73, respectively.

2.1.2. Identification of differentially expressed genes

The differentially expressed genes (DEGs) were identified from the normalized expression values by using the Linear Models for Microarray Data (LIMMA) package (version 3.45) (Smyth 2005) based on a pre-constructed statistical analysis approach (Kori et al. 2016). Raw data in each dataset was standardized using the Robust Multi-Array Average (RMA) expression measure (Bolstad et al. 2003) and Affy package (Gautier et al. 2004) as implemented in the R/Bioconductor (ver.3.6.3) (Gentleman et al. 2004).

To identify DEGs associated with treatment response, comparative analyses of treatment response groups, i.e., responders and non-responders, were performed on both datasets. Similarly, high-survival and low-survival patient groups in GSE8970 dataset were compared to identify DEGs associated with overall survival outcomes. The Benjamini-Hochberg method was used to control the false discovery rate. Fold changes determined the down and upregulation pattern of each DEG, and at least $\log FC > 1.5$ (upregulation) or $\log FC < -1.5$ (downregulation) and adjusted p-value < 0.05 were accepted as significant. The information of gene products was obtained from GeneCards: The Human Gene Database (Safran et al. 2010).

2.1.3. Functional enrichment analysis

The functional annotation and enrichment analyses were performed using the ConsensusPathDB database to reveal the biological functions and processes associated with DEGs (Kamburov et al. 2011). GO (The Gene Ontology Consortium 2015) and the KEGG (Kanehisa et al. 2014) were used as resources for molecular functions, biological processes, and pathways. P-values were obtained via Fisher's Exact Test. Benjamini-Hochberg's correction was used as the multiple testing correction technique, and enrichment results with adjusted $p < 0.05$ were considered statistically significant.

2.1.4. Identification of reporter metabolites associated with Acute Myeloid Leukemia

The statistically significant changes in gene expression profiles in each dataset were mapped onto the Human Metabolic Reaction (HMR 2.0) (Mardinoglu et al. 2014) model using the reporter metabolites algorithm (Patil and Nielsen 2005) implemented in the BioMet Toolbox (ver.2.0) (Garcia-Albornoz et al. 2014) to identify reporter metabolites. Benjamini-Hochberg's method was used for the correction of the p-values. Metabolites Biological Role (MBRole) database (ver.2.0) (López-Ibáñez et al. 2016) was used to determine the enrichment of reporter metabolites in metabolic pathways. Adjusted $p < 0.05$ were considered statistically significant.

2.1.5. Identification of reporter receptors, transcription factors, and miRNAs

The adapted version of the reporter features algorithm (Kori and Arga 2018) was used to identify reporter receptors, TFs, and miRNAs. The combinatorial human transcriptional regulatory interaction network and Human Transcriptional Regulation Interactions database (HTRIdb) (Bovolenta et al. 2012) were used to obtain experimentally validated TF-target gene interactions, and to create a TF-target gene network. Likewise, the experimentally confirmed miRNA-target gene interactions were acquired from our previous study (Gov and Arga 2016; Kori and Arga 2018) and miRTarbase (release 6.0) (Chou et al. 2016) to generate the miRNA-target gene network. To reconstruct a receptor-protein interaction network, the proteins with receptor activity (GO: 0004872) were screened in the Protein Analysis Through Evolutionary Relationship (PANTHER) (Mi et al. 2019), database for Annotation, Visualization and Integrated Discovery (DAVID) (D. W. Huang et al. 2007), and GeneCodis (Tabas-Madrid et al. 2012) databases, and the physical protein interactions of these receptors were retrieved from the BIOGRID database (v.3.5.167) (Chatr-aryamontri et al. 2017). The p-values were converted to z-scores and integrated with the molecular interaction networks to determine a score for each biomolecule (receptor, transcription factor, or miRNA) based on the z-scores of its network neighbors using the inverse cumulative distribution. Then, the scores from a standard normal distribution were converted to p-values, and statistically significant features ($p < 0.05$) were designated as reporter biomolecules.

2.1.6. Cross-validation of the reporter biomolecules

The prognostic power of reporter biomolecules (i.e., hubs, TFs, receptors, and miRNAs) was analyzed at the transcriptome level using an independent RNA-Seq and miRNA-Seq dataset from The Cancer Genome Atlas (TCGA). The TCGA-AML dataset consists of 149 samples with their clinical information (including overall survival data). Considering the expression profiles of the biomolecules of interest, patients were classified into low- and high-risk groups according to their prognostic indices. Multivariate survival analyses and risk assessments were performed using the SurvExpress tool (Aguirre-Gamboa et al. 2013). Boxplots were used to show differences in expression levels between the risk groups. The t-

test was used to estimate the statistical significance of the differences. Kaplan–Meier plots were used to determine the survival signatures of reporter biomolecules. As a cut-off, a log-rank p-value < 0.05 was considered statistically significant in all analyses.

2.2. Identification of Differential Co-Expression Networks Associated with Acute Myeloid Leukemia Prognosis

2.2.1. Transcriptome datasets

Deciphering individual differences in disease development and treatment response was the goal of the current differential co-expression analysis. The inclusion criteria were datasets relevant to these goals, datasets with adult patient groups, and datasets with at least 25 samples each.

To prevent microarray platform differences, datasets obtained from the same microarrays and Affymetrix microarray platforms were employed. Two transcriptome datasets (GSE5122 and GSE8970) were downloaded from the Gene Expression Omnibus (GEO) (Barrett et al. 2012). Individuals in dataset GSE5122 (Raponi et al. 2007) were patients with relapsed or refractory AML, whereas the other dataset, GSE8970 (Raponi et al. 2008), consisted of previously untreated patients. Samples in both datasets were collected from patients before treatment. To discover gene signatures related to treatment response, both datasets were employed in the comparative analysis of groups responding to treatment, i.e., responders and non-responders.

The Cancer Genome Atlas (TCGA) provided independent RNA-Seq datasets that contained clinical information about patients and were used for prognostic analyses (Table 2.1). Patients with a complete or hematologic response were classified as "treatment response" in both datasets. On the other hand, "stable disease" was defined as no hematologic response but no disease progression (Raponi et al. 2007). Patients with stable disease were not included in the analysis because they neither responded nor did not respond to the study target. After preprocessing of the data in the GSE5122 and GSE8970 datasets, the male-to-female ratio was 26:27 and 18:8, respectively, and the mean age was 60 and 73 years, respectively.

Tipifarnib, a farnesyltransferase inhibitor, was used in the treatment of patients in both datasets.

Table 2.1 Transcriptome Datasets Employed in the current study

<i>Source-ID</i>	<i>Disease</i>	<i>Purpose</i>	<i># of samples</i>	<i>References</i>
<i>GEO-GSE5122</i>	Acute Myeloid Leukemia	Network identification	53	(Raponi et al. 2007)
<i>GEO-GSE8970</i>	Acute Myeloid Leukemia	Network identification	34	(Raponi et al. 2008)
<i>TCGA-LAML</i>	Acute Myeloid Leukemia	Prognostic performance	200	(Cancer Genome Atlas Research Network et al. 2013)
<i>TCGA-DLBC</i>	Diffuse Large B-cell Lymphoma	Prognostic performance	58	(Lohr et al. 2012)
<i>TCGA-CLL</i>	Chronic lymphocytic leukemia	Prognostic performance	201	(Landau et al. 2015)

2.2.2. Differential gene expression analysis

The DEGs were characterized using a previously developed statistical analysis method (Kori et al. 2016). Each dataset was normalized using the Robust Multi-Array Average (Bolstad et al., 2003) implemented in the "affy" package (Gautier et al. 2004) of the R/Bioconductor platform (Gentleman et al. 2004). Multiple testing options of LIMMA (Smyth 2005) were used to determine DEGs from normalized log expression values. Benjamini-Hochberg's method was used to control for false discovery rate. An adjusted p value of 0.05 was used to determine the statistical significance of DEGs. Fold changes were used to evaluate the regulatory pattern of each DEG, and at least $\log FC > 1.5$ (upregulation) or $\log FC < -1.5$ (downregulation) was considered significant.

2.2.3. Correlated gene pairs in response status

Pearson's correlation coefficients (PCCs) were used to calculate the correlation patterns of each core gene pair in response status as responders and non-responders. In the co-expression analysis, we used the threshold value of 0.7 (Cicek 2017; L. Liu et al. 2014; Willsey et al. 2013), which is generally accepted as a significant PCC level. When the associated PCC is greater than 0.7, the gene pair was considered positively co-expressed, and the PCC between genes less than -0.7 was considered negatively co-expressed.

To identify differential co-expression profiles between two conditions, the following formula was applied:

$$|(PCC_{Response} - PCC_{Nonresponse})/PCC_{Response}| \geq 1$$

where $PCC_{Response}$ and $PCC_{Nonresponse}$ are the PCC of DEG pair in response and non-response states, respectively.

2.2.4. Construction of co-expression networks based on response status

To construct differential gene co-expression networks around differentially co-expressed gene pairs, four different conditions were considered; (1) gene pairs with positive correlations in the responded state but no correlation in the non-responded state (PO), (2) gene pairs with negative correlations in the responded state but no correlation in the non-responded state (NO), (3) gene pairs with no correlations in the responded state but with positive correlation in the non-responded state (OP), (4) gene pairs with no correlations in the responded state but with negative correlation in the non-responded state (ON). Two co-expression networks (PONO and OPON) were constructed within the significantly co-expressed DEGs. PONO shows the co-expression networks of the response state, whereas OPON represents correlations in the non-responded state. Functional enrichment analyses were performed for the gene sets in the networks using the ConsensusPathDB database (Kamburov et al. 2011). An adjusted $p < 0.05$ was used to describe statistical significance.

2.2.5. Determination of differential co-expression network modules

Cytoscape software (Shannon et al., 2003) (v3.9) and Cytohubba plug-in (Chin et al. 2014) were used to visualize the differential gene co-expression networks and determine hub genes regarding global and local topological metrics. Hub genes are defined as genes with high correlation, and the highly connected hubs that form an integral part of the network were accepted as a module.

2.2.6. Identification of transcriptional regulatory networks

Experimentally validated TF-target gene interactions from the current version of the Human Transcriptional Regulation Interactions database (HTRIdb) (Bovolenta et al. 2012) and the combinatorial human transcriptional regulatory interaction network (Gov and Arga 2016) were used to analyze the connection of TFs with network genes. The TFs that regulated the genes were presented in the analysis for each significant network.

2.2.7. *In silico* Validation and Prognostic performance of the networks in AML and related malignancies

The genes of differentially co-expressed networks were studied in AML and two different tumor types (chronic lymphocytic leukemia-CLL and diffuse large B-cell lymphoma-DLBCL) to determine the specificity of the response and non-response networks of AML and to analyze the expression pattern of the network in the different hematologic malignancies. Cox proportional hazards regression analysis in the SurvExpress validation tool (Aguirre-Gamboa et al. 2013) and RNA-Seq datasets obtained from TCGA were used to determine the prognostic capabilities of the genes in the differentially co-expressed networks. Samples were classified into low- and high-risk categories based on their prognostic index in SurvExpress. Kaplan-Meier plots, the log-rank test, the p-value, and the hazard ratio were used to determine the prognostic performance of the networks.

2.2.8. The evaluation of the immune microenvironment of the networks in AML

Patient prognosis and treatment response were associated with the presence of tumor-infiltrating leukocytes. Immunohistochemistry and flow cytometry can be difficult to implement and standardize due to their limited phenotypic markers. To gain better insight into the respective protein levels, the expression levels of network genes in immune cells were estimated using the CIBERSORT tools (Newman et al. 2015). This method provided a visual comparison of the network landscape of immune cells.

2.3. Transcriptomic Based Drug Repurposing Unraveled Potential Candidate Drugs for Acute Myeloid Leukemia

2.3.1. Selecting Transcriptomic Datasets

To understand why certain patients respond differently to therapy than others and to use this information to develop new therapies with a repurposing approach, the current integrative multi-omics studies were developed. Relevant datasets were selected that included at least 25 samples per dataset and adult patient groups. To investigate gene expression profiles and response mechanisms in AML, we searched Gene Expression Omnibus (GEO) (Barrett et al. 2012) and ArrayExpress datasets (Athar et al. 2019). We selected GSE5122 (Raponi et al. 2007) and GSE8970 (Raponi et al. 2008) from the GEO database, which are two separate transcriptome datasets from two consecutive studies. Gene expression profiles are included in both the GSE8970 and GSE5122 datasets. Both datasets were used to compare groups that responded to treatment (responders and non-responders) to find gene signatures related to treatment efficacy. In both samples, patients were treated with the farnesyltransferase inhibitor tipifarnib. All patients who achieved either an objective response (complete remission, complete remission with incomplete platelet recovery, or partial remission) or a hematologic response (reduction > 50% of leukemic blast cells in the bone marrow) were considered to have responded to treatment. Without hematologic response, but also without disease progression, was considered "stable disease" (Raponi et al., 2007). Patients whose

condition remained stable were not counted as either responders or non-responders for the purposes of the study. GSE5122 originally included 58 patients, but we had to exclude five of them because they did not respond to treatment. GSE8970 originally included 38 patients, but due to exclusions based on response, only 34 remained. In both GSE5122 and GSE8970, the ratio of males to females after preprocessing was 26:27, and the average age of the sexes barely varied between 60 and 73 years.

2.3.2. Identification of differentially expressed genes

DEGs were identified using a previously created statistical analysis method (Kori et al. 2016). The "affy" (Gautier et al. 2004) package of the R/Bioconductor platform Robust Multi-Array Average (Bolstad et al. 2003) was used to normalize each dataset (Gentleman et al. 2004). DEGs were calculated from the normalized log expression values using multiple LIMMA testing methods (Smyth 2005). The false discovery rates were controlled using the Benjamini-Hochberg method. Statistical significance of DEGs was determined using an adjusted p value < 0.05 . The regulatory pattern of each DEG was assessed by fold changes, with at least $\log FC > 1.5$ (upregulation) or $\log FC < -1.5$ (downregulation) considered important.

2.3.3. Drug Repositioning Based on DEGs

For the network-based DR, the up- and down-DEGs associated with the reactions were considered using GeneXpharma (Turanli et al. 2017), a publicly available platform that provides 50,304 gene-drug interactions between 4,344 genes and 11,939 drugs using statistical tests for the disease-gene-drug triad. The query lists from up-regulated and down-regulated DEG were used as input to determine if there were interactions between the reaction mechanism and the major drug candidates. Simulation results with a p-value of less than 0.05 were considered statistically significant, and drugs were linked to the corresponding DEGs using a hypergeometric test. To find drugs already associated with AML, additional searches were performed in publicly available databases such as PubMed and DrugBank.

2.3.4. Cell Culture and *In Vitro* Assays of Repurposed Drugs

HL-60 (ATCC, cat. no. CCL-240) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% Pen-Strep glutamate. Cells were maintained at a density between 1×10^5 and 1×10^6 viable cells/mL at 37°C and 5% CO₂. KG-1 (ATCC, cat. no. CCL-246) cells were cultured in IMDM supplemented with 20% fetal bovine serum and 1% Pen-Strep glutamate. Cells were maintained at a density between 2×10^5 and 1×10^6 viable cells/mL at 37°C and 5% CO₂. Cells were treated for 24 hours with various concentrations of aniracetam (Selleckchem), desipramine (Selleckchem), doxepin (Selleckchem), estramustine (Selleckchem), hydrochlorothiazide (Selleckchem), leucovorin (Selleckchem), nortriptyline (Sigma-Aldrich), and risedronate (Selleckchem). Desipramine, doxepin, estramustine, leucovorin, and nortriptyline were dissolved in water; hydrochlorothiazide and risedronate were dissolved in NaOH, whereas aniracetam was dissolved in DMSO. Control groups were treated with different concentrations of 0.1% water, 0.1% NaOH, and 0.1% DMSO for 24 hours.

2.3.5. Cell Viability Assays

The effect of the drug candidates on AML cell proliferation was determined by the WST-1 method according to the manufacturer's instructions (Sigma-Aldrich). Briefly, 1×10^4 cells were seeded in 96-well plates, treated with defined concentrations of drugs and controls, and incubated at 37°C for 24 hours. WST-1 reagent (10 µl/well) was added and incubated at 37°C for 4 hours, and absorbance was measured at 420 nm using an EnSpire multimode plate reader (PerkinElmer). Each experiment was repeated three times with triplicate samples. Results were expressed as percentage of viable cells compared to control.

2.3.6. Immunoblotting Analysis

Total protein isolation was performed with cell lysis buffer (Cell Signaling Technology, CST)-containing a protease inhibitor cocktail and PMSF after drug treatment. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (23225 Thermo

Fisher Scientific Inc.). Proteins from cell extracts in reducing Laemmli buffer (40 µg) were loaded onto SDS-PAGE (8% acrylamide) and then blotted onto nitrocellulose membranes (Thermo Fisher Scientific). Membranes were blocked with 5% nonfat dry milk TBST and incubated with primary antibodies PARP (Cell Signaling Technology, 9542, 1:1,000) and Akt (Cell Signaling Technology, 9272, 1:1,000) at 4°C overnight. After incubation with HRP-conjugated rabbit secondary antibody (Calbiochem, D0016365, 1:10,000) for 2 h at room temperature. HRP-conjugated secondary antibody was used to detect the bands, and then Pierce™ ECL Western Blotting Substrate (32106 Thermo Fisher Scientific Inc.) was used to view the bands (Cell Signaling Technology). The ChemiDoc™ MP system was used for imaging (Bio-Rad Laboratories, Inc.). ChemiDoc™ MP System software Image Lab software was used to determine the band density of the lanes. GAPDH (Santa Cruz, sc-365062, 1:10,000) was used as a loading control.

2.3.7. Cell Death Assays

Caspase activity was determined using colorimetric assay kits following the manufacturer's instructions. Briefly, 3×10^6 cells/mL were incubated with the drugs at 37°C for 24 hours. After washing the cells with ice-cold PBS, they were lysed in ice-cold cell lysis buffer for 30 minutes, and the supernatant was separated by centrifugation (10,000 g at 4°C for 15 minutes). Caspase-8 assay plates were prepared by adding cell lysate and then incubated at 37°C in the dark for 2 hours after addition of the reaction buffer containing acetyl-Ile-Glu-Thr-Asp p-nitroanilide. The amount of p-nitroanilide formed was then quantified at 400 nm using a microplate reader, and the percentage increase in caspase-8 activity was determined by comparing the drug-treated HL-60 and KG-1 cells with the control group.

AML cells were analyzed for apoptosis and cell death using the Cell Death Detection ELISA kit (Roche, Cat. NO. 11544675001), which detects cytoplasmic nucleosome fragments generated during cell death. Drug-treated AML cells (1×10^5 cells/well) were seeded onto the plates for 24 hours. Cells were collected with the incubation buffer and then homogenized. DNA fragments were isolated from the cytoplasmic fractions and then transferred to microtiter plates coated with streptavidin and treated with a biotinylated anti-histone

monoclonal antibody. The peroxidase-conjugated anti-DNA monoclonal antibody with ABTS as substrate was used at 405 nm to measure the amount of fragmented DNA bound to the anti-histone antibody. Absorbance data are in arbitrary units after normalization with control cell lysate data.

2.3.8. Statistical Analysis

Statistical significance of responses to treatments was determined by two-way tests ANOVA using Prism software (GraphPad Software, Inc., San Diego, CA, USA). All experiments were performed in triplicate. Results were expressed as mean $\pm$ standard error of the mean. Results were considered statistically significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. New Multi-Omics Molecular Signatures and Implications for Systems Medicine Diagnostics and Therapeutics Innovation for Acute Myeloid Leukemia

3.1.1. The transcriptomic codes of Acute Myeloid Leukemia

We retrieved gene expression profiles and associated clinical data from two datasets (GSE5122 and GSE8970) from the GEO database. AML patients were classified into two groups, responders and non-responders, based on their response to tipifarnib treatment, and comparative analysis were performed on both datasets to identify DEGs associated with treatment response. On the other hand, patients in the GSE8970 dataset were divided into high and low survival groups based on their overall survival, and comparative analyzes were performed to identify DEGs associated with overall survival. In both cases, upregulated and downregulated DEGs were identified.

As a result of the comparative analyzes, we identified 1472 upregulated and 1135 downregulated DEGs associated with treatment response. In addition, 583 upregulated and 323 downregulated genes were found to be associated with overall survival of AML patients.

The up and downregulated genes were classified based on their activities and functions. The functional annotations of the upregulated DEGs associated with treatment response were significantly enriched with cell cycle checkpoints, apelin signaling pathway, MAPK signaling pathway, RHOH-GTPase cycle, and leukocyte activation. In contrast, genes associated with Rheumatoid arthritis, glucose metabolism, hemostasis, and neutrophil degranulation were downregulated (Figure 3.1). The enrichment analyses of upregulated DEGs associated with overall survival indicated signaling receptor binding, immune system development, response to the hormone, and hemoglobin binding. Enrichment of downregulated DEGs was carried out with the establishment of localization, enzyme binding, pancreatic adenocarcinoma pathway, response to wounding, and AGE-RAGE pathway (Figure 3.1).

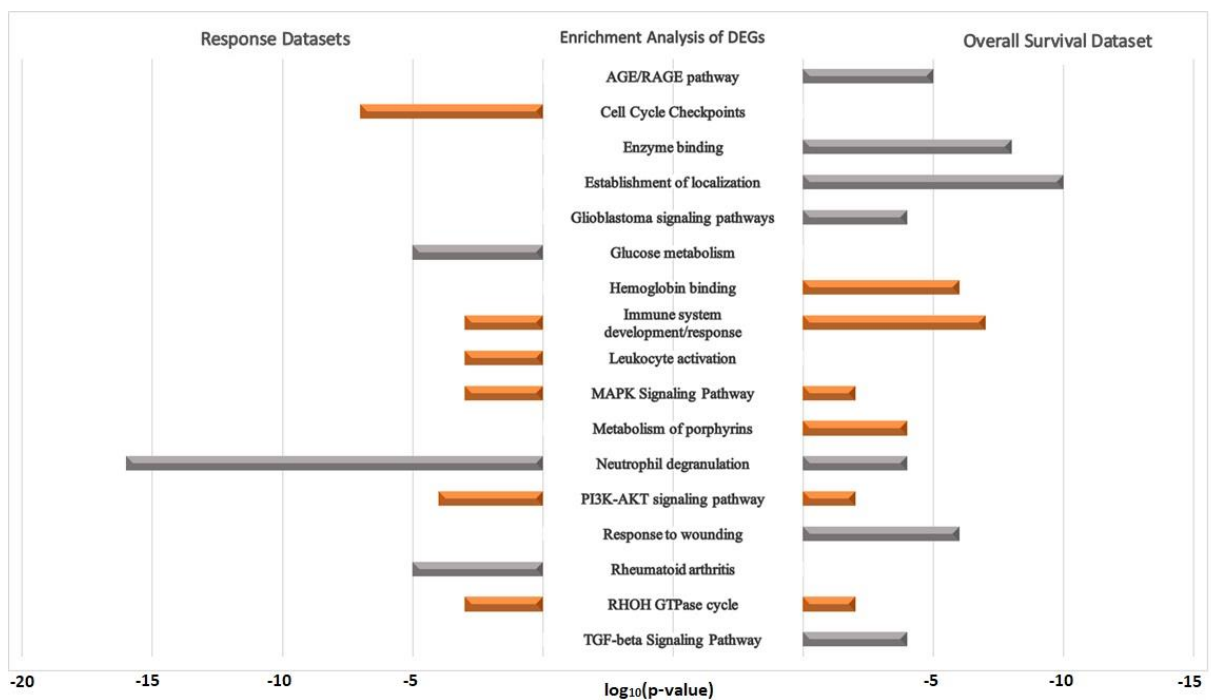


Figure 3.1 Gene set enrichment analysis of the treatment response (GSE5122 and GSE8970) and survival (GSE 8970) datasets. The orange bars represent upregulation of the pathway or process, whereas the gray bars represent downregulation.

3.1.2. The metabolic codes of Acute Myeloid Leukemia

The genome-scale human metabolic network (HMR 2.0) was used to identify reporter metabolites with the integration of transcriptome data of each dataset. The pathway enrichment analyses were performed using MBROLE 2.0 to understand the metabolic activities of reporter metabolites better.

The most remarkable metabolic pathways were amino acid metabolisms such as alanine, asparagine and glutamine metabolism, cysteine and methionine metabolism, valine, leucine, and isoleucine biosynthesis, arginine, and proline metabolism. Also, arachidonic acid metabolism was associated with several metabolites such as 10,11-dihydro-20-dihydroxy-LTB₄, 10,11-dihydro-20-trihydroxy-LTB₄, 12(S)-HETE, 12-oxo-20-dihydroxy-LTB₄, 12-oxo-20-hydroxy-LTB₄, 12-oxo-20-trihydroxy-LTB₄, 20-hydroxy-5S-HETE, 20-hydroxy-LTB₅, 20-OH-10,11-dihydro-LTB₄, 5(S)-HETE, leukotriene B₄, leukotriene B₅ in enrichment analysis of reporter metabolites (Figure 3.2).

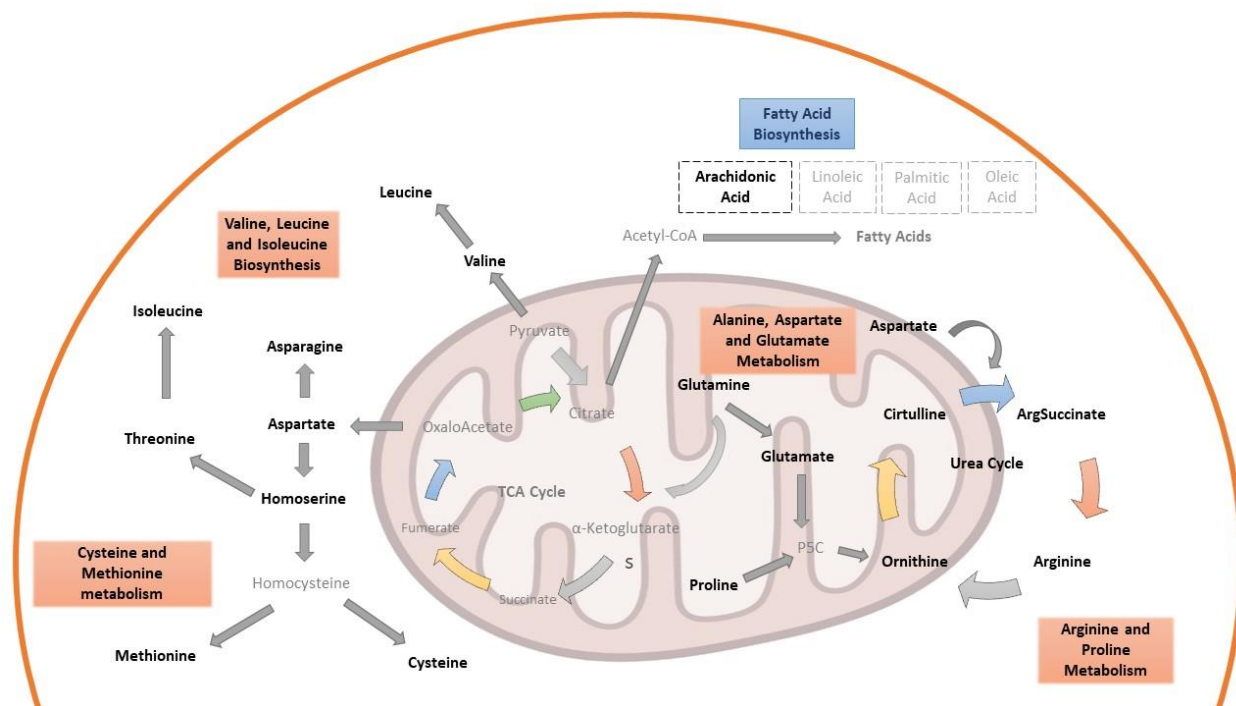


Figure 3.2 A conceptual summary of reporter metabolites highlighted as potential molecular signatures in AML. Black represents detected metabolites; gray indicates the metabolites which were not detected in the present study

3.1.3. The receptor codes of Acute Myeloid Leukemia

In reviewing the literature, no data was found on the reporter receptors of AML. It is crucial to identify reporter receptors that initiate transcriptional responses, providing valuable data on identifying effective biomarkers and drug targets. Differential expression patterns of their physically interacting partners were used to establish the relevance of receptors. The results showed that five reporter receptors (ACVR1, CSF3R, EGFR, PTPRC, PTPRG) (Table 3.1) were common in all datasets.

Table 3.1 Reporter receptors and transcription factors of AML datasets ($p < 0.05$)

Reporter biomolecules (Receptors and TFs)	Name	Functioning in human diseases
ACVR1	Activin A Receptor Type 1	Mutations in the ACVR1 can be detected in endometrial cancers (Zhao and Pritchard 2016).
CSF3R	Colony Stimulating Factor 3 Receptor	It is frequently associated with abnormalities of RUNX1, CBFB, CEBPA, and NPM1 genes in AML (Braun et al. 2019; Y. Zhang et al. 2018).
EGFR	Epidermal Growth Factor Receptor	Its expression in AML patients is associated with a poor prognosis (Nath et al. 2020).
PTPRC	Protein Tyrosine Phosphatase Receptor Type C	Increased PTPRC expression was connected with a poor prognosis (Ruela-de-Sousa et al. 2010)
PTPRG	Protein Tyrosine Phosphatase Receptor Type G	It is frequently deleted in the lung (Galvan et al. 2015) and renal cell carcinoma (Kastury et al. 1996).
AR	Androgen Receptor	High expression was also associated with favorable overall survival of Adenoid cystic carcinoma, Liver hepatocellular carcinoma, AML, Ovarian cancer (Hu et al. 2020).
E2F4	E2F Transcription Factor 4	Essential modulator of AML proliferation and differentiation via regulation of the MAPK signaling pathway in AML (Y. Feng et al. 2020).
ESR1	Estrogen Receptor 1	Constituted an independent outcome predictor in AML (Hess et al. 2006).
ETS1	ETS Proto-Oncogene 1	The proto-oncogene regulated the expression of chemokine and cytokine genes and was necessary for activating the Ras/ERK pathway (Plotnik et al. 2014).
FOXA1	Forkhead Box A1	Abnormally expressed in AML patients with FLT3-TKD and NRAS-PM mutations (Neben et al. 2005)
FOXP3	Forkhead Box P3	Higher expression was detected in bone marrow samples of AML patients (Wang et al., 2020).
GATA1	GATA Binding Protein 1	Regulates differentiation and maturation of erythroid cells and megakaryocytes, and epigenetic deregulation of GATA1 causes the development of AML with NPM1 and FLT-ITD mutations (Drissen et al. 2010)
GATA2	GATA Binding Protein 2	Deletion caused AML blast cell differentiation, increased apoptosis in leukemic stem cells (lscs), enriched for a pro-apoptotic and myeloid differentiation signature in lscs (Menendez-Gonzalez et al. 2019).

GATA3	GATA Binding Protein 3	Involved in multiple tumor-related pathways in B-ALL to impact leukemogenesis and endothelial-specific knockout of GATA3 leads to impairment of Hematopoietic stem cell generation (Hou et al. 2017).
PRDM14	PR/SET Domain 14	Highly expressed and correlated with poor survival in breast cancer (Casamassimi et al. 2020) and partner with CBFA2T3 on DNA and participates in T-ALL development (Tracey et al. 2019).
TFAP2C	Transcription Factor AP-2 Gamma	Transcriptionally activates p21 expression, decreases clonogenic survival, and delays breast cancer cell growth (Li et al., 2006).
YBX1	Y-Box Binding Protein 1	Involved in erythroid cell development and highly expressed in myeloid leukemic cell lines, and downregulation was observed during myeloid differentiation (Bhullar and Sollars 2011).

3.1.4. The regulatory codes of Acute Myeloid Leukemia

The TFs and miRNA, which are regulatory elements in transcriptional expression, are associated with the development and progression of various diseases and play vital roles in biological processes. Thus, it is necessary to understand the mechanism of those regulatory elements in different physiological and disease conditions. Regarding the study, we identified the reporter transcriptional regulators by utilizing the combinatorial human transcriptional regulatory interaction network. According to the results, the twelve TFs were detected in all datasets (Table 3.1). Additionally, the reporter miRNAs were examined, and the sixteen miRNAs were common in all datasets (Table 3.2)

Table 3.2 Reporter micro-RNAs associated with AML datasets ($p < 0.05$)

miRNA	Functioning in human diseases
let-7b	Overexpression downregulates AML1-ETO oncogene expression in t(8;21) AML by targeting its 3'UTR (Johnson et al., 2021).
miR-106b-5p	Potential diagnostic biomarker for ischemic stroke and was ranked as one of the most prominently overexpressed miRNAs in AML (Verboon et al., 2016).
miR-122-5p	Suppresses cell migration and invasion in gastric cancer via downregulating DUSP4 (Xu et al., 2018).
miR-124-3p	Highly promoted by overexpression of SOCS3 in HL-60 cells (Valiollahi and Behravan, 2016).

miR-16-5p	Expression positively associated with VEGF in AML (L. Li et al., 2017).
miR-17-5p	Downregulated in m t(15;17)-positive APL patients (Marcucci et al., 2011), whereas it was significantly overexpressed AML with t(11q23)/MLL (Z. Li et al., 2008).
miR-181a-5p	The elevated expression was connected with longer overall and disease-free survival, indicating as a possible prognostic marker in AML patients treated with intensive induction chemotherapy and auto-stem cell transplantation (Seipel et al., 2020).
miR-192-5p	Suppressed the progression of lung cancer bone metastasis via targeting TRIM44 (Zou et al., 2019).
miR-20a-5p	Upregulated in nasopharyngeal cancer (Chen et al., 2016). Moreover, it promotes colorectal cancer invasion and metastasis (Cheng et al., 2016).
miR-20b-5p	Upregulated and induces the malignant behavior of breast cancer stem cells (Xia et al., 2020) and overexpression is detected in CLL as an indicator of a favorable prognosis (Papageorgiou et al., 2018).
miR-26a-5p	Downregulated, could be a good prognostic marker in patients with bladder cancer (Miyamoto et al., 2016).
miR-335-5p	Tumor suppressor, represses estrogen receptor α in breast cancer cells, implying that it may be involved in estrogen signaling abnormalities (Martin et al., 2017).
miR-484	Diagnostic specificity for CLL (Brown et al., 2017) and high expression was remarkably correlated with a favorable prognosis in ALL (Schotte et al., 2012) and highly overexpressed in the serum of patients with early breast cancer (Zearo et al., 2014).
miR-519d-3p	Overexpression inhibits breast cancer cell growth and motility and arrests those cells into the G0/G1 cell cycle (D. Li et al., 2018).
miR-92a-3p	Downregulated in AML and negatively correlated with the MCL1 that is highly implicated in the pathogenesis of AML (Gado1 et al., 2019).
miR-93-5p	Increased in the exosomes of patients with ovarian cancer (H. Zhang et al., 2019).

3.1.5. Prognostic performance of the reporter biomolecules

The analysis of the prognostic power of reporter biomolecules using RNA-Seq or miRNA-Seq datasets obtained from independent studies resulted in the datasets' samples divided into low- and high-risk groups. The box plots were used to demonstrate the variations in gene expression levels between the risk groups. Evaluation of prognostic capabilities was performed using the log-rank test and Kaplan-Meier plots based on survival results. The differential expression profiles and valuable prognostic power of the reporter receptor CSF3R (p-value = 0.04, hazard ratio = 1.52), PTPRG (p-value = 0.01, hazard ratio = 2.57), and AR (p-value = 0.01, hazard ratio = 1.9), and are shown in Figure 3.3.

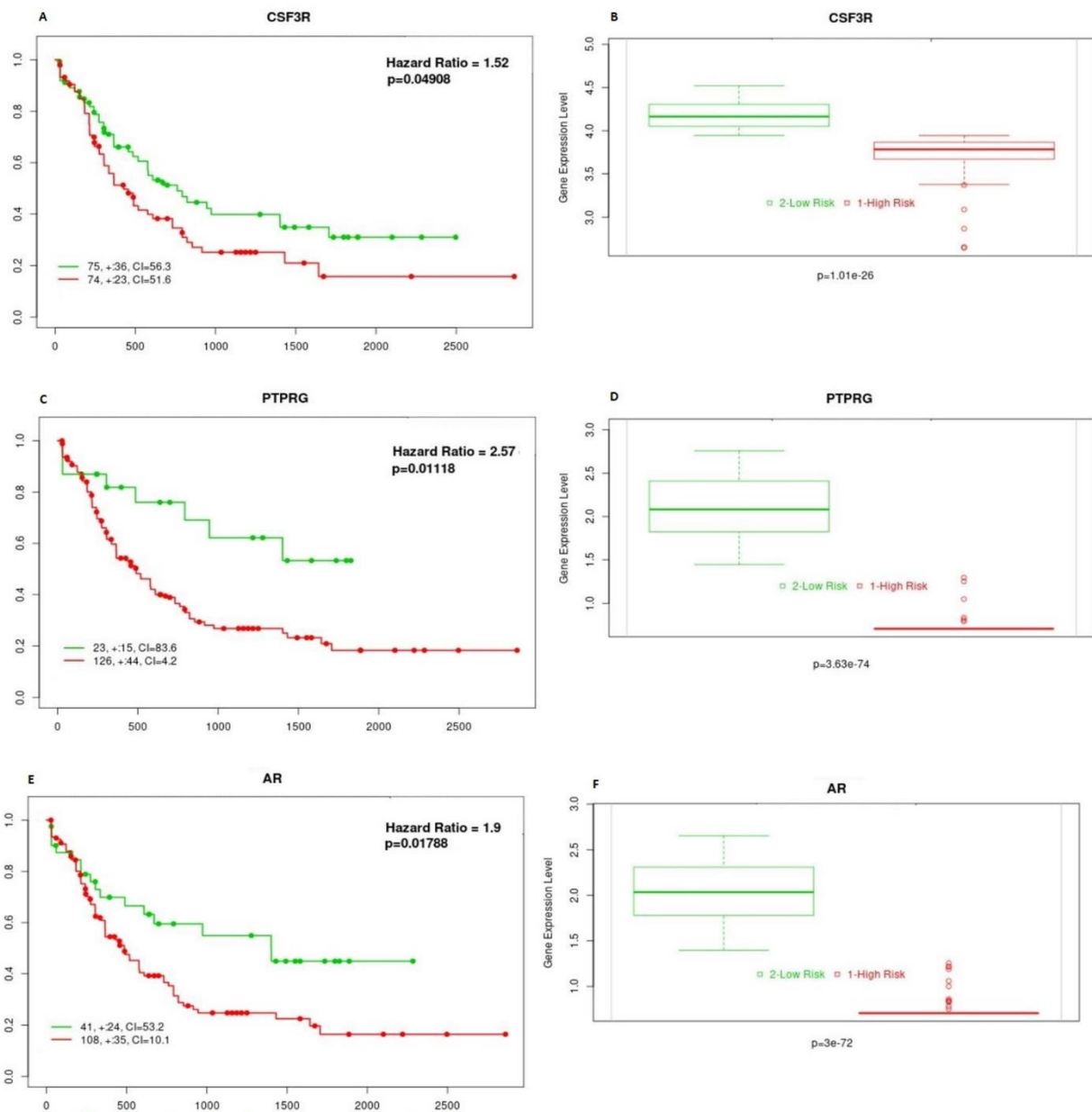


Figure 3.3 The cross-validation results for reporter biomolecules. The Kaplan-Meier (KM) curves demonstrate the prognostic power of (A) CSF3R, (C) PTPRG, and (E) AR. Box plots representing the expression levels of (B) CSF3R, (D) PTPRG, and (F) AR between the low- and high-risk groups. The total size of each group is shown at the left corner, and the number of censored samples is marked with dots.

3.1.6. Discussion

Recent advances in understanding the pathogenesis of AML have paved the way for the development of novel targeted therapies that help to go beyond current standard treatment and promise effective antileukemic activity. The identification of efficient predictive biomarkers and therapeutic targets could improve the sensitivity and specificity of diagnostic/prognostic methods and lead to the development of novel therapeutics and effective drug delivery strategies. Although there are numerous studies comparing healthy control and patient datasets to analyze the mechanism behind the disease, we took a different approach in this study. This research aims to evaluate the datasets from the perspective of treatment response to better understand the complexity of drug response and overall survival of patients during treatment of the disease within the multi-omics approach.

Assessment of response to treatment requires that the patient's condition can be scientifically evaluated at baseline and after a given treatment period. During therapy, treatment response and survival present significant unpredictable obstacles. Therefore, we sought here to make comparisons between groups of patients representing differences in response to treatment or overall survival rather than comparisons between healthy individuals and patients. Samples from two data sets were collected from patients before treatment with tipifarnib. The differences in expression levels in samples from patients prior to treatment will allow us to discover biomarkers that can give us a significant indication of treatment and allow for more effective determination of overall survival status during treatment. Distinguishing between patients who may respond to rationally planned targeted therapies and those who are unlikely to do so would help ensure that the right patients receive the right treatment, leading to better patient care and thus higher response and survival rates. In addition, mechanisms of action or resistance could be elucidated and potential new targets for antineoplastic therapy could be identified through the results of this research.

By individual analyses of each transcriptome dataset, hundreds of genes were statistically identified and further analyzed. The analysis indicated that 1472 DEGs were upregulated, and 1135 DEGs were downregulated in treatment response datasets. Furthermore, 582 upregulated DEGs and 346 downregulated DEGs were detected in the overall survival

dataset. The upregulated genes of treatment response datasets were shown to encode proteins in various molecular functions such as cell cycle checkpoints, RHOH GTPase cycle, MAPK Signaling Pathway, apelin signaling pathway, and immune system. In contrast, downregulated genes of those datasets were observed to be associated with the generation of rheumatoid arthritis, glucose metabolism hemostasis, neutrophil degranulation, peptide hormone metabolism, and TCR signaling.

The enrichment analyses demonstrated that most of the upregulated DEGs in the overall survival dataset were associated with signaling receptor binding, response to hormone, hemoglobin binding, heme biosynthesis, metabolism of porphyrins, glutamate binding, activation of AMPA receptors, synaptic plasticity, and calcium signaling pathway. While the downregulated DEGs were related to the pancreatic adenocarcinoma pathway, the AGE-RAGE pathway, signaling by NTRKs, glioblastoma signaling pathways, and the TGF-beta signaling pathway.

The reporter metabolites and substantially enriched metabolic pathways associated with the most critical transcriptional changes were discovered. Amino acid and arachidonic acid metabolism with several metabolites had come into prominence according to reporter metabolite analysis. Most hematologic malignancies have been linked to the metabolism of particular amino acids such as cysteine, glutamine, arginine, and branched-chain amino acids (Tabe et al. 2019). The previous study showed that L-asparagine treatment inhibited cell proliferation, induced apoptosis in the AML cell lines such as U937, HL-60, and KG-1a, and could be a promising new treatment option for AML (Chen et al., 2020). Glutaminase (GLS) inhibitor CB-839 targets the glutamine pathway, prevents glutathione production, and FLT3 inhibitor corporate to induce reactive oxygen species and inhibit AML cell growth via activation of apoptosis (Gregory et al. 2018). BCT-100, a pegylated human recombinant arginase, causes rapid degradation of intracellular and extracellular arginine concentrations, leading to inhibition of AML blast proliferation and a reduction in AML engraftment (Mussai et al. 2015).

Many kinds of research showed that an engineered human cyst(e)inase enzyme is highly effective in cysteine and cystine degradation in many cancers such as AML, chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and multiple myeloma

(Konopleva et al. 2018). Phosphoglycerate dehydrogenase (PHGDH) controls the serine biosynthesis pathway (SSP), and its inhibitor WQ-2101 sensitizes AML cells with FLT3 mutation to cytarabine (Bjelosevic et al. 2021). These findings disclose new perspectives into how FLT3 mutations re-program metabolism in AML and a combination therapy strategy to improve AML treatment.

Certain lipids were shown to be lower in AML cells than in normal leukocytes in many studies. Recent experiments have discovered wide-ranging alterations in the plasma and bone marrow lipidomes of AML patients (Tabe et al. 2020). Sphingolipids, phosphocholine, triglycerides, and cholesterol esters in AML patients were declined in the plasma (Pabst et al. 2017). However, gamma-linolenic acid 18:3 n-6 and 8,11,14-eicosatrienoic acid 20:3 n-6 were increased, although many prostaglandins such as PGE2 and 15-keto-PGF2 α were decreased in the plasma analyses (Pabst et al. 2017). The findings of this study figured out the importance of arachidonic acid metabolism highlighted with 12 metabolites following amino acids pathways. The plasma arachidonic acid and precursor's correlation with unfavorable prognostic risk was also observed. The importance of the arachidonic acid cascade in AML can be explained by the association of high arachidonic acid concentrations in AML plasma, especially with high BM blasts and high peripheral blasts. Moreover, PGF2 α was found to be elevated in AML with low BM and peripheral blast and in the favorable prognostic risk patients (Pabst et al. 2017).

Due to their genetic heterogeneity, myeloblasts and leukemic stem cells may be highly dependent on specific metabolic pathways in AML diseases (Mesbahi et al. 2022). Metabolic alteration in AML cells has been spotlighted as a potential therapeutic target, and promising findings have been shown in preclinical leukemia models using reagents that target metabolic pathways. The activation and the significant roles of the amino acid and arachidonic acid pathway in AML carcinogenesis were demonstrated by many clinical studies, and these results confirm our findings in that perspective.

The reporter features algorithm was adapted to identify reporter molecules such as receptors, transcription factors, and miRNAs. In this study, five proteins (namely, ACVR1, CSF3R, EGFR, PTPRC, and PTPRG) were identified as reporter receptors in AML. The previous studies reported frequent mutations of CSF3R associated with abnormalities of RUNX1,

CBFB, CEBPA, and NPM1 genes in AML (Braun et al. 2019; Y. Zhang et al. 2018). In addition, EGFR expression in AML patients has been associated with a poor prognosis (Nath et al. 2020); however, the exact mechanism of EGFR in AML progression is still unknown and has to be deeply investigated in further studies.

In AML and ALL, increased PTPRC expression is related to a poor prognosis (Ruela-de-Sousa et al. 2010) and elevated in cells expressing an oncogenic FLT3 mutant FLT3-ITD (Arora et al. 2012). PTPRG is frequently deleted in the lung (Galvan et al. 2015) and renal cell carcinoma (Kastury et al. 1996) and hypermethylated in breast cancer (Sherry et al., 2010), childhood ALL (Xiao et al, 2014), and cutaneous T-cell lymphoma (van Doorn et al. 2005). While the expression and function of PTPRG have been the focus of studies and well-explained in CML (Drube et al. 2018), PTPRG functions in the AML were not studied. ACVR1/BMPR1 pathway was found to be preferentially engaged to induce MIXL1 in hematopoietic stem cells or progenitors but was not proposed as a prognostic marker for AML (Raymond et al., 2014). Moreover, in the same study, ACVR1/BMPR1 signaling is suggested to stimulate endogenous MIXL1 expression in KG-1, OCI-ML2, ML3, and K562 cell lines, and LDN-193189, a ACVR1/BMPR1 inhibitor, is shown to target these cells. However, MIXL1 expression was unaffected by LDN-193189 in U937 and HL-60 cells, suggesting either a MIXL1-independent BMP induction or non-existence of this pathway in HL-60 and U937 cells that are resistant to LDN-193189 (Raymond et al. 2014).

Consequently, to our knowledge, the functional association of receptors PTPRG and ACVR1 with AML is being proposed for the first time in this study, which needs *in vitro* or *in vivo* validation. The differential expression of ACVR1, EGFR, PTPRC, PTPRG, and CSF3R between high and low-risk groups was also cross-validated here using an independent dataset (TCGA-AML), and the prognostic power of PTPRG and CSF3R was demonstrated (Figure 3.3). We showed that the expression of PTPRG and CSF3R are associated with a low risk of AML. Therefore, PTPRG and CSF3R warrant further mechanistic and functional investigation and may have great potential for predicting both treatment response and long overall survival rate for AML patients.

Since transcriptional regulation plays such an essential role in cellular homeostasis, pathogenesis can result from disturbances in transcriptional regulatory mechanisms is not a

surprise. AR, E2F4, ESR1, ETS1, FOXA1, FOXP3, GATA1, GATA2, GATA3, PRDM14, TFAP2C, and YBX1 were common in all datasets, and these transcription factors may play an essential role in the regulation of AML.

High AR expression was reported to be associated with favorable overall survival of AML (Hu et al. 2020). E2F4 was found to be an essential modulator of AML proliferation (Y. Feng et al. 2020). It was found that methylations of tumor suppressor genes and ESR1 constituted an independent outcome predictor in AML (Hess et al. 2008). Elevated levels of ETS1 were detected in AML, but the functional consequences of ETS1 over-expression in AML cells have not been observed (Lulli et al. 2010). FOXA1 is often found to be abnormally expressed in AML patients with FLT3-TKD and NRAS-PM mutations (Neben et al. 2005). The decreased level of FOXP3 was found to delay the leukemia progression and prolong the survival of AML mice (Wang et al., 2020). The mechanisms in the deletion or deregulation of GATA1 (Drissen et al, 2010) and GATA2 (Menendez-Gonzalez et al. 2019) were found to be associated with the development of AML. However, GATA3 was found to involve multiple tumor-related pathways in B-ALL to impact leukemogenesis. Endothelial-specific knockout of GATA3 leads to impairment of hematopoietic stem cell generation; its role is not identified in AML (Hou et al. 2017). PRDM14 acts in the maintenance of embryonic stem cells (ESCs) and the reacquisition of pluripotency in primordial germ cells (PGCs) (Tracey et al. 2019). PRDM14 was noticeably expressed and correlated with poor survival in breast cancer (Casamassimi et al. 2020) and can partner with CBFA2T3 on DNA and participate in T-ALL development (Tracey et al. 2019). Although PRDM14 has been widely studied in many cancers such as lung (Zhang et al., 2013), breast (Taniguchi et al. 2017), and lymphoblastic leukemia (Dettman and Justice 2008), the association of AML with PRDM14 was not specified. miR-10a is strongly overexpressed in AML cells and modulates TFAP2C by downregulating (T. T. Vu et al. 2020). Expression of YBX1 was found to be upregulated in myeloid leukemia cells, coupled with reduced proliferation, and impaired leukemic capacity in AML cell lines (Feng et al., 2021).

Our study highlighted PRDM14 and GATA3 as potential effective biomarkers in a patient's treatment response and survival conditions during the disease and can be a therapeutic target in AML. Moreover, we performed survival analyses to test the prognostic capability of the

reporter TFs through Kaplan-Meier curves, log-rank p-values, and hazard ratios. The prognostic power of AR was demonstrated in the RNA-Seq dataset obtained by an independent AML study (Figure 3.3).

The results of reporter miRNA analysis represented various miRNAs; sixteen reporter miRNAs were associated with treatment response. Previous studies have showed that most of the reporter miRNAs are associated with carcinogenesis and have altered expression levels in different cancers, including AML, CLL, ALL, gastric cancer, lung cancer, colorectal cancer, breast cancer, bladder cancer and ovarian cancer. These include: let-7b (Johnson et al. 2021), miR-106b-5p (Verboon et al. 2016), miR-122-5p (Xu et al. 2018), miR-124-3p (Liu et al., 2016), miR-16-5p (Li et al., 2017), miR-17-5p (Marcucci et al. 2011), miR-181a-5p (Seipel et al. 2020), miR-192-5p (Zou et al. 2019), miR-20a-5p (Cheng et al. 2016), miR-20b-5p (Papageorgiou et al., 2018; Xia et al., 2020), miR-26a-5p (Miyamoto et al. 2016), miR-335-5p (Martin et al. 2017), miR-92a-3p (Gado et al. 2019), and miR-93-5p (Zhang et al., 2017).

Overexpression of miR-519d-3p inhibits breast cancer cell growth and motility and arrests those cells into the G0/G1 cell cycle. By targeting LIMK1, which is a serine/threonine kinase, it plays a crucial role in actin and microtubule dynamics (Li et al., 2018). However, the association of miR-519d-3p in AML was not found. miR-484 has been implicated in diagnostic specificity for CLL (Brown et al. 2017), and its high expression was remarkably correlated with a favorable prognosis in ALL (Schotte et al. 2012). Besides, miR-484 has already been associated with various cancers, but the association of miR-484 was not identified in AML previously. As a result, the results indicated miR-519d-3p and miR-484 deserve more mechanistic and functional study and highlighted their potential as promising biomarkers for both therapy response and overall survival outcomes in AML.

3.2. Elucidation of Genes Associated with Prognosis of Acute Myeloid Leukemia Through Differential Co-Expression Network Analysis

3.2.1. Identification of differentially expressed genes in AML

Gene expression profiles and associated clinical data from two datasets (GSE5122 and GSE8970) were retrieved from the GEO database. Patients with relapsed and refractory AML were included in the GSE5122 dataset, whereas patients with previously untreated AML were included in the GSE8970 dataset. All AML patients were categorized into response/non-response status, and up and downregulated DEGs were identified by adjusted p-value < 0.05 and fold change > 1.5 (for upregulation) or < 0.5 (for downregulation). DEGs were identified as 141 up- and 281 down-regulated genes in GSE5122 and 1351 upregulated and 881 downregulated genes in GSE8970. Between the two datasets, 82 mutual DEGs were identified.

3.2.2. Detection and functional association of differential gene co-expression profiles in AML

To define the biological activities, enrichment analysis was performed for up and down DEGs. The upregulated genes were significantly enriched in MAPK signaling pathway, RHOH-GTPase cycle, immunoregulatory interactions between a lymphoid and a non-lymphoid cell, response to an inorganic substance, leukocyte-mediated immunity, and regulation of immune system processes (Figure 3.4A). In addition, membrane trafficking, TCR signaling, neutrophil degranulation, peptide hormone metabolism, tube development, embryonic morphogenesis, and response to nerve growth factors are conspicuous in the enrichment analysis of downregulated DEGs (Figure 3.4B). The possible co-expression profiles of 82 common DEGs were analyzed with PCC in response and non-response status. Four differential gene co-expression networks were created around the differentially co-expressed gene pairs. To reveal the co-expression networks in the responded state, the gene pairs with positive correlations (PO) were combined with gene pairs with negative correlations (NO). In contrast, the co-expression network of the nonresponsive state was analyzed by combining gene pairs with positive correlation (OP) and negative correlation

(ON). The response network (PONO) included 51 genes and 88 correlations, whereas the non-response network (OPON) included 65 connections between 43 genes.



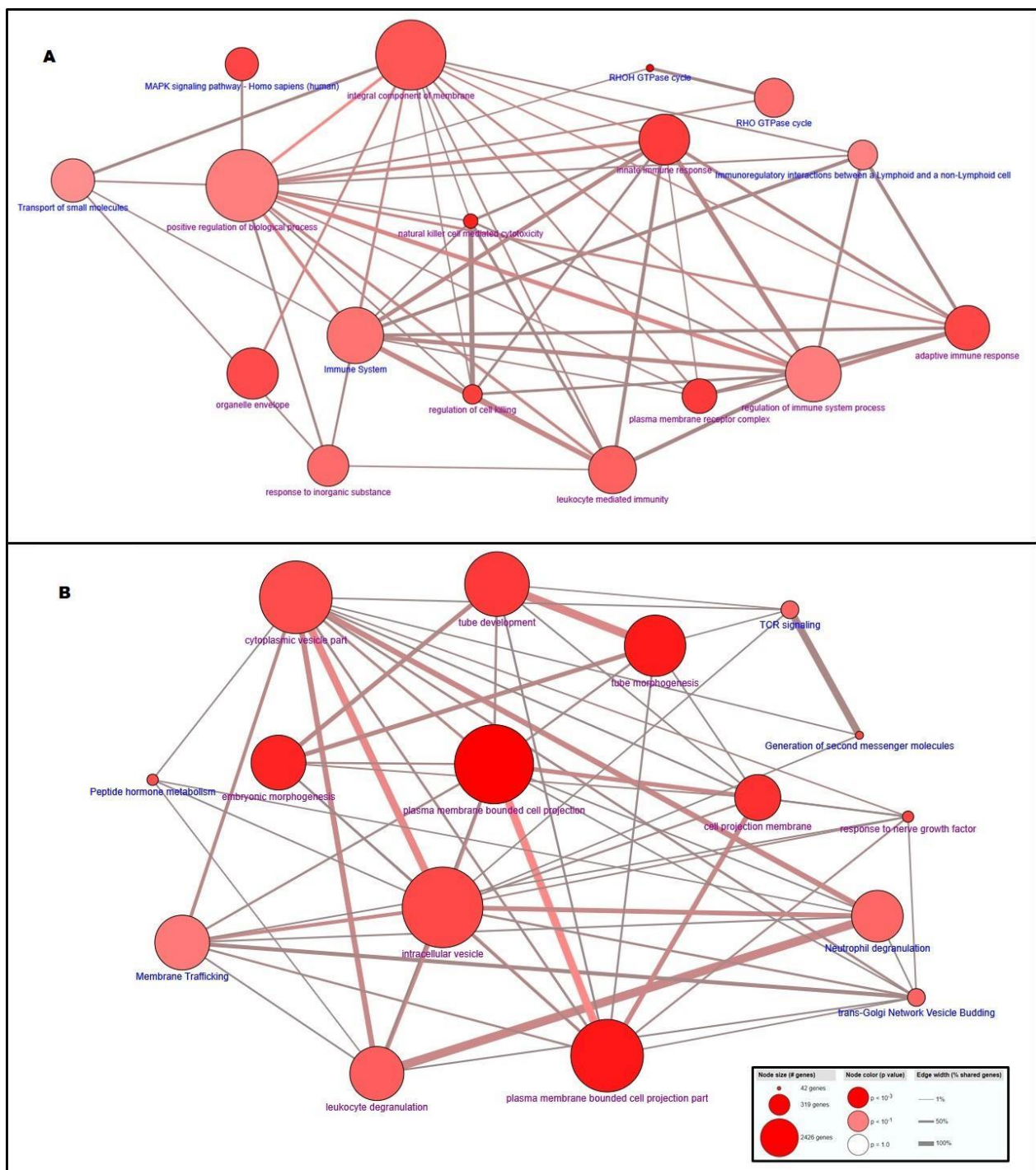


Figure 3.4 Biological process and pathway enrichments of (A) upregulated and (B) downregulated genes

Because we are searching for a prognostic gene module that could represent signatures for treatment response in AML, we focused on the hub genes of the networks and determined them to be modules (Figure 3.5).



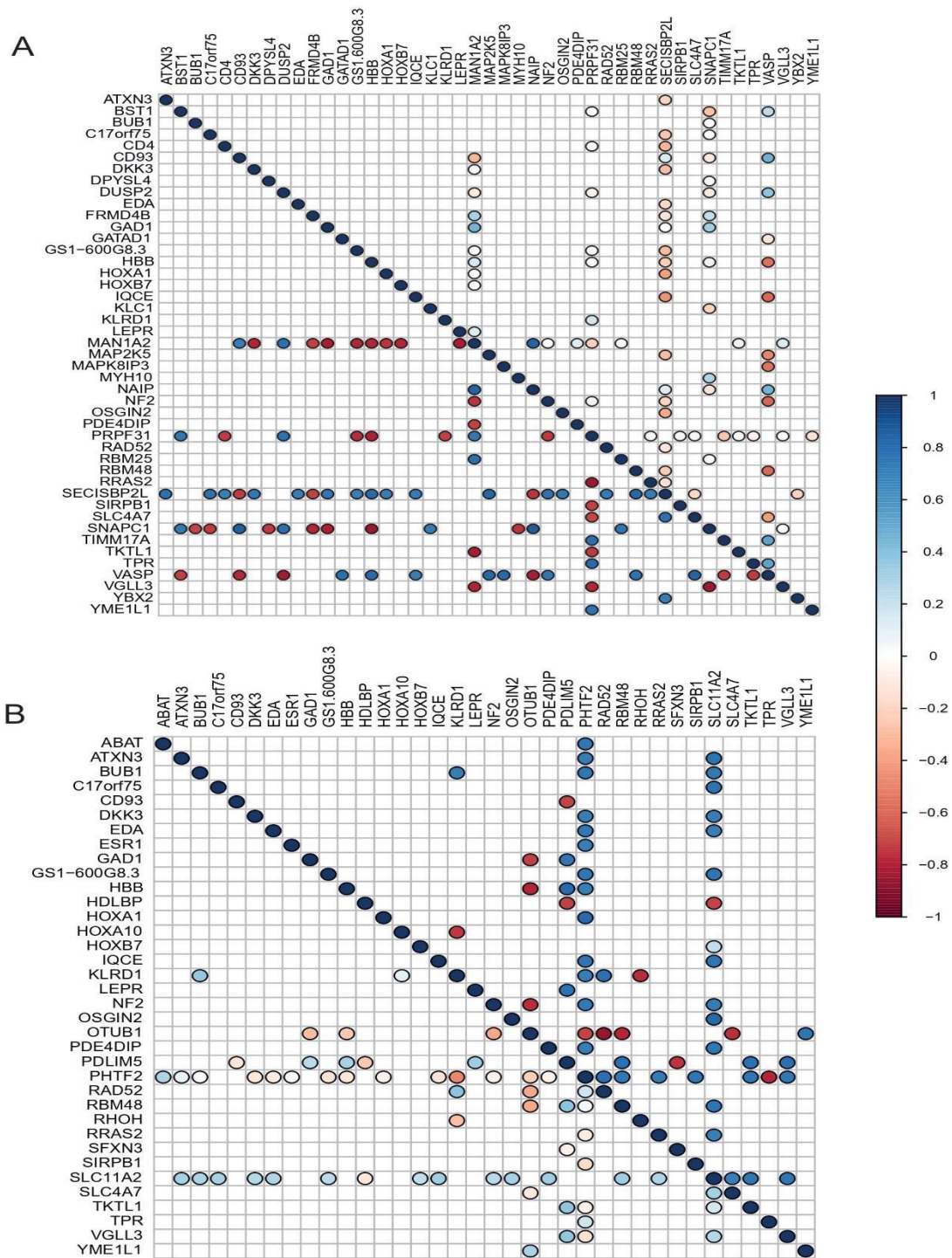


Figure 3.5 The correlation plots of hub genes were determined as modules. In the correlation plots, the lower triangle represents the co-expression patterns in response state whereas the upper triangle represents the co-expression patterns of genes in non-response state. (A) PONO (B) OPON

Moreover, we assume that the potential prognostic modules include a substantial number of genes and a high degree of connectivity within the module to obtain high precision in their predictive ability. MAN1A2, SNAPC1, PRPF31, SECIS2BPL, and VASP were found to be highly connected hub genes in the PONO networks (Figure 3.6). The prominent hub genes in the OPON network were PHTF2, SLC11A2, PDLIM5, OTUB1, and KLRD1 (Figure 3.7). It is important to keep in mind that this study used both datasets, which contained only patient data and no healthy samples; therefore, some genes were common in both networks. Interestingly, GAD1, NF2, HBB, and SLC4A7 appeared in four (PO-NO-OP-ON) differential gene co-expression networks. These genes are closely associated with hub genes in each network. A high proportion of PONO and OPON network genes were prognostic markers in renal and liver cancer (Uhlén et al. 2015; Uhlen et al. 2017). In addition, six genes of the response and five genes of the non-response network were prognostically associated as biomarkers in endometrial cancer.

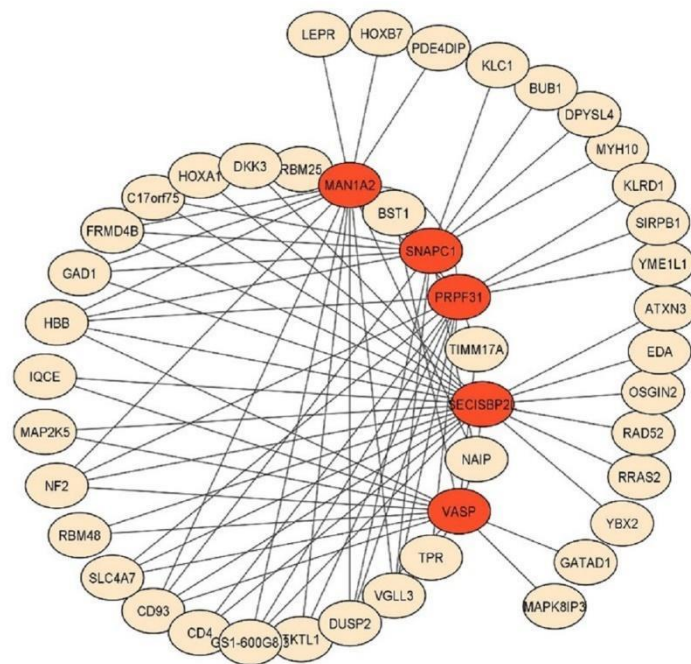


Figure 3.6 Co-expressed hub genes module of response network

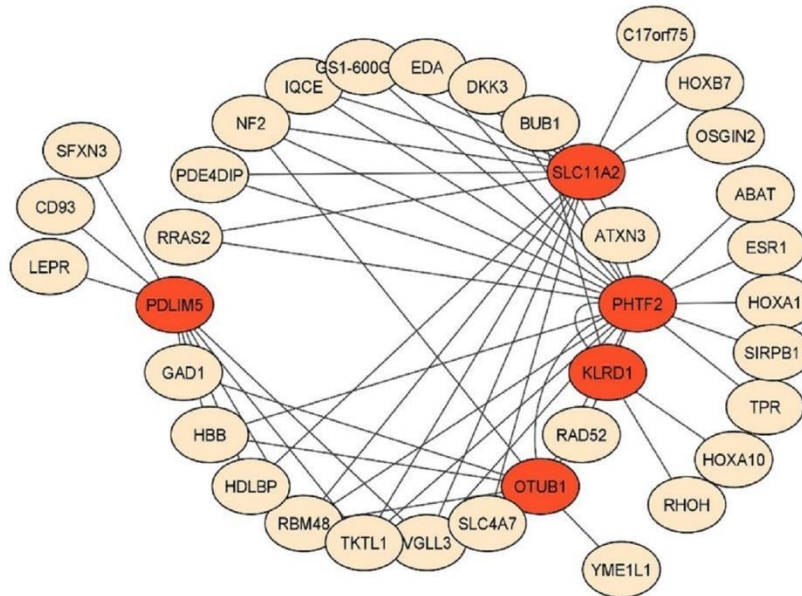


Figure 3.7 Co-expressed hub genes module of non-response network

3.2.3. Transcriptional Regulators of network genes

We performed sequential evaluations to link the critical regulators of transcriptional control in the networks to elucidate the mechanism in the differential co-expression pattern of drug response. A total of 17 TFs were found in the PONO networks, and the six TFs (ETS1, GATA2, AR, YBX1, FOXP3, and PRDM14) regulated the majority of genes in the network. A similar result was seen in the OPON networks; the first five TFs were the same. GATA1 was found instead of PRDM14 in the PONO network. In addition, ETS1, GATA2, and AR co-regulated 27 genes in the PONO network, whereas 25 genes in the OPON network were co-regulated by the same TFs.

3.2.4. Prognostic performance of response and non-response networks

Cox proportional hazards regression analysis was performed to evaluate the prognostic performance of response and non-response AML networks. A comprehensive RNA-seq

dataset was used in the studies, and samples were classified into low- and high-risk groups based on their prognostic index. Kaplan-Meier plots and log-rank tests were used. The prognostic performance of the PO (positively regulated) and NO (negatively regulated) response network was analyzed, and the hazard ratios were estimated as 2.19 ($p=3.29 \times 10^{-4}$) and 2.04 ($p=1.07 \times 10^{-3}$), respectively (Figure 3.8A and 3.8B). Moreover, prognostic analysis for the OP (positively regulated) and ON (negatively regulated) non-response network showed that the hazard ratio was estimated to be 2.5 ($p=3.1 \times 10^{-5}$) and 2.73 ($p=6.65 \times 10^{-6}$), respectively (Figure 3.8C and 3.8D).

Conversely, prognostic performance was higher when the response network was analyzed together (positive and negative regulation) than when it was analyzed alone. Similar results were obtained for the nonresponse network. The analysis showed the high performance of PONO (HR=4.12, $p=1.46 \times 10^{-9}$) and OPON (HR=3.66, $p=9.02 \times 10^{-9}$) networks as prognostic network biomarkers (Figure 3.8E and 3.8F). Since they are not independent networks, understanding their interaction may shed light on their regulation. It may provide clues to the right therapy for the disease by understanding what kind of mechanism it has in different treatment techniques and during treatment.

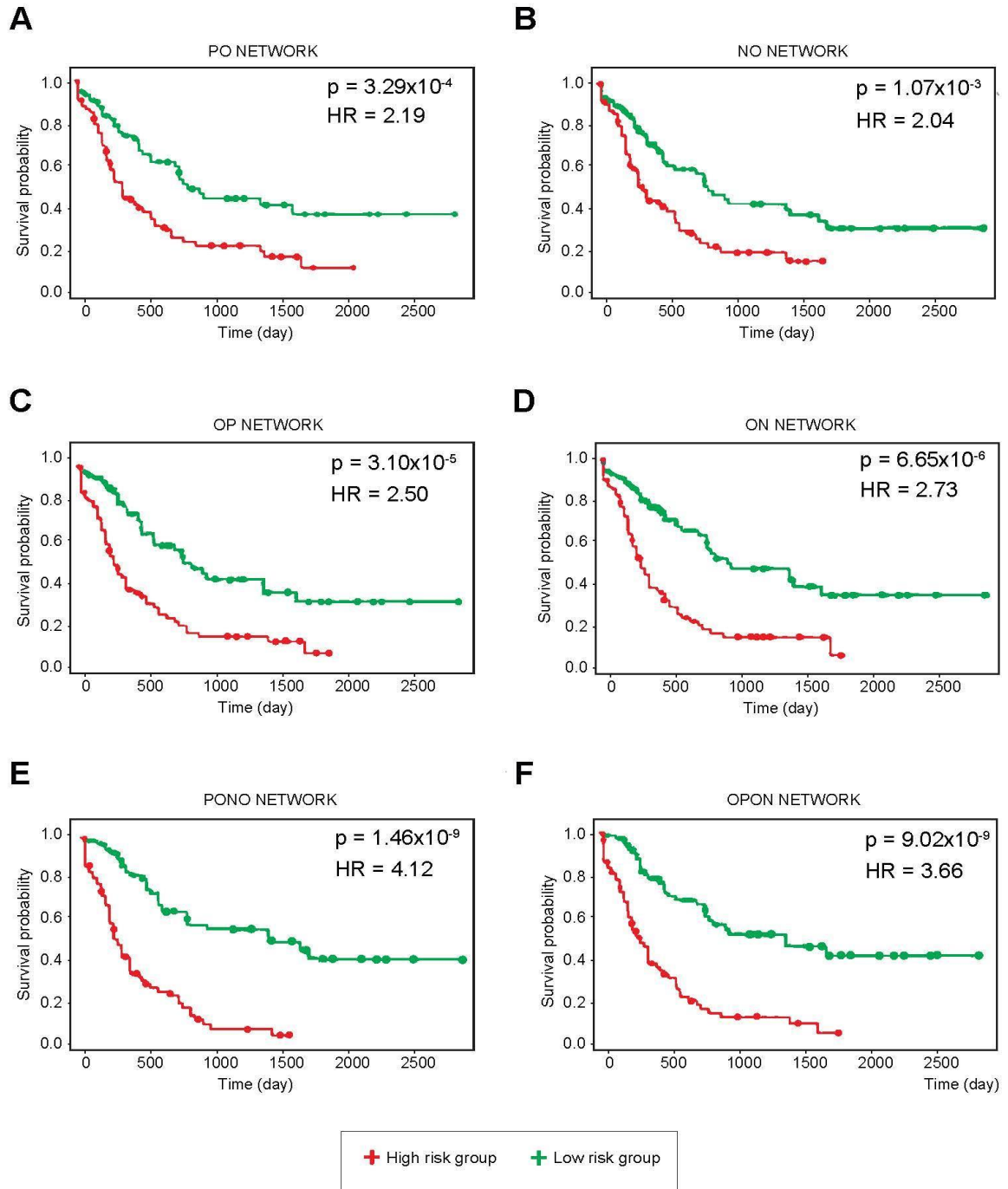


Figure 3.8 Prognostic power of hub related module genes when analyzed separately (A) PO (B) NO (C) OP (D) ON and together (E) PONO (F) OPON. The samples were partitioned into low- and high- groups according

to their prognostic index. Kaplan Meier plot of co-expressed of genes was drawn. The p-values were calculated via the log-rank test ($p < 0.05$)

Differentially co-expressed networks were studied in two different tumor types (CLL and DLBCL) types to determine the specificity of response and non-response networks in AML and to analyze the expression patterns of the networks in the different hematological malignancies. The response network (PONO) and non-response network (OPON) showed high prognostic performance in CLL ($HR=7.57$, $p=2.8 \times 10^{-7}$ and $HR=4.29$, $p=2.9 \times 10^{-5}$, respectively), while both PONO and OPON networks showed insignificant performance in DLBCL ($p=0.99$ $HR>100$ for PONO and OPON) (Figure 3.9). This result indicates that both networks can be considered prognostic for myeloid and lymphocytic leukemia, but not for DLBCL.

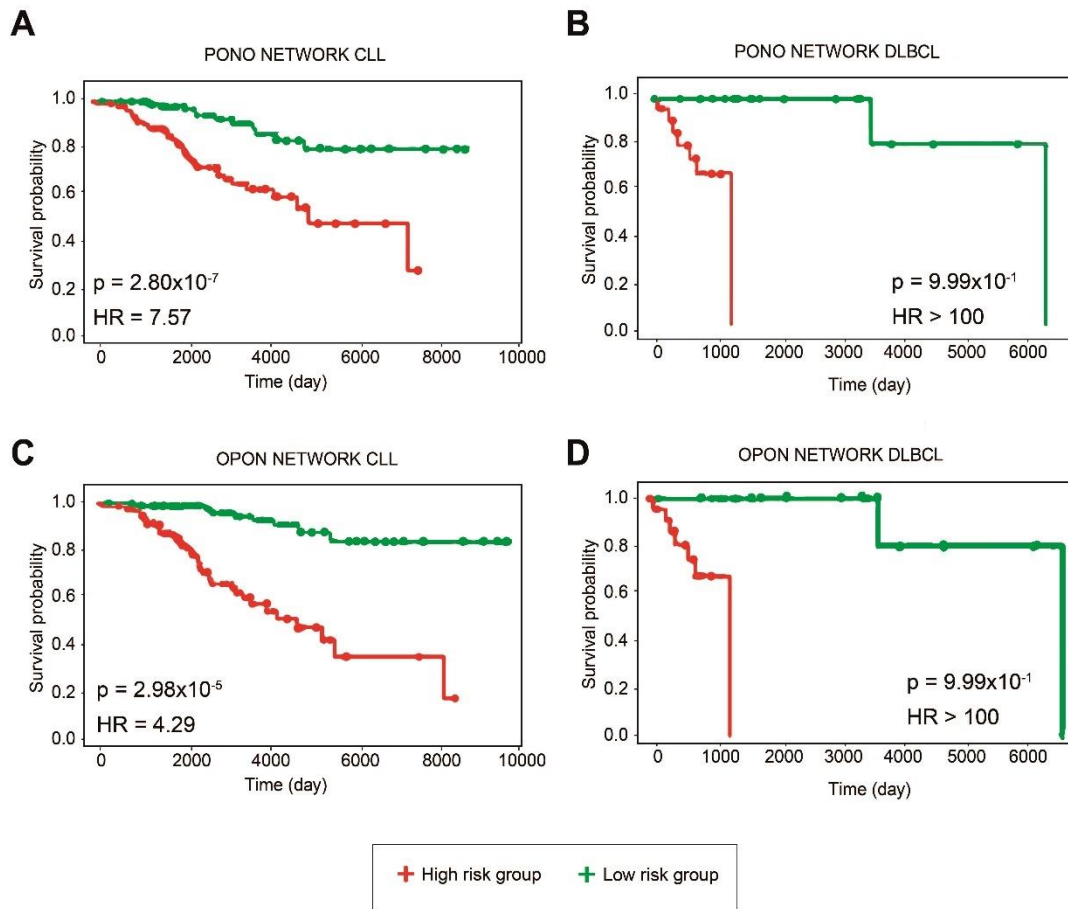


Figure 3.9 Prognostic power of hub related module genes in two types of cancer. Cox survival analysis was performed of hub genes using two types of hematological cancer. Hub genes of PONO were shown in (A) CLL (B) DLBCL and OPON in (C) CLL and (D) DLBCL

3.2.5. Immune Microenvironment analysis of networks

Many attempts have been made to redirect the immune system against malignant blasts because it is well known that leukemic blasts acquire the ability to evade immune surveillance and promote disease progression by disrupting their immunological environment (Jin and Jin, 2020). Therefore, we investigated how the immunological microenvironment would alter the treatment response. Immune infiltration of the PONO and OPON networks was computationally estimated using CIBERSORT (Figure 3.10). The results showed that the abundance of CD4 T cells (memory resting and activated) was

significantly different in the PONO and OPON networks (Figure 3.10). Plasma cells, eosinophils, memory cells, and naïve B cells were highly enriched in the PONO network. In addition, M1 macrophages, activated dendritic cells, neutrophils, and activated mast cells were found to be closely associated with immune infiltration in the OPON network.

Tumor morphology is determined by the neoplastic cell component and the immunological microenvironment within the tumor cells, which can undermine the host immune response. From this perspective, the tumor environment represents a promising and viable field for the development of new biomarkers. In any case, single cell analysis of AML samples is required to fully understand the significance of hub genes in the tumor microenvironment.

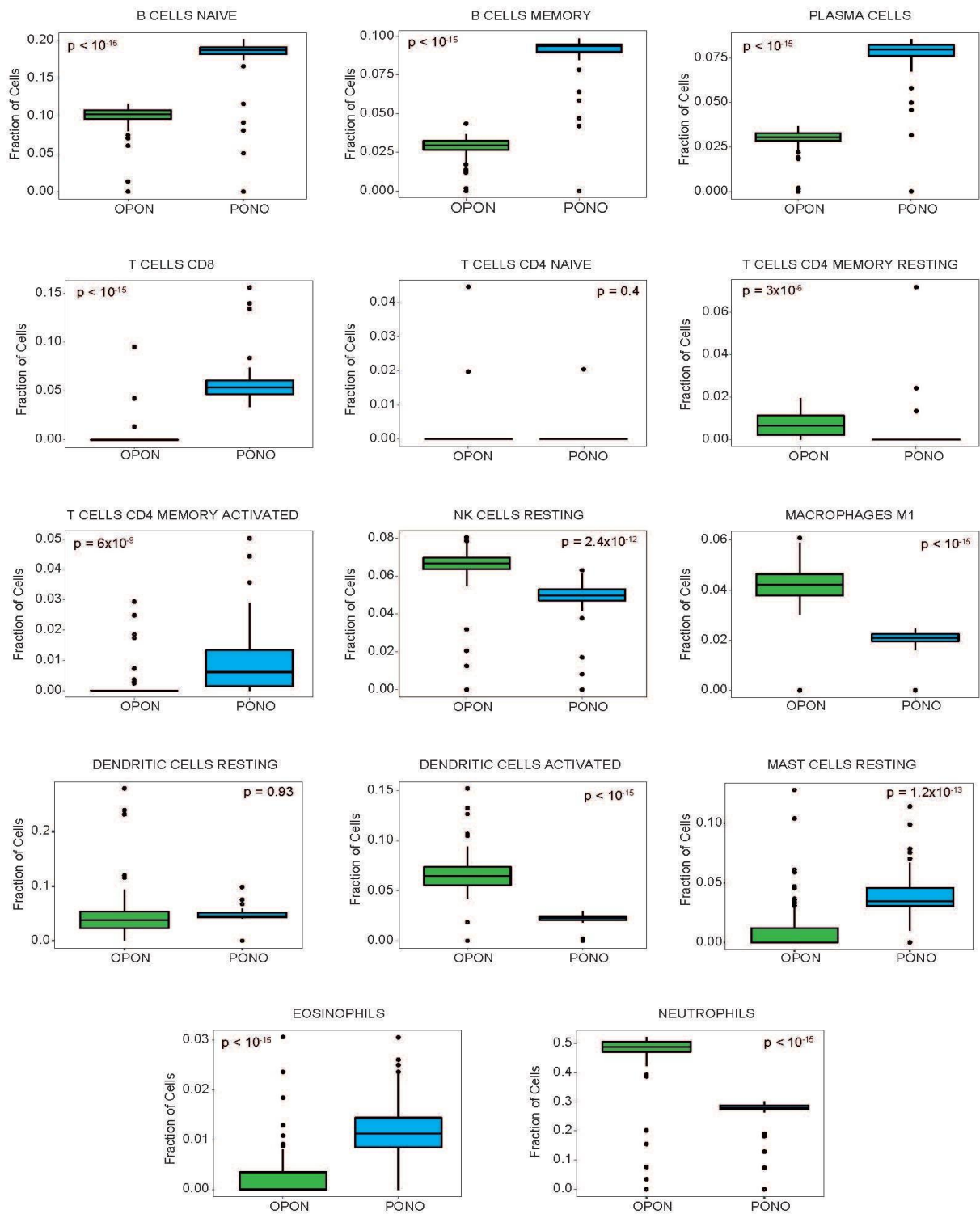


Figure 3.10 Immune cell specific expressions of hub genes

3.2.6. Discussion

AML is a clinically diverse disease characterized by various chromosomal abnormalities and gene mutations that result in significant differences in response and survival after therapy. Overcoming tumor relapse and drug resistance is a critical obstacle in the treatment of AML. The farnesyltransferase inhibitor tipifarnib has been used to inhibit farnesyltransferase function by blocking prenylation of the CAAX tail motif. It prevents Ras from binding to the membrane and renders it inactive. The Ras signaling pathway is a potential therapeutic target for AML, as many AML patients have mutations in NRAS, KRAS, or genes that stimulate Ras signaling (DiNardo and Cortes 2016).

The main goal of this study was to build networks around genes based on variations in differential co-expression patterns of treatment status. It is important to remember that genes involved in complex diseases such as cancer never work alone but interact in a complicated and interconnected system. We used comparative and integrative analysis to create a co-expression network for drug response and non-response. Data from refractory, relapse, and previously untreated AML patients were extracted from datasets and analyzed. Identification of changes in co-expression patterns of genes in patients could provide information about possible treatment-related genes and their co-expressed network for the treated patients who did not respond to previous therapy and/or for the patients who were untreated. Consequently, the unique co-expressed gene networks uncovered here could be considered as "systems biomarkers" that pave the way to identify patients most likely to respond to and benefit from therapy. Most research that has used methods to analyze differential co-expression networks has focused primarily on positive correlations and ignored the biological implications of negative correlations (Ahmed et al. 2012). In this study, negative and positive correlations in a network (PONO for patients who respond to therapy, OPON for patients who do not respond) were examined based on response status to determine how different correlations interact and influence drug response.

The genes in the PONO network were mainly enriched in the MAPK pathway, the cycle of Rho GTPases, and semaphorin interactions. Ras and Rho family GTPases play essential roles in signal transduction during MAPK cascade activation (Kant et al. 2011). In semaphorin

interactions, it was discovered that the MAPK family is activated in response to different axon guidance molecules. MAPK activation in semaphorin signaling has been found to be associated with cytoskeletal control (Jeroen Pasterkamp et al. 2003). Semaphorins regulate cytoskeletal structure via modulation of Rho family proteins and cytoskeletal attachment via R-Ras signaling (Aurandt et al. 2006). Genes in the OPON network were enriched in alanine and aspartate metabolism, TCR signaling, GABA, and leptin signaling. Leptin receptors were highly expressed in AML cell lines and contributed to proliferation. Moreover, the receptors were constitutively expressed in primary leukemic cells, newly diagnosed, refractory, and relapsed AML patients (Gorska et al., 2013; Han and Wang, 2015).

The topological metrics of the networks were considered to determine a highly associated gene group in AML based on response and non-response status and to define hub gene-related modules. SECISBP2L (selenocysteine incorporation), MAN1A2 (metabolic process), PRPF31 (mRNA splicing), VASP (cell junction assembly), and SNAPC1 (transcriptional regulation) were identified as highly connected gene groups in the PONO network. In addition, PHTF2 (transcriptional regulation), SLC11A2 (heme process), PDLIM5 (cell-cell adhesion), OTUB1 (adaptive immune system), and KLRD1 (immune response regulation) were found in the OPON network (Table 3.3).

Table 3.3 The biological meaning and descriptions of network related Hub genes proposed in the current study

<i>Gene List</i>	<i>Name</i>	<i>Functioning in human diseases</i>
<i>SECISBP2L</i>	SECIS Binding Protein 2 Like	It is highly expressed in the central nervous system (Su et al. 2004) and was suppressed in lung cancer, slows down the cell proliferation (Yu et al., 2011).
<i>MAN1A2</i>	Mannosidase Alpha Class 1A Member 2	It has key role in cancer and immune-related activity such as inflammation and pathogen infection (Loke et al. 2016; Schwarz and Aebi 2011) and was identified as a prognostic factor for B cell lymphoma (Kim et al., 2014) and the participation of α -1,2 mannosidases in cancer has been described, its function remains unknown.
<i>PRPF31</i>	Pre-mrnas Processing Factor 31	It has been associated with Retinitis Pigmentosa (RP) (Rio Frio et al. 2008) and its genetic variants have been found as worse metformin response in type 2 diabetes (Retroff et al. 2018).
<i>VASP</i>	Vasodilator-Stimulated Phosphoprotein	Phosphorylation of VASP on Ser157 was recovered in Imatinib-responsive individuals but not in those who were resistant (Bernusso et al. 2015)
<i>SNAPC1</i>	Small Nuclear RNA Activating Complex Polypeptide 1	It can interact with Rb and p53, suggesting that it may play a role during the cell cycle (Baillat et al. 2012) and has been associated with breast cancer tumorigenesis (Xie et al., 2002).
<i>PHTF2</i>	Putative Homeodomain Transcription Factor 2	It was reported that fatty acid metabolism mediated by PHTF2 can greatly impact the tumorigenic potential of gastric cancer cells both <i>in vivo</i> and <i>in vitro</i> studies (Chi et al. 2020).
<i>SLC11A2</i>	Solute Carrier Family 11 Member 2	It was associated in iron uptake as a transmembrane iron transporter, plays crucial roles in transferrin cycle endosomes in erythroid precursors, hepatic iron overload, iron absorption in the intestinal system, and many other processes (Gunshin et al. 2005).
<i>PDLIM5</i>	PDZ and LIM Domain 5	The upregulation of PDLIM5 was reported in many cancer types, such as prostate (Shui et al. 2014), and papillary thyroid carcinoma (X. Wei et al. 2018) and was associated with poor outcomes in lung cancer, and its existence in lung tissue causes cell adhesion, migration, invasion, and metastasis (Shi et al., 2020).

<i>OTUB1</i>	OTU domain-containing ubiquitin aldehyde-binding protein 1	It functions critically in the DNA damage response, cell apoptosis, proliferation, and cancer development. It stimulates the activation of RAS in lung cancer (Baietti et al. 2016), plays a role in deubiquitination and stabilization of FOXM1 in ovarian (Wang et al., 2016) and breast cancer (Karunaratna et al. 2016), promotes invasion in the colon (Liu et al., 2014) and activates RhoA-mediated invasion in prostate cancer (Iglesias-Gato et al., 2015).
<i>KLRD1</i>	Killer Cell Lectin Like Receptor D1	The low expression and the inhibiting and activating form was detected in AML patients compared to healthy subjects (Sanchez-Correa et al. 2011). The higher expression of neural cell adhesion molecule (NCAM) and CD94 was associated with early death in pediatric AML (LoNigro et al., 2008).

SECISBP2L plays a role in selenoprotein expression but does not promote Sec incorporation *in vitro* (Donovan and Copeland 2009). It is likely the result of whole genome duplication early in the vertebrate lineage (Donovan and Copeland 2009). Discovery of the function of this protein is still in the early stages. MAN1A2 encodes a protein that belongs to a mannosyl oligosaccharide family and has a function in the maturation of Asn-linked oligosaccharides in the Golgi complex (Schwarz and Aeby 2011). Members of this family play key roles in the folding, maturation, and transport of glycoproteins and are involved in cancer and immunological activities such as inflammation and infection with pathogens (Loke et al. 2016; Schwarz and Aeby 2011). PRPF31 encodes part of the spliceosome complex and is essential for cell metabolism and survival. This study showed that PRPF31 protein plays a direct role in the mitotic process and is associated with spindle microtubules (Pellacani et al. 2018). VASP, a family member of the Ena-VASP protein, has a proline-rich domain that binds SH3- and WW-domain-containing proteins. Zyxin and VASP are associating proteins that play a role in cellular adhesion and movement. Their involvement and the interaction of VASP and zyxin have been studied in K562 cells, and their inhibition led to a decrease in the expression of anti-apoptotic proteins such as BCL-XL and BCL2 (Bernusso et al. 2015). SNAPC1 functions as a basal transcription factor to promote snRNA transcription. In

addition to snRNA genes, SNAPC1 chromatin also occupied many transcriptionally active protein-coding genes.

PHTF2 is an evolutionarily conserved gene expressed primarily in muscle tissue (Manuel et al. 2000) and has been associated with mediating stress transcription. Although its essential paralog PHTF1 has been overexpressed in T- ALL cell lines to regulate cell proliferation and apoptosis (X. Huang et al. 2015), the association of PHTF2 with other hematological cancers has not been reported. SLC11A2 is associated with iron uptake as a transmembrane iron transporter and plays a critical role in transferrin cycle endosomes in erythroid progenitor cells (Gunshin et al. 2005). AML patients have been found to have higher serum ferritin at diagnosis, which stimulates leukemia cell development while suppressing normal colony formation of progenitor cells (Lebon et al., 2015; Wang et al., 2019). Moreover, in patients with hematologic malignancies after allogeneic hematopoietic stem cell transplantation, an elevated serum ferritin level is a poor prognostic indicator of overall survival and non-relapse mortality (Armand et al. 2014). Although direct associations between SLC11A2 and AML prognosis have not been reported, there may be a causal effect of dysregulation of SLC11A2 on AML prognosis. PDLIM5 functions in cell proliferation and differentiation in many cell types and tissues. It was identified as a protein kinase that binds to PKC, PKD, AMPK, and PKA. The study showed that the expression of PDLIM4 was lower in AML patients than in healthy controls. The lower PDLIM4 expression resulted in longer overall survival than AML patients without PDLIM4 (Li et al., 2013). Another study claimed that high expression of PDLIM2 and PDLIM7 are negative prognostic factors in AML patients, but their influence on survival was not found in allo-HSCT recipients (Longzhen Cui et al. 2019). Although the interactions and functions of various PDLIM family members have been demonstrated in AML studies, the association of PDLIM5 has not yet been reported.

OTUB1 is characterized by an ovarian tumor (OTU) domain and encodes a protein that is a specific ubiquitin iso-peptidase that cleaves ubiquitin from branched poly-ubiquitin chains. OTUB1 may play a critical role in cancer treatment because cancer cells rely on a functioning ubiquitin-proteasome system (UPS), making it an attractive target for developing new therapies with selectivity for tumor cells. KLRD1 encodes an antigen (CD94) expressed mainly on natural killer (NK) cells. It forms a heterodimer with NKG2A (inhibitory form),

NKG2C, or NKG2E (activating form) and binds to the non-classical MHC I molecule and HLA-E in humans. Expression of HLA-E in tumors suppresses NK cell function via the inhibitory receptor CD94/NKG2A on the surface of NK cells, resulting in immune cell resistance to tumor (Kamiya et al. 2019). After treatment of AML patients with haplo-mismatched SCT, NK cells were rapidly generated, and higher expression of CD94/NKG2A was detected in the NK cells than in healthy donors (Nguyen et al. 2009). Moreover, uncovering other possible KLRD1 (CD94) interactions in AML blast cells may reveal predisposing factors for disease prognosis and treatment resistance.

Transcriptional regulators of genes were examined, and five TFs, ETS1, GATA2, AR, YBX1, and FOXP3, were found to be significant in both networks. ETS1 expression has been found to be increased in childhood AML; however, the functional implications of ETS1 overexpression in AML are not yet known (Bolouri et al. 2021). GATA2 plays a critical role in hematopoietic stem and progenitor cell formation, and overexpression is associated with poor prognosis in AML patients (Katsumura et al. 2016). Hu et al. demonstrated the comprehensive pan-cancer analysis of AR in various tumor types and found that AML patients with high AR expression had a higher survival rate (Hu et al. 2020). FOXP3 is critical for the development or function of Tregs; its expression was increased in newly diagnosed and relapsed/refractory patients (Tian et al. 2015). The recent study showed that YBX1 is a particular dependency and therapeutic target in AML, required for oncogenic protein production (Perner et al. 2022).

The malignancy of various cancers has been associated with the presence of tumor-infiltrating immune cells. Determining the status of the immune response in individual patients at each stage of the disease is critical for deciding subsequent clinical diagnosis and treatment. On the other hand, immune profiling offers the potential to identify biomarkers of response to immunotherapy, providing important information to improve clinical trial design by using techniques to modify anti-leukemia immunity for AML treatment. Several clinical studies have shown that T cell immunity is impaired in AML in multiple ways, including an increase in T-regulatory cells and a decrease in T helper cells, T cell exhaustion, and abnormal transcription factor activity (Pyzer et al. 2017; Tan et al. 2020). AML patients have impaired immunological responses due to immunosuppressive circuits activated by soluble

factors and checkpoint molecules, including PD-L1, TIM-3, and IDO-1. CD8⁺ T lymphocytes with increased PD-1 expression may contribute to cytotoxic T cell dysfunction and immune response suppression in patients with advanced AML.

B cells can mature into plasma cells that generate antibodies, but they can also perform several additional roles that help the immune system eliminate tumor cells. The number of CD19⁺ B cells has been shown to decrease substantially and significantly in relapsed and refractory AML patients compared to healthy donors (Goswami et al. 2020). Another study indicated that the number of B cells recovered in patients who fully responded to AML treatment, demonstrating that B cells may play a role in the development of AML (Dong et al. 2020). Survival analysis performed in the study showed that mast cell quiescence had a significant association with survival of AML patients (Jia et al. 2021). Nevertheless, patients with AML may benefit from treatment that targets their mast cells. Macrophages may play a role in treatment resistance in AML, but the role in the overall mechanism is still unclear. Interaction between macrophages and AML cells and/or substances secreted by macrophages may reduce AML cell sensitivity to drugs, and macrophages may be retrained by AML blasts to support leukemia, according to preliminary findings (Mussai et al. 2013). Recent research by Al-Matary et al. has greatly improved our knowledge of how leukemia-associated macrophages (LAMs) protect AML cells (Al-Matary et al. 2016). They discovered that AML cells were responsible for the invasion of LAMs into the bone marrow and spleen of leukemia patients as well as mice. LAMs supported *in vitro* development of AML cell lines better than macrophages from non-leukemic mice in various gene mutation-induced AML mouse models. LAM infiltration was found to be correlated with mouse survival.

Antigens are presented by dendritic cells (DCs) that trigger specific T cell responses. They are also responsible for the recruitment and activation of NK cells and the differentiation of naive T cells. Tumor-forming plasmacytoid DCs (TF-PDCs) from AML patients have been shown to express leukemia-associated proteins in several studies. The study showed that patients with TF-PDCs had median lower sensitivity to standard chemotherapy regimens and resistance to pinarubicin and sorafenib (Zhu et al. 2020). They suggested that TF-PDC positive AML patients may have primary chemotherapy resistance and TF-PDC in the AML microenvironment may be responsible for drug resistance and disease relapse. Within the

scope of that research, the overall survival of TF-PDC positive AML patients was found to be shorter than that of TF-PDC negative patients (Zhu et al. 2020). Although neutrophils have an anti-inflammatory effect, they also secrete cytokines that promote cancer progression, such as interleukin and tumor necrosis factor α .

On the other hand, lymphocytes are crucial components of the immune system in fighting cancer cells. Recent studies have shown that neutrophil to lymphocyte ratio (NLR) is a biomarker of the patient's immune response in the tumor microenvironment (Zhang et al., 2021). Zhang et al. indicated that AML patients with a high NLR may have a decreased antitumor response, leading to an increased risk of death. In addition, Mushtaq et al. showed that a high NLR in relapsed/refractory AML patients indicates a poor prognosis (Mushtaq et al. 2018).

This study allows us to shed light on the prognosis of AML based on response status. In any case, more needs to be done to confirm this clinically, and the relevant underlying process is still being elucidated. We identified the potential prognostic biomarkers in AML based on the treatment responses of patient data on gene co-expression networks. Two networks were identified based on response and were found to be predictive for both AML and CLL. As a result, novel genes were identified as highly connected in the networks and may be associated with patient response to therapy.

The application of targeted therapy in AML has been difficult because of the complexity of mutational processes within and between individuals and the lack of pharmacological drugs for most mutational events. Identifying therapeutically relevant pharmacogenomic interactions by determining gene-drug interactions is a powerful method. By applying bioinformatics approaches, we have constructed co-expression networks that can be used to clinically assess drug response and classify patients who respond. It should be remembered that genes associated with cancer function together in the complex networks of cells. From this point of view, in our study, we looked at negative and positive correlations in a response status- based network to see how different correlations interact and influence drug response. SECISBP2L, MAN1A2, PRPF31, VASP, SNAPC1 in the response network and PHTF2, PDLIM5 and OTUB1 in the non-response network were proposed as novel molecular biomarkers.

3.3. Drug Repurposing Analysis to Unveil the Potential Candidate Treatment Strategies For Acute Myeloid Leukemia

3.3.1. Identification of DEGs

Two datasets, GSE5122 and GSE8970, were extracted from the GEO database, each containing gene expression profiles and accompanying clinical data. While GSE5122 includes individuals with relapsed and refractory AML, GSE8970 includes patients with newly diagnosed AML. Upregulated and downregulated DEGs in AML patients were determined using an adjusted p-value < 0.05 and a fold change > 1.5 (for upregulation) and < 0.5 (for downregulation). DEGs were found to consist of 141 upregulated genes and 281 downregulated genes in GSE5122 and 1351 upregulated genes and 881 downregulated genes in GSE8970, respectively.

3.3.2. Identification of Candidate Drugs for AML Treatment Through Drug Repositioning

The transcriptome-guided drug repositioning tool geneXpharma (Turanli et al., 2017) was used to determine whether or not the diseases and drugs that interacted with our DEG query list were unique in our studied dataset based on patient responses to treatment. Each drug was assigned a hypergeometric p-value by the tool, and only those with a p-value less than 0.05 were considered significant. Thus, we identified 109 different drugs for AML patients' response mechanisms. The drugs belong to different groups, including antineoplastics (16%), steroids and steroid derivatives (15%), antidepressants (10%), amino acids, peptides and proteins (6%), antimicrobials (antimalarials, antiparasitics, antibiotics and antifungals) (4%), antibodies (3%), antiplatelet agents (2%), and others (44%) (antacids and adsorbents, anthracyclines, antianemic agents, antiarrhythmic agents, antibiotics, anticholinergics, antihypertensives, antiinfectives, antiplatelet agents, and flavonoids). After identifying potential drug candidates, we searched PubMed and DrugBank to determine whether any of them had been used previously to treat AML. An analysis of the available literature revealed an association between several drugs and AML.

Results from repurposed drugs that have been approved for treating AML (11%), tested in experimental research to investigate the mechanism of AML (66%), and are currently being studied in clinical trials (16%) were discovered. Eight medications account for the remaining 7% that have never been tested before for treatment of AML. The drugs doxorubicin (Vu et al., 2020), vorinostat (Schaefer et al. 2009), thioridazine (Aslostovar et al. 2018), digoxin (Laverdière et al. 2018), and dasatinib (Sigal Tavor et al. 2020) have previously been associated with AML. A total of eight newly developed drugs-aniracetam, desipramine, doxepin, estramustine, hydrochlorothiazide, leucovorin, nortriptyline, and risedronate-were used in our subsequent experiments (Table 3.4).

3.3.3. Repositioned Drug Candidates Inhibited the Proliferation of AML Cell Line

We used the WST-1 assay to examine how repurposed drugs affected the viability of the AML cell lines. To determine the IC₅₀ values for each, the HL-60 and KG-1 were subjected to various doses of these drugs. After 24 hours, we evaluated the viability of the cells and compared how these drugs inhibited the growth of the AML cell lines. The results showed that treatment with 6 drugs including desipramine (HL-60: 48 μ M, KG-1: 52 μ M, at 24h), doxepin (HL-60: 175 μ M, KG-1: 157 μ M at 24h), estramustine (HL-60: 72 μ M, KG-1: 69 μ M at 24h) , hydrochlorothiazide (HL-60: 401 μ M, KG-1: 306 μ M at 24h), nortriptyline (HL-60: 46 μ M, KG-1: 81 μ M at 24h), and risedronate (HL-60: 387 μ M, KG-1: 391 μ M at 24h) reached the IC₅₀ values and significantly reduced cell viability of cell lines following the treatment (Figure 3.11). The inhibitory impact of leucovorin was not observed at any dose, although the aniracetam dosage required to produce an inhibitory effect was quite high. Therefore, they were not tested in western blot and apoptosis experiments.

Table 3.4 Repurposed drug candidates for treatment of AML

<i>Repositioned Drugs</i>	<i>Function</i>	<i>Chemical Taxonomy</i>	<i>Mechanism of Action</i>
<i>Aniracetam</i>	<i>Nootropic- memory and attention disturbances for cerebrovascular</i>	<i>Benzoic acids and derivatives</i>	<i>Glutamate receptor 2 Glutamate receptor 3 5-hydroxytryptamine receptor 2A Dopamine D2 receptor</i>

	diseases and degenerative brain disorders		
<i>Desipramine</i>	<i>Tricyclic antidepressant</i> -used in the treatment of depression	Dibenzazepines	Sodium-dependent noradrenaline/serotonin transporter 5-hydroxytryptamine receptor 2A/2C/1A Sphingomyelin phosphodiesterase Histamine H1 receptor Muscarinic acetylcholine receptor Beta-1/2 adrenergic receptor Dopamine D2 receptor Alpha-1/2 adrenergic receptors
<i>Doxepin</i>	<i>Tricyclic antidepressant</i> -used in the treatment of depression	Dibenzoxepines	Histamine H1/H2/H4 receptor Muscarinic acetylcholine receptor M1/M2/M3/M4/M5 Alpha-1A/1B/1D adrenergic receptor Sodium-dependent serotonin transporter Sodium-dependent noradrenaline/serotonin transporter 5-hydroxytryptamine receptor 2A/2B/2C
<i>Estramustine</i>	<i>Antineoplastic</i> - used for progressive prostate cancer	Estrane steroids	Microtubule-associated protein 1A/2 Estrogen receptor alpha/beta
<i>Hydrochlorothiazide</i>	<i>Diuretic</i> - used for edema and hypertension	1,2,4-benzothiadiazine-1,1-dioxides	Solute carrier family 12 member 3 Calcium-activated potassium channel subunit alpha-1
<i>Leucovorin</i>	<i>Folate analog</i> -used for toxic effects of methotrexate	Glutamic acid and derivatives	Folate Metabolism Methotrexate Action Pathway
<i>Nortriptyline</i>	<i>Tricyclic antidepressant</i> -used in the treatment of depression	Dibenzocycloheptenes	Sodium-dependent noradrenaline/serotonin transporter 5-hydroxytryptamine receptor 2A/2C/1A Sphingomyelin phosphodiesterase Histamine H1 receptor Muscarinic acetylcholine receptor Beta-1/2 adrenergic receptor Dopamine D2 receptor Alpha-1A/1B/1D adrenergic receptors
<i>Risedronate</i>	<i>Bisphosphonate</i> -used for osteoporosis and Paget's disease.	Bisphosphonates	Farnesyl pyrophosphate synthase

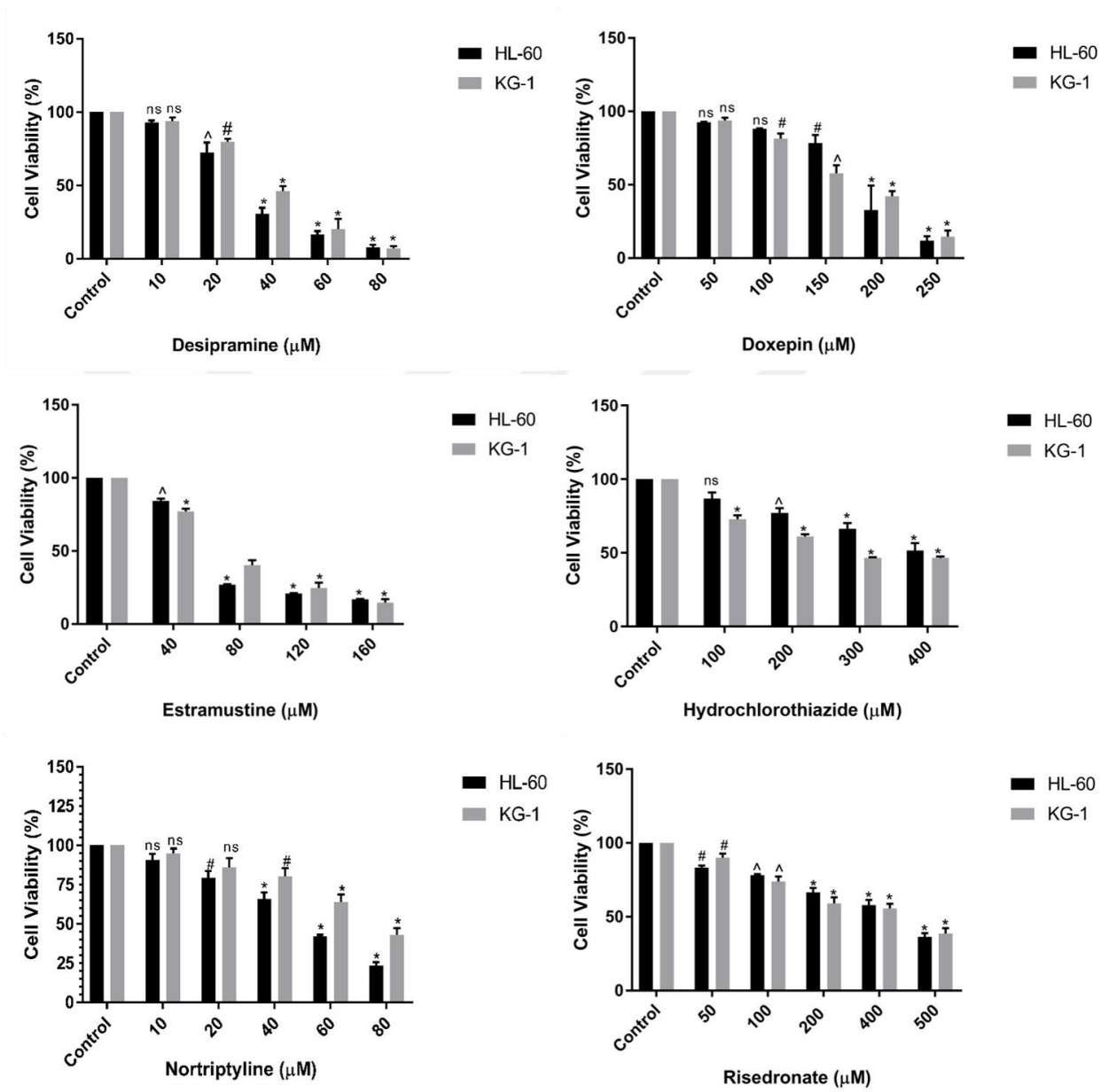


Figure 3.11 Experimental demonstration of the inhibitory effect of desipramine, doxepin, estramustine, hydrochlorothiazide, nortriptyline, and risedronate on the proliferation of tumor cell lines HL-60 and KG-1. Cell lines were exposed to different concentrations of these six drugs and cell viability was tested after 24 hours. Cell proliferation was measured using the WST-1 assay. Boxes show the standard error of the mean for cell viability calculated from triplicate experiments. # $p < 0.05$; ^ $p < 0.001$; * $p < 0.0001$; ns: not significant.

Furthermore, we tested the expression of Akt and Parp/cleaved Parp proteins after treatment with the drug. Cell lysates of each treatment were prepared at doses that resulted in cells reaching the proposed IC50 value. Nortriptyline, desipramine, and doxepin significantly increased Akt expression compared with control groups, whereas risedronate and hydrochlorothiazide decreased Akt expression in HL-60 cells. In addition, the inhibitory effect of nortriptyline, desipramine, and estramustine on Akt level was observed in KG-1 cell line (Figure 3.12). Cleavage of Parp was used to detect apoptosis in the cell. Increased expression of cleaved Parp was observed in both AML cell lines upon exposure to newly developed drugs, except for desipramine in HL-60 cells (Figure 3.13).

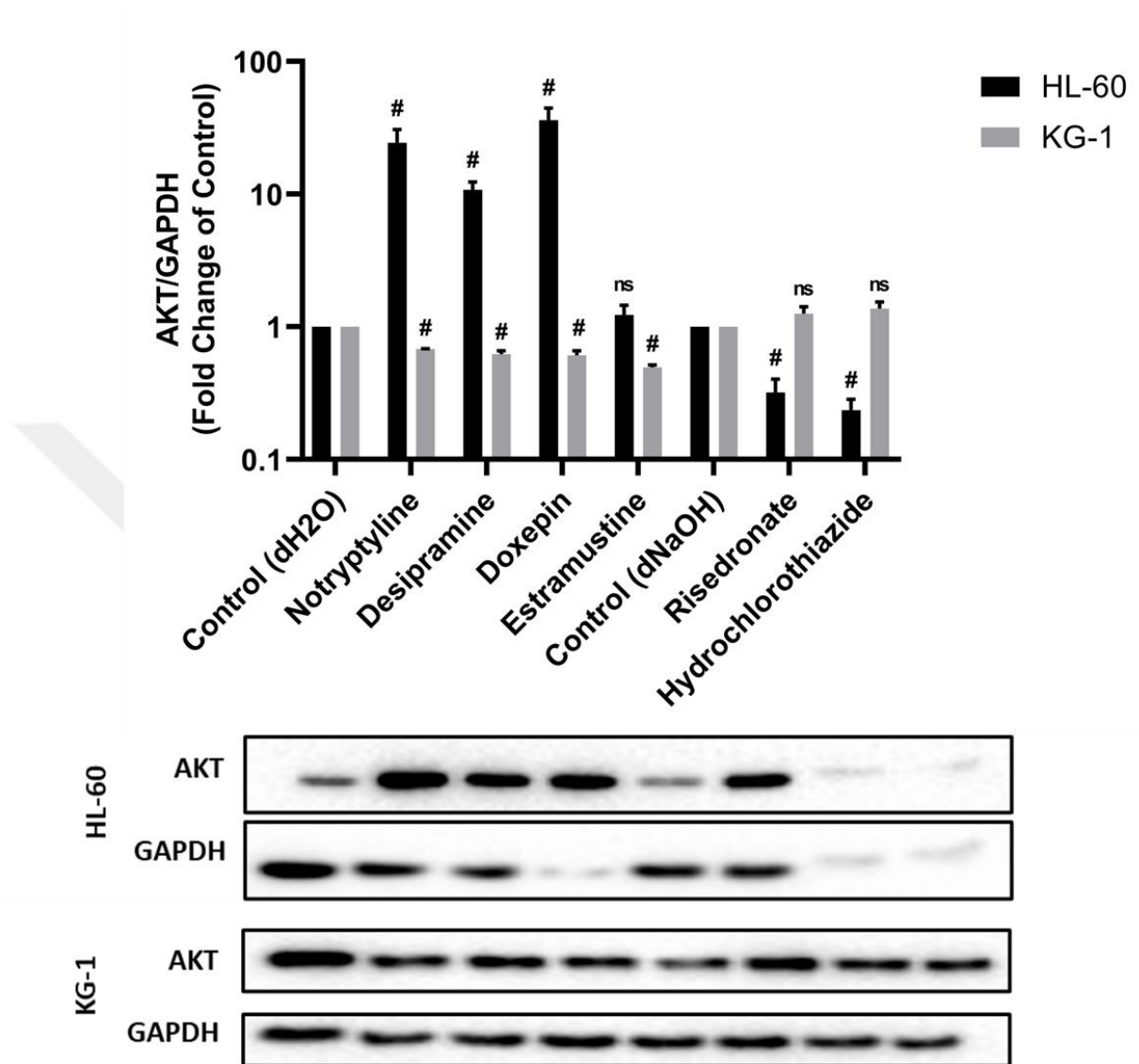


Figure 3.12 Western blot results of each repurposed drugs displayed how drugs affected AKT protein expressions. Data are the representation of at least three independent experiments. # $p < 0.05$; ^ $p < 0.001$; * $p < 0.0001$; ns: not significant.

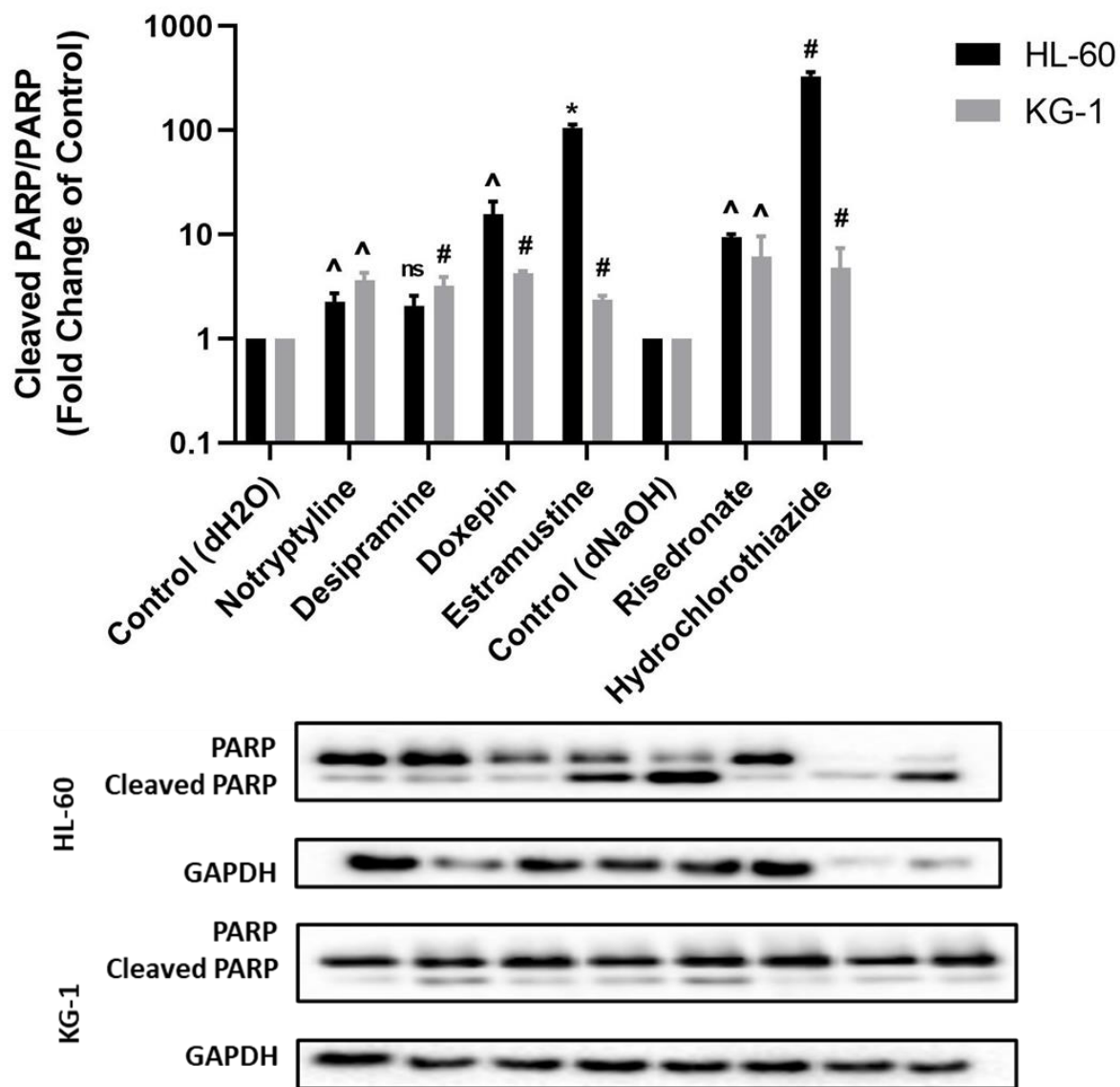


Figure 3.13 Western blot results of each repurposed drugs displayed how drugs affected PARP and Cleaved PARP protein expressions. Data are the representation of at least three independent experiments. # $p < 0.05$; ^ $p < 0.001$; * $p < 0.0001$; ns: not significant

3.3.4. Candidate Drugs Reduces Cell Number by Activation of Cell Death

Many studies have shown that the two major pathways are involved in triggering cell apoptosis, including the death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway. Caspase family members play critical roles in two key apoptotic

pathways. Among them, caspase-8 acts as the initiator of the extrinsic pathway. To further confirm whether caspase-8 is involved in apoptosis, caspase-8 activation was analyzed by a fluorometric assay. As shown in Figure 3.14, treatment with estramustine increased the activation of caspase-8 in HL-60 cells compared to the control group ($p < 0.0001$). However, estramustine decreased the activation of caspase-8 in KG-1 cells. We did not observe activation of caspase-8 in either AML cell with the other drugs.

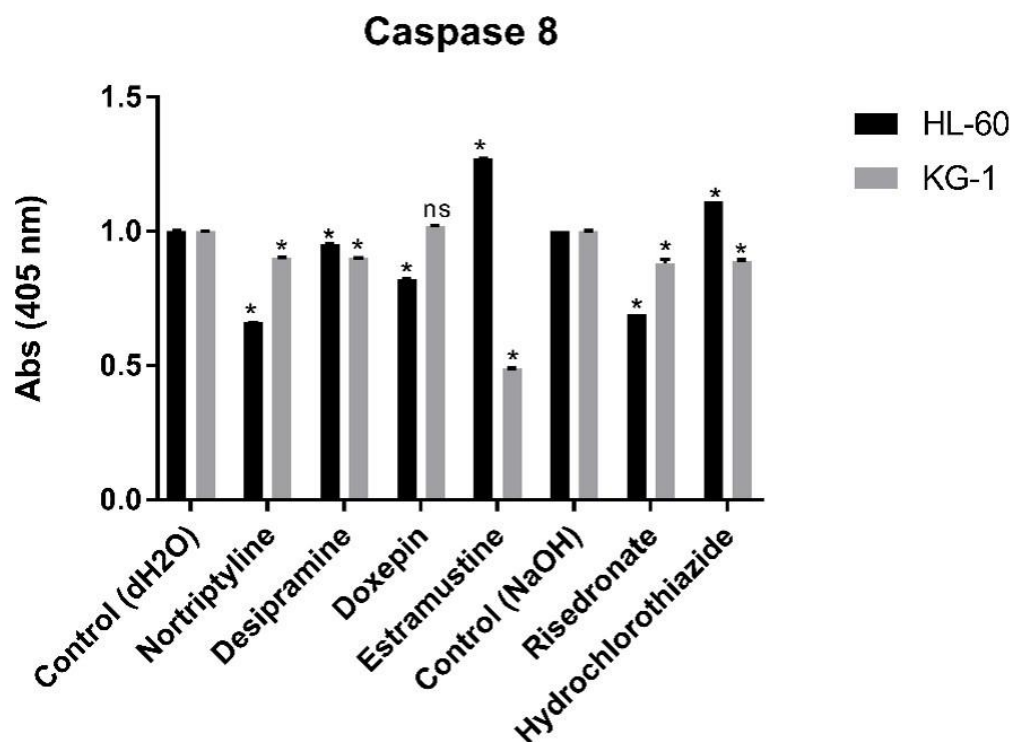


Figure 3.14 Effect of drugs on the Caspase 8 activity in HL-60 and KG-1 cells. # $p < 0.05$; ^ $p < 0.001$; * $p < 0.0001$; ns: not significant.

ELISA-mediated detection of cell death more clearly indicated activity between individual AML cells (Figure 3.15). Increased cell death was observed with nortriptyline, desipramine, doxepin and estramustine at HL-60 and KG-1 cells ($p < 0.0001$). Cell death was dramatically reduced in HL-60 compared to control with exposure to risedronate and hydrochlorothiazide. In addition, cell death was increased in KG-1 cells with both risedronate and hydrochlorothiazide treatment. Table 3.5 summarizes the effects of the repositioned drugs on

AML cell lines by reporting IC-50 values, AKT, cPARP/PARP, caspase 8, and cell death ELISA results for each drug tested.

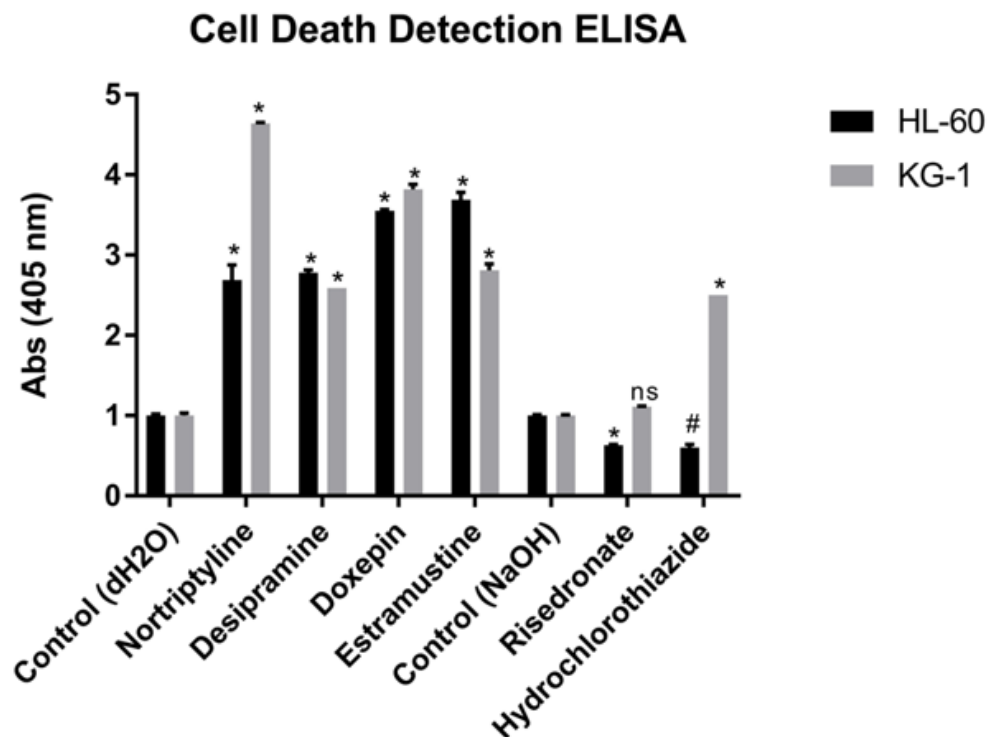


Figure 3.15 Repurposed drugs promote cell death that was determined using the Cell Death Detection ELISA assay. # $p < 0.05$; ^ $p < 0.001$; * $p < 0.0001$; ns: not significant.

Table 3.5 Table showing the impact of drugs on AML cells in experimental studies

Drugs/Cells	IC-50		AKT		cPARP/PARP		Caspase 8		Cell Death ELISA	
	HL-60	KG-1	HL-60	KG-1	HL-60	KG-1	HL-60	KG-1	HL-60	KG-1
Nortriptyline	46 μ M	81 μ M	↑	↓	↑	↑	↓	↓	↑	↑↑
Desipramine	48 μ M	52 μ M	↑	↓	Ns	↑	↓	↓	↑	↑
Doxepin	175 μ M	157 μ M	↑	↓	↑	↑	↓	↓	↑	↑
Estramustine	72 μ M	69 μ M	Ns	↓	↑↑	↑	↑	↓↓	↑	↑
Risedronate	387 μ M	391 μ M	↓	Ns	↑	↑	↓	↓	↓	Ns
Hydrochlorothiazide	401 μ M	306 μ M	↓	Ns	↑↑	↑	↑	↓	↓	↑

3.3.5. Discussion

Despite advances in molecular biology specifically relevant to AML and a better understanding of the mechanisms of leukemogenesis, standard treatment for AML has remained largely unchanged in recent decades. High development costs, competition from generic drugs that have a long path to clinics that overlaps with patent protection, increasingly conservative regulatory regimens, and a lack of breakthrough technologies have hampered *de novo* drug research. Drug repositioning is a viable method for drug development because it involves the process of identifying new therapeutic indications for already licensed drugs or drug candidates. An *in silico* technique was used to search for an already approved differentiation inducer that inhibits the ability of leukemic stem cells to self-renew.

In our research, the integration of mRNA expressions was further improved by using mRNA-integrated drug repurposing, which led to the identification of eight different drug candidates. The IC₅₀ values for desipramine, doxepin, estramustine, hydrochlorothiazide, nortriptyline, and risedronate were determined. After IC₅₀ values were determined, these drugs were subjected to Akt protein analysis. Each drug was analyzed for its effects on Akt levels, which play an important role in cellular processes such as cell survival, cell cycle progression, cell proliferation, and angiogenesis.

All multicellular organisms rely on programmed cell death. Apoptosis is the most thoroughly studied form of programmed cell death and involves the activation of caspase proteases. Other mechanisms such as cleavage of PARP by caspases are also considered hallmarks of apoptosis. Necroptosis and pyroptosis are two examples of non-apoptotic cell death processes that have become the focus of scientific interest. These other methods of cell death, termed "non-apoptotic cell death mechanisms," are either independent of apoptosis or are activated when apoptosis fails. Therefore, we investigated whether the use of repositioning drugs in AML cell lines has an impact on cell death by examining caspase-8 activity, cleaved Parp content, and photometric enzyme immunoassay.

The tricyclic antidepressant nortriptyline has been shown to be effective in the treatment of various mental disorders such as migraine (Punay and Couch 2003), Parkinson's disease, and

depression (Ghazi-Noori et al. 2003). A significant antiproliferative effect against glioblastoma has been noted with nortriptyline treatment (Lee et al. 2016), and also against melanoma (Parker et al. 2012). In addition, the multiple myeloma cell line U266 has been reported to undergo apoptosis when treated with nortriptyline (Biber et al. 2018). Osteosarcoma (Hsu et al. 2004) and prostate (Pan et al. 2010) tumors are also inhibited by the antineoplastic properties of nortriptyline. Caspase-dependent apoptosis is induced by nortriptyline treatment in human TCCSUP and MBT-2 mouse bladder cancer cells (Yuan et al. 2015). In addition, nortriptyline has been shown to restore oxidative stress-induced corticosteroid sensitivity via direct inhibition of PI3K, making it a promising treatment for diseases that do not normally respond to corticosteroids, such as asthma (Mercado et al. 2011). Our study showed that nortriptyline increased Akt expression in HL-60 cells, whereas it decreased Akt levels in KG-1 cells compared with controls. Moreover, we observed profound induction of cell death in both cells, not through activation of caspase 8, but through upregulated expression of cleaved Parp and increased levels of cytoplasmic histone-associated DNA fragments.

Both *in vivo* and *in vitro* studies have shown that desipramine affects a variety of molecular levels. Desipramine inhibits cytochrome P450 enzymes and enhances membrane steroid transporter activity, thereby reducing tumor necrosis factor- α production in the brain (Pariante et al. 2001; Reynolds et al. 2005; Shin et al. 2002). The study showed that desipramine inhibited cell proliferation in Hep3B cells through mechanisms such as enhanced apoptosis, activated MAPK signaling, and increased intracellular Ca^{2+} (Yang and Kim 2017). It was reported that desipramine caused mitochondrial membrane potential to remain unchanged while inducing the characteristic apoptotic morphology of chromatin condensation in rat glioma C6 cells (Ma et al. 2011). In addition to increasing p53 levels, the desipramine/cisplatin combination also increased mitochondrial damage, caspase activation, and poly(ADP ribose) polymerase cleavage. This suggests that desipramine synergizes, at least in part, with cisplatin by activating various apoptotic signaling pathways. In HCT116 cells treated with cisplatin, desipramine dramatically increased PARP cleavage (Kabolizadeh et al. 2012). Neither wild-type nor db/db (leptin receptor knockout) mice showed

phosphorylation of Akt or ERK1/2 in the hippocampus or prefrontal cortex in response to desipramine (Guo and Lu 2014). Our results suggest that desipramine activated Akt levels in HL-60, whereas Akt expression was decreased in KG-1 cells. Desipramine treatment had differential effects on the induction of apoptosis via the Parp pathway in the two cell lines. Cleaved Parp activities were not detected on HL-60, but desipramine significantly increased cleaved Parp expression on KG-1 cells. Apoptosis was induced after 24 hours compared to control. Desipramine did not enhance the increase in apoptosis via the caspase-8 pathway but by a different mechanism.

Tricyclic antidepressants such as doxepin work by blocking the reuptake of serotonin and norepinephrine in the brain (Vermeeren and Coenen 2011). Doxepin is effective as a topical treatment for atopic dermatitis due to its anti-inflammatory effects and is also used as a secondary treatment for chronic urticaria (Drake et al. 1994). Doxepin protects against oxidative stress by inhibiting calcium signaling, which is a major contributor to oxidative stress-induced damage (Ji et al. 2004). It also reduces lipid peroxidation and boosts antioxidants such as superoxide dismutase. Doxepin treatment has been found to promote apoptosis of spinal ganglion cells and malignant glioma cells (Haller et al. 2007; Higgins and Pilkington 2010). Another study showed that doxepin had a double-edged effect on PC3 cell survival. Doxepin increased cell viability by 30% at 100 M. However, at 200 and 250 M doxepin, cell death occurred in a dose-dependent manner (Lu et al., 2015). It was demonstrated that the expression of pAkt was significantly increased by doxepin compared to the control group at a concentration of 10^{-7} μ M. In another study, doxepin-treated mice were shown to have decreased p-Akt expression in their skeletal muscles. They suggested that decreased expression of p-Akt and GLUT4 in skeletal muscle may have played a role in slowing glucose metabolism (Chang et al. 2021). In our study, the effect of doxepin on Akt expression in KG-1 cells was not detected. Nevertheless, doxepin significantly increased Akt levels in HL-60 cells. Doxepin has been shown to promote cell death in both AML cell lines.

The anticancer drug estramustine phosphate sodium has a distinct dual mechanism of action that makes it particularly effective against cancer. The antigonadotropic activity of the metabolites of estramustine phosphate sodium, estrone and estradiol, is responsible for the

decrease in testosterone levels (Kuhl 2005). Specifically, it causes microtubule depolymerization by binding to microtubule-associated proteins and/or tubulin, which prevents cell division in G2/M phase (Inoue 2018). The cytostatic drug estramustine, used to treat metastatic prostate cancer, has been shown to reduce disease severity and make cancer cells more sensitive to radiation treatment (Bergenheim and Henriksson 1998). According to the results of another study, glioma cells were driven into apoptosis by estramustine phosphate via phosphorylation of bcl-2 (Yoshida et al. 2001). Inhibition of miR-31 expression by estramustine phosphate leads to apoptosis in a prostate cancer cell line (Wei et al., 2018). In malignant gliomas, the antimicrotubule medication estramustine, but not irradiation, is responsible for inducing apoptosis through pathways involving AKT and caspase. AKT levels were decreased after estramustine treatment, which induces apoptosis in glioma cells (Vallbo et al. 2002). In our study, Akt expression was decreased in KG-1 cells but undetectable in HL-60 cells. In addition, treatment with estramustine promoted cell death at a high rate compared to the control group. Moreover, it is the only drug that exhibits caspase 8 activity in HL-60 cells.

Bisphosphonates are used to treat a number of conditions characterized by increased bone loss, such as postmenopausal osteoporosis and tumor bone metastases (Russell et al. 2008). This study showed that the cleaved form of caspase 9 was significantly increased in osteoclasts after 16 hours of risedronate therapy, whereas the 18-kd-active fragment of caspase 8 was barely detectable. In contrast to the activation of apoptosis and suppression of ERK, bone resorbing activity of osteoclasts was inhibited at risedronate doses similar to those required to suppress Akt activity (Matsumoto et al. 2011). Our result showed that risedronate induced apoptosis only through the cleaved Parp pathway. Risedronate also significantly decreased Akt protein expression in HL-60, while no activity was detected in KG-1 cells.

Hydrochlorothiazide is used to treat hypertension and water retention due to its diuretic properties. In addition, it has the potential to treat diabetic insipidus, renal tubular acidosis, and reduce the risk of kidney stones in individuals whose urine has a high calcium content (Akbari and Khorasani-Zadeh 2022; Carter et al. 2004). Hydrochlorothiazide had no effect on vascular smooth muscle cell proliferation or apoptosis or MAPK phosphorylation (Wang

et al., 2017). Administration of hydrochlorothiazide to rats results in destruction of distal tubule epithelium followed by significant apoptotic cell death and peritubular inflammation (Loffing et al. 1996). Hydrochlorothiazide treatment increased cell death compared with the control group in both AML cells. Although no activity of the drug on Akt levels was detected in KG-1 cells, an inhibitory effect was obtained in HL-60 cells by exposure to hydrochlorothiazide.

This is the first time that these drugs (desipramine, doxepin, estramustine, hydrochlorothiazide, nortriptyline, and risedronate) have been proposed in the literature as drug candidates for the treatment of AML. The major limitation of the study is that it considers only two data sets, as there is no data from treated patients in the databases. In addition, the mechanism of action of the newly developed drugs should be thoroughly investigated to understand the signaling pathways involved.

There are newly developed drugs in the literature for the treatment of AML and trying to repurpose drugs that do not work can still teach researchers something about how AML works. Some molecules have a lot of potential but are inadequate in terms of efficacy. It is possible that future studies will be conducted to examine the impact of the proposed drugs on the pathways of action of the various cancer mechanisms. While this study provides an attractive framework for future evaluation of these drugs in the therapy of AML, both as single agents to limit cell proliferation and as potential participants in chemotherapeutic agents, the most important extension of this work would be to determine whether the observations of the *in vitro* studies can be experimentally verified by *in vivo* studies and clinical trials.

It has been shown that the drugs that successfully induced cell death, although they did not cause apoptosis via the caspase-8 pathway. The uncontrollable increased activity of leukemic cells is one of the reasons contributing to the difficulties in treating AML in this way. Therefore, when researching the mechanism of AML, it is necessary to conduct research with different AML cell lines. Multicellular organisms are composed of a variety of cells, each of which has its own morphology and performs a specific function. Therefore, this method is very effective to compare the effects of drugs on AML-related cellular processes.

Consequently, studying the drug mechanism in cells with different characteristics in cancer research will provide an effective pathway in drug development for future steps. In addition, alternative dose combinations can be investigated to determine how the interaction alters the form and intensity of the combined effect. Future efforts should be made to incorporate the method of "system-level drug repurposing" into the practice of developing successful clinical treatments for AML.

In summary, we have mapped the transcriptomic landscape of AML by integrating data at the mRNA level and proposed novel drug candidates on this basis. We validated our findings by testing cell viability, cell death mechanisms, and Akt protein expression. The effects of the doxepin, estramustine and nortriptyline on apoptosis and Akt provided us with promising results for further studies on the use of these drugs in the treatment of AML. To make future studies on the treatment of AML meaningful, the synergistic mechanisms between these newly developed drugs and other anticancer drugs need to be uncovered.

4. CONCLUSION

AML is a heterogeneous clonal disease characterized by hematopoietic progenitor cells with deregulated proliferative activity and impaired ability to develop into mature blood cells. Treatment has improved in younger people, but survival in the elderly is very poor, averaging only a few months. There are a number of factors that have led to a dramatic increase in interest in AML among both basic and clinical researchers. As with AML, most patients experience disease relapse, usually with resistant disease, despite significant tumor reduction after intensive combination chemotherapy. This suggests that in many cases, leukemia stem cells are not reached by current combination therapy. However, leukemogenesis is also influenced by a variety of other genetic processes, including oncogene activation and haploinsufficiency. Despite significant advances in our understanding of the biology of the disease, clinicians still face significant obstacles in the regular treatment of patients with AML.

The ability to identify biomarkers at different stages of a biological process is a major advantage of the systems biology approach. Due to systems biology techniques that facilitate

the processing of huge amounts of data, we can now look at the system as a whole. The use of systems biology approaches immediately comes to mind when thinking about how to develop new and successful treatment strategies for AML. Numerous studies have been conducted to determine the molecular processes underlying AML pathogenesis and to elucidate the dysregulations of disease signatures at many biological levels.

Based on these considerations, the main objective of this dissertation was to identify biomarkers and drug-responsive molecules such as genes, transcription factors, proteins, and signaling pathways that play an important role in AML from a systems biomedicine perspective using currently used algorithms. In addition, potential candidate molecules and promising newly developed drugs for AML were discovered as a result of these studies.

This thesis consists of three topics and aims to identify effective prevention and/or treatment strategies for AML using systems biology approaches. As the first topic of the work, we aimed to contribute to the development of potential systems-level biomarkers that can be used to predict AML. Based on an integrative multi-omics approach, we present molecular codes of AML at RNA (mRNA, miRNA), protein (receptor, TF) and metabolite levels. The applied methodology provided significant evidence for novel molecular signatures in AML such as PTPRG and ACVR1 as receptors, PRDM14 and GATA3 as TFs, miR-519d-3p and miR-484 as miRNAs, which require further validation studies *in vitro* and *in vivo*. Amino acid and arachidonic acid metabolism were also identified as potential therapeutic targets. In addition, the high prognostic power of CSF3R, PTPRG, and AR was demonstrated. These biological molecules not only demonstrate the interaction of AML with specific biological processes, but also have the potential to be used as systems-level biomarkers for AML in the context of disease prognosis. Reliable biomarkers that can accurately predict the prognosis of patients with AML will be helpful for therapeutic outcomes. This multi-omics study lays the foundation for future research into better therapeutic trials in the treatment of AML.

The second goal of the dissertation was to present the techniques that provide a framework for differential co-expression analysis to elucidate treatment mechanisms. The resulting co-expressed gene networks have prognostic power to discriminate patients by response mechanism and have the potential to allow clinicians to differentiate patient prognosis before

and during disease treatment. The main goal of the research was to create networks focused on genes by analyzing differences in treatment-related co-expression patterns. Remember, in complex diseases like cancer, genes never act alone, but as part of a complex and interconnected system. We built a co-expression network linking genes involved in drug response using comparative and integrative analysis. The data extracted and analyzed included patients with AML who were refractory, had relapsed, or had never been treated. Patients who have not responded to previous therapy and/or untreated patients may benefit from learning about potentially treatment-relevant genes and their co-expressed network by identifying changes in the co-expression patterns of genes in patients. With this in mind, our study examined negative and positive correlations in a response status-based network to see how different correlations interact and influence drug response. Eventually, SECISBP2L, MAN1A2, PRPF31, VASP, SNAPC1 in the response network and PHTF2, PDLIM5 and OTUB1 in the non-response network were proposed as novel molecular biomarkers.

As the third objective of this work, we presented the transcriptomic landscape of AML by integrating data at the mRNA level and proposed new drug candidates based on this integration. We validated our results by assays of cell viability, apoptosis, and Akt and Parp protein expressions. It was shown that the drugs that successfully induced cell death, although they did not cause apoptosis via the caspase-8 pathway. The uncontrollable increased activity of leukemic cells is one of the reasons contributing to the difficulties in treating AML in this manner. Therefore, it is important to conduct studies with multiple AML cell lines to investigate the mechanism. Multicellular organisms are composed of a variety of cells, each of which has its own morphology and performs a specific function. Thus, comparing the effects of drugs on cellular processes associated with AML is greatly facilitated by this strategy. Cancer research can greatly benefit from studying drug mechanisms of action in cells with different characteristics, as this may provide a promising avenue for future drug development. In addition, other dose combinations can be evaluated to determine how the interaction affects the overall effect. Our investigation into the effects of nortriptyline, doxepin, and estramustine on AML cell lines has yielded promising outcomes, which could lead to significant advances in the treatment of AML. These findings suggest that these drugs

could be viable candidates for further investigation and potential use as therapeutic agents in the management of AML. This represents an encouraging development in the ongoing effort to improve the efficacy of treatments for this challenging disease. Successful therapeutic interventions for AML should be developed in the future using the systemic approach to drug repurposing.

Finally, the study concluded that a systems biomedicine-mediated, multi-level omics assessment of AML based on response mechanisms provides new insights into genes, proteins, reporter biomolecules, co-expressed elements, prognostic biomarkers, and potential candidates for new drugs to be developed. This enabled objective and prudent prioritization of drugs currently in use, leading to effective outcomes. Only by integrating data from multiple omics could drug repositioning for AML occur with the precision and originality required for effective treatment. This entire work is the first report of systems biomedicine-oriented drug repositioning for AML through the integration of multi-omics data and critical extensions of *in silico* and *in vitro* confirmations. This is an important step towards the development of precision oncology.

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ÖZGEÇMİŞ

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Uluslararası hakemli dergilerde yayımlanan makaleler:

1. **Kelesoglu N**, Kori M, Turanlı B, Arga KY, Yilmaz BK, Duru OA. Acute Myeloid Leukemia: New Multiomics Molecular Signatures and Implications for Systems Medicine Diagnostics and Therapeutics Innovation. OMICS: A Journal of Integrative Biology, 26(7): 392-403, 2022.
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Yazılan kitaplardaki bölümler:

1. Kelesoglu N, Karademir-Yilmaz B, Arga K.Y. (2022). Molecular Signatures in Acute Myeloid Leukemia: From Diagnosis to Targeted Therapy and Drug Repositioning. In: Interdisciplinary Cancer Research. Springer, Cham.
2. Kelesoglu N, Caliskan A, Aydın B, Arga KY. (2023) Büyük veri ekosistemi. Yeni Nesil Dizileme ve Klinikteki Uygulamaları, Ed: Prof. Dr.Ahmet Okay Çağlayan.

Uluslararası bilimsel toplantılarda sunulan ve bildiri kitaplarında (proceedings) basılan bildiriler:

1. Kelesoglu N, Korkmaz N.S, Turanlı B, Arga K.Y, Yilmaz B.K, Duru O.A. Transcriptomics-Based Drug Repurposing Unravels Novel Therapeutic Strategies In AML. 4th Eurasia Biochemical Approaches & Technologies (Ebat) Congress, 3-6 November, 2022 Antalya
2. Korkmaz N.S, Kelesoglu N, Duru O.A, Turanlı B, Kori M, Gorel A. M.Y, Arga K.Y, Yilmaz B.K. İlaç Yeniden Konumlandırma İle Belirlenen Aday İlaçların Akut Myeloid Lösemi Hücrelerinde Proteazom İnhibitörü İle Kombinasyon Tedavisinin İncelenmesi. 4th Uluslararası Katılımlı Hücre Ölümü Araştırma Derneği Kongresi, 17-19 Mart, 2022 Online.

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4. Kelesoglu N. Tumor Suppressors Primary Transcript Levels And Correlation With Bcr-Abl Transformation In The Chronic Myelogenous Leukemia Patients, Otto Warburg International Summer School and Research Symposium, Max Planck Institute for Molecular Genetics, Berlin, Germany, September 2015
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6. Kelesoglu N. Mechanism Involved In Regulation Of Expression Of The Tumor Suppressor Phlpp In Chronic Myelogenous Leukemia, European Conference of Technology and Society Sarajevo, 27-28.06.2013
7. Kelesoglu N, Ler D, Efremov D.G, Suljagic M. Loss Of PHLPP1 Tumor Suppressor Enhances BCR-Induced Pro-Survival Signaling In Chronic Lymphocytic Leukemia, PERIODICUM BIOLOGORUM, Vol.114, Suppl 1, 2012, Second Meeting Of The Croatian Association For Cancer Research Zagreb, 8-9.11.2012