



UNIVERSITÀ
DEGLI STUDI
DELL'AQUILA



DISCAB
Dipartimento di Scienze
Cliniche Applicate
e Biotecnologiche



UNIVERSITY OF L'AQUILA

DEPARTMENT OF BIOTECHNOLOGICAL AND APPLIED CLINICAL SCIENCES

PhD in Experimental Medicine

Curriculum: Biotechnology and Biochemical Sciences

XXXVIII cycle

Targeting CES1 in human cancers

SSD: MEDS-26/A

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a.y. 2024/2025

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ABSTRACT

Acute Myeloid Leukemia (AML) is a highly lethal disease often characterized by a profound metabolic reprogramming that enables leukemic cells to survive under conditions of energy and environmental stress, contributing to chemotherapy resistance. Specifically, AML cells can flexibly rely on oxidative phosphorylation (OXPHOS) and reprogrammed lipid metabolism to meet their high energy demands and sustain survival and proliferation under stress. Moreover, constitutive activation of the NF- κ B pathway is a frequent feature of AML, promoting survival, inflammation, and treatment resistance.

Recently, carboxylesterase 1 (CES1) has been identified as an NF- κ B-regulated lipase that regulates lipid catabolism, thereby facilitating energy production under nutrient-depleted conditions. However, how NF- κ B-driven programs and CES1-dependent lipid metabolism intersect with bioenergetic adaptation in AML has not been defined.

This study aimed to characterize the contribution of CES1 to metabolic adaptation under energy stress (ES) in AML and to evaluate the antileukemic effects of CES1 inhibition.

We demonstrated that in basal conditions, all AML cell lines displayed a predominantly glycolytic phenotype, whereas ES induced a metabolic shift towards OXPHOS and upregulated CES1.

Indeed, pharmacological inhibition of CES1 with the commercially available inhibitor GR-148672X resulted in impaired OXPHOS and induced ferroptosis or apoptosis in all AML lines analyzed. Genetic knockdown of *CES1* confirmed the reduced cell survival observed with pharmacological inhibition. In vivo, GR-148672X slowed the tumor growth in the THP1 xenograft model. Furthermore, the combined treatment with GR-148672X and BCL-2 inhibitor, venetoclax, revealed heterogeneous interactions across the cell line panel, with synergistic cytotoxicity in specific AML cells.

Collectively, these data reinforce the concept that targeting metabolic adaptation, specifically through CES1 inhibition, may provide a novel and effective therapeutic approach in AML.

1. INTRODUCTION

1.1 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a genetically and clinically heterogeneous hematologic malignancy characterized by the clonal proliferation of hematopoietic stem cells and progenitor cells, accompanied by impaired differentiation. As a result, immature myeloid precursors (myeloblasts) accumulate in the bone marrow and peripheral blood, leading to reduced production of mature blood cells, including red blood cells, platelets, and white blood cells (DiNardo et al., 2023). In some cases, AML patients may also develop extramedullary disease (EMD), defined as infiltration of leukemic blasts into tissues outside the bone marrow. EMD at diagnosis occurred in 23.7% of patients, most commonly involving lymph nodes (11.5%), spleen (7.3%), liver (5.3%), skin (4.5%), gingiva (4.4%), and the central nervous system (CNS) (1.1%) (Ganzel et al., 2016).

AML is the most common form of acute leukemia in adults, accounting for about 25-30% of all leukemia cases. Based on SEER data, the annual incidence is estimated at approximately 4-5 cases per 100,000 inhabitants, with increasing incidence with age; the median age at diagnosis is around 68-70 years (*SEER, 2024*). Moreover, despite therapeutic progress, the prognosis remains poor, with an overall 5-year survival of less than 30% in the general population, which is even lower in elderly patients or patients with comorbidities (*SEER, 2024*). Regarding risk factors related to the onset of AML, in addition to age, other factors such as exposure to benzene, ionizing radiation, or alkylating chemotherapeutic agents, as well as genetic predisposing conditions (e.g., myelodysplastic syndromes and germline mutations), may contribute to the development of the disease (Shallis et al., 2019). In addition, lifestyle factors have also been associated with the risk of AML; for example, being overweight (body mass index 25-29.9 kg/m²) or obese (body mass index ≥ 30 kg/m²) is associated with an increased risk of AML (Li et al., 2017).

1.1.1 Clinical presentation and diagnosis

AML typically presents with a rapid onset of symptoms driven primarily by the replacement of normal hematopoiesis, leading to bone marrow failure. The resulting cytopenia manifests as anemia, thrombocytopenia, and leukopenia. Additional

symptoms result from the uncontrolled proliferation of leukemic blasts and their subsequent tissue infiltration (common signs and symptoms of AML are summarized in Table 1).

Table 1: Signs and symptoms of AML

Underlying Mechanism	Associated Specific Symptoms
Anemia	Tiredness or weakness, paleness of the skin, shortness of breath (dyspnea), and fatigue.
Thrombocytopenia	Easy bruising, excessive bleeding (e.g., from gums or nose), and petechiae (tiny red spots) on the skin.
Leukopenia	Frequent infections, fever, chills, and night sweats.
Systemic Effects and Tissue Infiltration	Bone pain, lymphadenopathy, weight loss, and acute emergencies like leukostasis (headache, visual changes, respiratory distress).

The diagnostic evaluation of AML requires a comprehensive, multimodal approach to establish diagnosis, classification, and prognostic risk stratification. The fundamental investigation is based on peripheral blood smear and bone marrow aspirate, and core biopsy to assess cellularity and morphology, which necessitate supplementary specialized analyses, such as immunophenotyping, cytogenetic, and molecular assays (DiNardo et al., 2023).

The diagnosis of AML hinges on the identification of an abnormal proliferation of myeloid blasts. Historically, based on morphological assessment, AML was defined by the presence of greater than 30% of blasts of myeloid lineage (myeloblasts, monoblasts, or megakaryoblasts) in the peripheral blood or bone marrow; however, this threshold has been lowered to 20% of myeloid blasts (DiNardo et al., 2023). Crucially, AML diagnosis can be established regardless of the blast percentage in cases of isolated extramedullary AML (*i.e.*, myeloid sarcoma), or in the presence of defining genomic changes (DiNardo et al., 2023). These AML-defining genomic changes include balanced translocations or gene fusions such as t(8;21) (RUNX1-RUNX1T1 fusion), inv(16) or t(16;16) (CBFB-MYH11), which characterize Core-Binding Factor AML (CBF-AML), and the t(15;17) fusion gene PML-RARA, which defines Acute Promyelocytic Leukemia (APL) (DiNardo et al., 2023). The

requirement of 20% blasts remains for AML with BCR-ABL1 fusion, to avoid overlap with chronic myeloid leukemia (CML), and for AML with CEBPA mutations.

In addition to morphological assessment, flow cytometry immunophenotyping is a fundamental diagnostic tool. In AML, it supports the detection and quantification of leukemic blasts, determines blast lineage, and reveals aberrant antigen-expression patterns that help distinguish malignant blasts from normal progenitors. For diagnostic purposes, antigens are typically classified as precursor-stem cell markers (CD34, CD117, HLA-DR); myeloid markers (cMPO, CD33, CD13); myeloid maturation markers (CD11b, CD15, CD64, CD4, CD38, CD11c); monocytic markers (CD14, CD36, CD64); megakaryocytic markers (CD41, CD36, CD61); and erythroid markers (CD235, CD71, CD36). Immunophenotyping is also pivotal for assessing treatment response and detecting minimal residual disease (MRD) by tracking leukemia-associated antigens defined at diagnosis during and after therapy (Pessoa et al., 2023).

Cytogenetic analysis and molecular profiling using next-generation sequencing (NGS) panels should follow morphology and immunophenotyping. At minimum, testing should include abnormalities that define specific AML subtypes (e.g., NPM1, CEBPA, RUNX1) and mutations with prognostic and therapeutic relevance (e.g., FLT3-ITD, TP53, IDH), which inform risk stratification and treatment selection (DiNardo et al., 2023).

1.1.2 Progress in the classification system and risk stratification

The classification of AML has undergone significant evolution over the past decades. The French-American-British (FAB) classification, introduced in 1976 (Bennett et al., 1976), represented the first substantial attempt to standardize the diagnosis of AML. The system set the blast number cut-off at 30% and was primarily based on morphology and cytochemistry. Over subsequent FAB revisions, AML was ultimately subdivided into eight subtypes designated M0 to M7 (Table 2).

Table 2: AML classification according to the FAB system

M0	Undifferentiated acute myeloid leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation

M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Erythroid leukemia
M7	Acute megakaryoblastic leukemia

Although the FAB system remains useful for describing morphology, this classification did not account for molecular features that have emerged over time as major prognostic determinants, so it was replaced by the World Health Organization (WHO) classification of hematologic neoplasms. Published in 2001 and updated in 2008, 2016, and 2022, this classification considers clinical features, morphology, immunophenotyping, cytogenetics, and molecular genetics (Khoury et al., 2022). The last update in 2022 (WHO-HAEM5) of myeloid proliferations and neoplasms include nine sub-categories: Myeloid precursor lesions; Myeloproliferative neoplasms (MPNs); Mastocytosis; Myelodysplastic neoplasms (MDNs, previously known as myelodysplastic syndrome, MDS); MDN/MPNs; AML; Secondary myeloid neoplasms; Myeloid/lymphoid neoplasms with eosinophilia and defining gene rearrangement; Acute leukemias of mixed or ambiguous lineage. AML is classified into two principal groups: (1) AML with defining genetic abnormalities; (2) AML, defined by differentiation. In addition, myeloid sarcoma is described as a distinct tissue-based manifestation of AML and requires comprehensive genetic characterization for appropriate classification and treatment planning (Table 3) (Khoury et al., 2022).

Table 3: WHO 2022 classification of AML

AML with defining genetic abnormalities
APL with PML::RARA fusion
AML with RUNX1::RUNX1T1 fusion
AML with CBFB::MYH11 fusion
AML with DEK::NUP214 fusion
AML with RBM15::MRTFA fusion
AML with BCR::ABL1 fusion
AML with KMT2A rearrangement

AML with MECOM rearrangement
AML with NUP98 rearrangement
AML with NPM1 mutation
AML with CEBPA mutation
AML, myelodysplasia-related
AML with other defined genetic alterations
AML defined by differentiation
AML with minimal differentiation
AML without maturation
AML with maturation
Acute basophilic leukaemia
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia
Myeloid sarcoma

Compared to previous versions, the latest update expanded the list of defined genetic abnormalities to better reflect the molecular landscape of the disease. Furthermore, the mandatory 20% blast count threshold was eliminated for most AML forms associated with specific genetic alterations, reflecting a shift towards a genetic-first definition. The exceptions to this rule remain AML with BCR::ABL1 fusion and AML with CEBPA mutations, for which the 20% cut-off is maintained (Khoury et al., 2022).

In the same year, a parallel classification system was introduced: the International Consensus Classification (ICC) of Myeloid Neoplasms and Acute Leukemias. A substantial difference between the WHO-5 and the ICC lies in the definition of the blast threshold for diagnosis. The ICC requires at least a 10% blast count in the bone marrow or peripheral blood to define AML with recurrent genetic abnormalities, with a few exceptions (e.g., $\geq 20\%$ for AML with BCR::ABL1 fusion or with prior MDS/MPN). The ICC also formalizes MDS/AML for cases with 10–19% blasts in selected molecular/cytogenetic settings. Another fundamental distinction is the handling of secondary AML cases. The ICC does not establish secondary AML as a

distinct entity; instead, it is used as a diagnostic qualifier to the disease entities that are primarily defined by their genetic profile (Arber et al., 2022; H. Döhner et al., 2022).

This classification system is integrated with the European Leukemia Network (ELN) guidelines, which incorporate insight from novel molecular findings and recent trial results, enabling the stratification of AML patients into three different prognostic groups: favorable, intermediate, and adverse risk. AML with chromosomal translocations t(8;21), or inv(16), and mutated NPM1 without FLT3-ITD, as well as bZIP-CEBPA mutations, characterize favorable risk disease. Intermediate-risk disease includes, for example, NPM1-mutated AML with FLT3-ITD and most cases with a normal karyotype that lack adverse-risk markers. Cytogenetic alterations, such as complex karyotype (defined as more than 3 chromosomal abnormalities), monosomy 5 or 7, t(6;9), inv(3), AML with myelodysplasia-related gene mutations, and mutated p53, were associated with a poor prognosis (H. Döhner et al., 2022).

1.1.3 Molecular landscape of AML

In the last decade, several genetic features, including both cytogenetic and molecular aberrations, have been identified as cooperating in leukemogenesis. Nowadays, these molecular abnormalities are pivotal for the diagnosis of genetically defined AML subtypes, for prognostic risk assignment according to ELN guidelines, and for therapeutic strategy choice. Notably, the identification of recurrent mutations helped refine the prognosis of patients with normal cytogenetics (CN-AML), which represent nearly half of de novo AML cases.

The most frequently mutated genes include *Fms-like tyrosine kinase 3 (FLT3)*, *Nucleophosmin (NPM1)*, *DNA methyltransferase 3 alpha (DNMT3A)*, *Isocitrate dehydrogenase 1/2 (IDH1/2)*, *Ten-Eleven Translocation 2 (TET2)*, *Runt-related transcription factor 1 (RUNX1)*, *Tumor Protein P53 (TP53)*, *Neuroblastoma RAS viral oncogene homolog / Kirsten rat sarcoma viral oncogene homolog (NRAS/KRAS)*, *CCAAT/enhancer-binding protein alpha (CEBPA)*, and *Wilms tumor 1 (WT1)*, which may occur as single mutations or in conjunction with other mutations. These mutations are particularly enriched in CN-AML, but they are also observed in cases with cytogenetic abnormalities (Kantarjian et al., 2025).

A description of the most recurrent mutations associated with AML is reported below.

FLT3 mutations occur in approximately 30% of de novo AML cases, and are frequently associated with normal karyotype, *NPM1* mutations, and *DEK::NUP214*-AML. *FLT3* is a class III receptor tyrosine kinase that is physiologically expressed on hematopoietic stem and progenitor cells, where it regulates survival, proliferation, and differentiation in response to its ligand *FLT3L*, by activating PI3K/AKT/mTOR, RAS/MAPK/ERK, and JAK/STAT pathways (Gilliland & Griffin, 2002). Activating *FLT3* mutations in AML are internal tandem duplications (ITD) and point mutations within the tyrosine kinase domain (TKD), which occur in 20-25% and 7-10% of cases, respectively (Daver et al., 2019). Prognostically, *FLT3*-ITD has been consistently associated with higher leukocyte and blast counts at diagnosis, increased relapse risk, and reduced overall survival, particularly when present with a high allelic ratio (Gale et al., 2008). However, ELN 2022 no longer uses *FLT3*-ITD allelic ratio for genetic-risk assignment, reflecting both biological complexity and the changing therapeutic landscape. Subsequent work showed that the impact of *FLT3*-ITD is strongly modulated by co-mutations, especially *NPM1*, and by ITD allelic burden and insertion site (Papaemmanuil et al., 2016; Rücker et al., 2022). For *FLT3*-TKD activating mutations, the prognostic relevance is less consistent. Although some early and subset analyses suggested that *FLT3*-TKD mutations might confer an adverse prognosis comparable to *FLT3* internal tandem duplications (ITD) (Yanada et al., 2005), more recent large cohort studies and meta-analyses indicate that their prognostic impact is weaker and largely context-dependent, with *FLT3*-TKD often showing neutral or even favorable outcomes depending on co-occurring molecular lesions and patient population (Mead et al., 2007; Li et al., 2023). Clonal evolution studies have further shown that *FLT3* mutations can be gained or lost between diagnosis and relapse, or *FLT3*-mutant clones can also acquire secondary TKD mutations, supporting repeated *FLT3* testing over the disease course (Smith et al., 2012).

NPM1 is a nucleolar chaperone protein that shuttles between the nucleus and cytoplasm and is involved in ribosome biogenesis, centrosome duplication, and genomic stability. In AML, *NPM1* mutations, primarily restricted to exon 12, disrupt the nucleolar localization signal, creating a novel nuclear export signal that causes aberrant cytoplasmic localization of the protein (Falini et al., 2005). These mutations occur in about one-third of adult AML and in 45-60% of cytogenetically normal AML, defining a biologically distinct entity often characterized by monocytic or

myelomonocytic features, and low or absent CD34 expression (Falini et al., 2005). NPM1 mutations are frequently accompanied by co-mutations in FLT3 (especially FLT3-ITD), DNMT3A, TET2, and IDH1/2, and are associated with a characteristic HOX-high transcriptional program that is thought to play a central role in leukemogenesis (Falini et al., 2020). Clinically, a study on 300 CN-AML patients showed that NPM1 mutations were associated with higher complete remission rates and improved overall survival, but only when FLT3-ITD was absent (K. Döhner et al., 2005)

DNMT3A encodes a DNA methyltransferase essential for cytosine residue methylation of CpG dinucleotides and epigenetic regulation of hematopoietic stem cells differentiation and proliferation. DNMT3A mutations, including missense and nonsense mutations, most commonly at R882, occur in 20-30% of adult CN-AML and tend to be early, stable, pre-leukemic lesions that persist at remission and contribute to clonal hematopoiesis (Ley et al., 2010; Cole et al., 2017). Ley et al. found that DNMT3A mutations are associated with shorter OS and increased risk of relapse (Ley et al., 2010). More recent work has refined their prognostic impact in the context of co-mutations (e.g., FLT3-ITD, NPM1), generally confirming an adverse effect, particularly for R882 and high variant allele frequency mutations (Oñate et al., 2022). Recent studies also suggest that DNMT3A-R882 mutant clonal hematopoiesis relies on oxidative phosphorylation (OXPHOS), revealing metabolic vulnerabilities that may be exploitable at pre-leukemic and leukemic stem cell levels (Gozdecka et al., 2025).

IDH1 and *IDH2* encode enzymes involved in the citric acid cycle. Mutations at IDH1 R132 and IDH2 R140/R172 lead to a loss of normal enzyme function and enable the enzymes to convert α -ketoglutarate into 2-hydroxyglutarate. This product inhibits α -ketoglutarate-dependent dioxygenases, including TET2, resulting in a characteristic DNA and histone hypermethylation phenotype and impaired hematopoietic differentiation (Figueroa et al., 2010). IDH1/2 mutations are found in 15-20% of AML cases, often in cytogenetically normal and NPM1-mutated disease, and are often early events present in pre-leukemic cells (Figueroa et al., 2010; Paschka et al., 2010). Early studies suggested that IDH1 mutations and IDH2 R172 confer an adverse prognosis, while IDH2 R140 may be linked to a relatively favorable outcome; however, larger studies and meta-analyses indicate that the

overall prognostic impact is modest and highly dependent on the specific context (Thol et al., 2010; Green et al., 2011; DiNardo et al., 2015).

TET2 encodes a dioxygenase that catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, thereby promoting active DNA demethylation. Loss-of-function *TET2* mutations are common in myeloid malignancies and age-related clonal hematopoiesis, occurring in 10-20% of AML cases, and lead to global DNA hypermethylation and impaired hematopoietic differentiation (Gaidzik et al., 2012). In AML, *TET2* mutations often co-occur with *NPM1*, *FLT3-ITD*, and *RAS* pathway lesions, whereas they are mutually exclusive with *IDH1/2* mutations, reflecting convergent disruption of the same epigenetic axis (Chou et al., 2011).

RUNX1 encodes the α subunit of the core-binding factor (CBF) transcription complex and is crucial for definitive hematopoiesis, lymphocyte differentiation, megakaryocyte maturation, and regulation of hematopoietic stem cells (HSCs) (Nakagawa et al., 2011; Gaidzik et al., 2016). Somatic *RUNX1* mutations in AML comprise loss-of-function and dominant-negative alterations (*RUNX1-ETO*) and are enriched in older patients, secondary or therapy-related AML, and minimally differentiated (M0) subtypes. Early work in heterogeneous and cytogenetically normal AML cohorts identified *RUNX1* mutations as adverse, associated with lower complete remission rates and shorter survival (Greif et al., 2012). This has been largely confirmed by more recent meta-analyses, which highlight that *RUNX1*-mutated AML frequently co-harbors mutations in *ASXL1*, *SRSF2*, and *RAS* genes and generally falls into the adverse-risk category in modern classifications (Lew-Derivry et al., 2023; Ran et al., 2025).

TP53 encodes the tumor suppressor p53, a key regulator of DNA damage responses, cell cycle arrest, and apoptosis, and it controls several metabolic pathways. It is the most frequently mutated gene in human cancers. In AML, *TP53* mutations are strongly linked to complex karyotypes, therapy-related or secondary disease, and chromosomal aneuploidy, and they define a distinct biological and clinical entity with significant chemoresistance (Shahzad et al., 2024). Multiple studies indicate that AML with *TP53* mutations has a poor prognosis, with median overall survival typically around 4-6 months and 2-year survival less than 10%, even with intensive chemotherapy or venetoclax-based low-intensity treatments (N. G. Daver et al., 2023).

N-RAS and *K-RAS* encode small GTPases that transmit signals through the MAPK and PI3K pathways. Activating missense mutations cluster in codons 12, 13, and 61 and occur in 15-20% of AML cases. RAS-mutated AML is often associated with myelomonocytic or monocytic morphology, normal or non-complex karyotypes, and co-mutations in genes such as *NPM1* (Alawieh et al., 2024). Earlier series suggested heterogeneous frequency and spectrum of mutations but minimal influence on clinical outcome (Bowen et al., 2005). Still, more recent cohort analyses indicate that *KRAS* mutations are associated with inferior survival and higher relapse risk, especially in patients treated with hypomethylating agent (HMA)-based or venetoclax-HMA regimens, whereas *NRAS* mutations often show no clear adverse effect (Ball et al., 2021; Mustafa Ali et al., 2023).

CEBPA encodes the myeloid transcription factor CEBP α , which is critical for granulocytic differentiation. In AML, mutations typically affect the N-terminal region, introducing premature stop codons, and the C-terminal basic leucine zipper (bZIP) domain (Su et al., 2022). Multiple studies and meta-analyses have demonstrated that *CEBPA* mutations confer a favorable prognosis, particularly in cytogenetically normal, de novo AML, and this genotype is classified as favorable risk in ELN guidelines (Li et al., 2015; De la Torre et al., 2024).

WT1 encodes a transcription factor involved in hematopoietic differentiation, apoptosis, and stem-cell maintenance. *WT1* inactivating mutations, typically frameshift or nonsense mutations in exons 7 and 9, occur in 10-15% of AML, are enriched in cytogenetically normal and intermediate or adverse-risk disease, frequently co-occur with *FLT3*-ITD and *CEBPA* mutations, and associate with younger age (Virappane et al., 2008; Ho et al., 2010; Rampal & Figueroa, 2016). Most studies link *WT1* mutations to reduced event-free and overall survival, and higher relapse risk (Virappane et al., 2008; Ho et al., 2010). In addition, *WT1* overexpression is observed in the majority of patients with AML (70-90%) and is widely used as a minimal residual disease (MRD) marker (Lambert et al., 2021), thereby integrating *WT1* into both genetic risk and response-adapted management algorithms.

The comprehensive characterization of these recurrent genetic lesions has not only deepened the understanding of AML biology but also paved the way to the development of targeted therapeutic strategies.

1.1.4 Therapeutic Approaches

Nowadays, AML treatment selection is based on the patient's age, performance status, and the cytogenetic and molecular risk category.

Therapy is generally divided into two main phases: Induction, to achieve complete remission, and consolidation, to eliminate residual disease and prevent relapse.

For fit patients with AML, the standard of care remains induction chemotherapy with the "7+3 regimen", introduced in 1973. It consists of a 7-day continuous infusion of cytarabine plus 3 days of an anthracycline, such as daunorubicin or idarubicin. The consolidation therapy in patients who achieve complete remission (CR) is represented by high-dose cytarabine (HiDAC). Randomized and cooperative group studies over several decades have shown that 7+3 achieves CR rates of 60-80% in younger adults and that anthracycline dose intensification can further improve outcomes in selected subgroups, so 7+3 remains the standard induction for medically fit patients (Fernandez et al., 2009; Othus et al., 2023). Post-remission, allogeneic hematopoietic stem cell transplantation (allo-HSCT) is generally recommended for patients with adverse-risk genetics and considered for intermediate-risk disease, whereas favorable-risk AML is often managed with chemotherapy alone; meta-analyses and randomized clinical trials have confirmed that allo-HSCT reduces relapse, with a survival benefit most clearly in intermediate/adverse-risk AML (Cornelissen & Blaise, 2016; Bornhäuser et al., 2023). Supportive care, including transfusions, growth factor support, and intensive anti-infective prophylaxis and treatment, remains essential to safely deliver these myelosuppressive regimens.

For patients with secondary AML (s-AML), conventional 7+3 has shown limited efficacy. So, CPX-351, a liposomal formulation that co-encapsulates cytarabine and daunorubicin at a fixed 5:1 molar ratio, was developed to optimize pharmacokinetics and drug effect. In a randomized phase III trial in 309 adults aged 60-75 years with newly diagnosed high-risk s-AML, CPX-351 significantly improved overall survival, CR/CRi rates, and post-transplant outcomes compared with standard 7+3. These data led to regulatory approval and have made CPX-351 a preferred first-line option in this high-risk subgroup (Lancet et al., 2018, 2021)

Acute promyelocytic leukemia (APL) (PML::RARA fusion) represents a special case in which differentiation therapy has essentially replaced conventional chemotherapy. For low- and intermediate-risk APL, the combination of all-trans retinoic acid (ATRA)

plus arsenic trioxide (ATO) has been shown in randomized trials to be non-inferior or superior to ATRA plus anthracycline-based chemotherapy in terms of event-free and overall survival, with less hematologic toxicity; long-term follow-up confirms cure rates >90%, and ATRA+ATO is now considered standard of care across these risk groups (Lo-Coco et al., 2013; Sanz et al., 2019).

In older patients or those unfit for intensive chemotherapy, the standard of care has shifted toward low-intensity treatments, primarily involving hypomethylating agents (HMAs) (e.g., azacitidine, decitabine), often combined with BCL-2 inhibitors such as venetoclax (DiNardo et al., 2020). The AZA-AML-001 phase III trial showed that azacitidine, compared with conventional care regimens (low-dose cytarabine or intensive chemotherapy or supportive care), produced a significant improvement in median overall survival (10.4 vs 6.5 months) in patients ≥ 65 years with newly diagnosed AML and $\geq 30\%$ blasts, establishing azacitidine as a standard low-intensity option in this setting (Dombret et al., 2015). Similarly, a phase III study of decitabine in older patients with AML demonstrated higher response rates and a survival trend favoring a 5-day decitabine schedule over conventional therapies (Kantarjian et al., 2012). Subsequent work has explored extended (10-day) decitabine courses, particularly in high-risk patients (Short et al., 2019). These HMA-based approaches have provided a platform onto which new targeted agents are now frequently added.

The most profound change in low-intensity therapy has been the introduction of the BCL-2 inhibitor venetoclax. In the phase III VIALE-A trial, venetoclax plus azacitidine significantly increased CR/CRi rates and median overall survival (14.7 vs 9.6 months) compared with azacitidine alone in previously untreated AML patients ineligible for intensive chemotherapy, establishing venetoclax-azacitidine as a new standard of care in this population (DiNardo et al., 2020; Pratz et al., 2024). Based on these data, venetoclax combinations received regulatory approval in the US with azacitidine, decitabine, or low-dose cytarabine (FDA, 2020), and in the EU with azacitidine or decitabine for older/unfit AML patients (EMA, 2021). Venetoclax-based regimens are now being explored in fit patients, in combination with intensive chemotherapy and multiple targeted agents, although myelosuppression and infectious complications remain essential challenges.

Targeted therapies have transformed the management of genetically defined AML subsets. For FLT3-mutated AML, the phase III CALGB 10603/RATIFY trial

demonstrated that adding the multi-kinase FLT3 inhibitor midostaurin to standard 7+3 induction and consolidation, followed by one year of maintenance, significantly improved overall and event-free survival in adults aged 18–59 years with FLT3-ITD or FLT3-TKD mutations, establishing intensive chemotherapy plus midostaurin as the standard for fit newly diagnosed FLT3-mutated patients (Stone et al., 2017; Larson et al., 2021). In the relapsed/refractory (R/R) setting, the phase III ADMIRAL trial showed that the selective, type I FLT3 inhibitor gilteritinib improved median overall survival and CR rates compared with salvage chemotherapy in R/R FLT3-mutated AML (Perl et al., 2019, 2022), supporting its regulatory approval for this indication. An Italian real-world study further supported its effectiveness and documented its use as a bridge to allo-SCT (Cairoli et al., 2025). More recently, the highly selective type II inhibitor quizartinib, combined with intensive induction and consolidation therapy, and continued as maintenance, has improved survival in patients with newly diagnosed FLT3-ITD-mutated AML aged 18 to 75 years in the QuANTUM-First trial (Erba et al., 2023), supporting its approval and expanding the FLT3 inhibitor armamentarium.

Similarly, IDH1 and IDH2 mutations have become actionable targets. The IDH1 inhibitor ivosidenib and the IDH2 inhibitor enasidenib were initially approved as single agents in R/R IDH-mutated AML based on durable responses and acceptable safety in pivotal phase I/II studies (DiNardo et al., 2018). Subsequent trials have evaluated their integration into frontline intensive chemotherapy and azacitidine-based regimens (DiNardo et al., 2021; Stein et al., 2021). These agents exemplify how metabolic driver mutations can be exploited therapeutically in AML.

Other targeted and immunotherapeutic approaches have also entered clinical practice. Gemtuzumab ozogamicin (GO), an anti-CD33 antibody-drug conjugate, has been reintroduced after meta-analyses of randomized trials showed that adding low-dose GO to induction, and in some schedules to consolidation, modestly improves overall and relapse-free survival, particularly in patients with favorable or intermediate cytogenetics, albeit with increased risk of hepatotoxicity and veno-occlusive disease (Burnett et al., 2011, 2012; Appelbaum & Bernstein, 2017). Maintenance therapy has also become an essential component of the treatment continuum: the QUAZAR AML-001 trial showed that oral azacitidine (CC-486) given as maintenance to older patients in first complete remission with or without complete blood count recovery (CR/Cri) after intensive chemotherapy significantly prolonged

overall and relapse-free survival versus placebo, leading to its approval as the first broadly applicable maintenance therapy for AML patients not undergoing allo-HSCT (Wei et al., 2020).

Finally, menin inhibitors have emerged as a promising option for specific AML subtypes. The oral menin inhibitor revumenib has shown high rates of remission in Relapsed/Refractory (R/R) acute leukemia with KMT2A rearrangements or NPM1 mutations in the phase I/II AUGMENT-101 trial, with manageable toxicity and frequent MRD-negative responses (Arellano et al., 2025; Issa et al., 2025). Based on these data, the FDA has approved revumenib for R/R leukemia with KMT2A rearrangements and for R/R AML with susceptible NPM1 mutation in adults and children, making it the first menin inhibitor to reach clinical practice and illustrating the rapid translation of genomic insights into targeted therapies (FDA, 2024, 2025). Despite the substantial progress in risk-adapted chemotherapy, allogeneic transplantation, and targeted agents, long-term survival in AML remains poor, and relapse is still the leading cause of treatment failure. These observations suggest that, beyond the genetic landscape and currently targetable signaling pathways, additional biological characteristics contribute to leukemic persistence and therapy resistance. One emerging feature that has gained increasing attention as a complementary avenue to improve AML therapy is metabolic reprogramming. Indeed, AML blasts and leukemic stem cells (LSCs) may adapt their bioenergetic and biosynthetic demands to hostile conditions, such as nutrient deprivation and drug exposure (Kreitz et al., 2019).

1.2 NF- κ B signaling in cancer and AML: From survival signaling to metabolic reprogramming

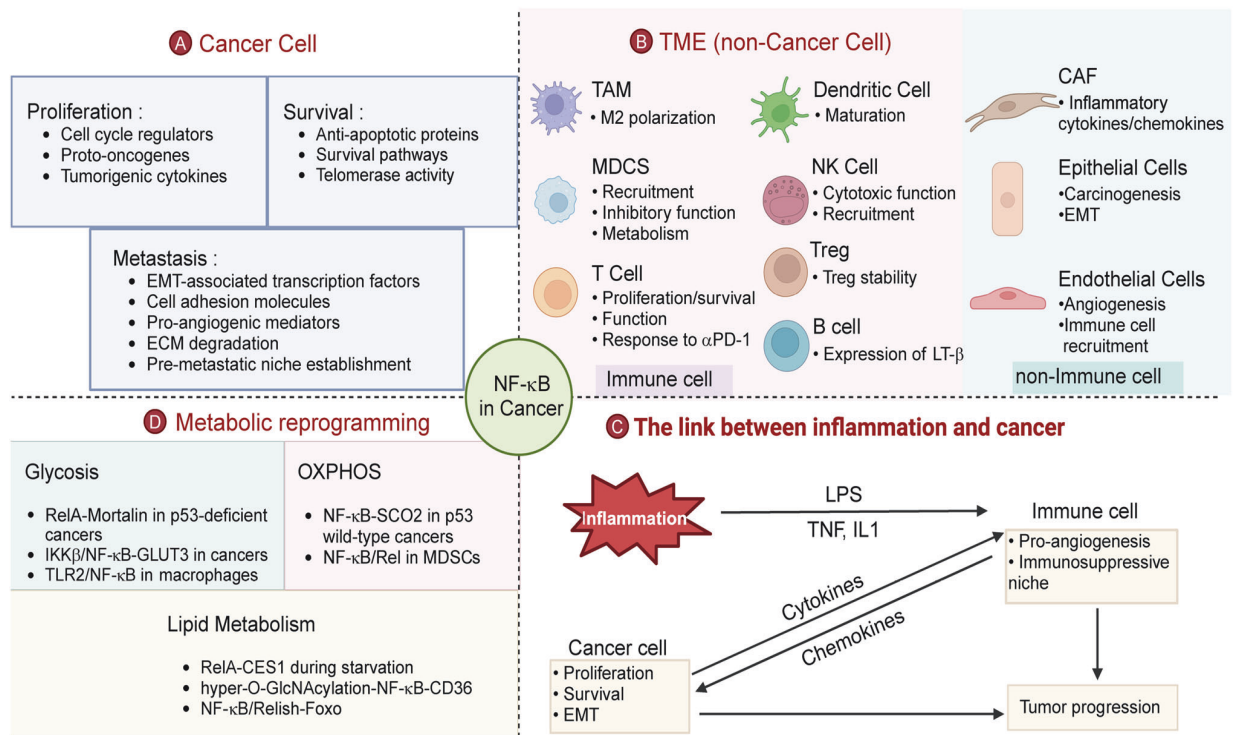
The nuclear factor kappa B (NF- κ B) transcription factors play an essential role in responding to stressors in the cellular environment and in regulating the expression of key regulatory genes involved in immune responses, inflammation, cell death, and proliferation (Mao et al., 2025).

The NF- κ B family comprises five structurally related transcription factors: RelA/p65, RelB, c-Rel, NF- κ B1 (p105/p50), and NF- κ B2 (p100/p52). These factors share a conserved N-terminal Rel homology domain (RHD) that enables DNA binding, dimerization, and interaction with ankyrin (ANK) repeat domains in the C-terminal of I κ B proteins. In resting cells, I κ Bs retain NF- κ B dimers in the cytoplasm by masking nuclear localization signals, thereby preventing transcriptional activity (T. Zhang et al., 2021). NF- κ B activation occurs via two major signaling pathways: the canonical and the non-canonical pathways. The canonical NF- κ B pathway responds to various extracellular stimuli, including pro-inflammatory cytokines (e.g., TNF- α , IL-1), Toll-like receptor ligands, pattern recognition receptors, and genotoxic stress. These stimuli activate the I κ B kinase (IKK) complex, composed of the catalytic subunits IKK α and IKK β and the regulatory subunit NEMO/IKK γ . Specifically, the first step in this signaling cascade is the activation of TAK1, which then activates the IKK complex. Once active, IKK phosphorylates I κ B proteins, especially I κ B α , thereby targeting them for ubiquitination and proteasomal degradation, allowing NF- κ B dimers, mainly RelA/p50, to translocate into the nucleus and regulate the transcription of genes involved in inflammation, immunity, cell survival, proliferation, and stress responses (Guo et al., 2024). In addition to this canonical pathway, a non-canonical NF- κ B pathway, typically activated by a subset of TNF family receptors (e.g., CD40 and RANK), relies on NF- κ B-inducing kinase (NIK), which induces processing of p100 to p52, via the activation of the kinase IKK α . Non-canonical signaling preferentially activates RelB/p52 heterodimers, with distinct roles in lymphoid organogenesis, B-cell maturation, dendritic cell activation, and bone morphogenesis (Sun, 2017).

Dysregulated NF- κ B activity has emerged as a hallmark of many solid tumors and hematologic malignancies, where it functions as a central hub integrating oncogenic signaling with chronic inflammation (Mao et al., 2025). Persistent NF- κ B signaling

can be driven by oncogenic mutations (e.g., RAS, BCR-ABL), core-NF- κ B mutations, receptor overactivation, chronic exposure to inflammatory mediators, or alterations in upstream regulators such as NIK and IKK (Mao et al., 2025). In diverse cancers, including colorectal, lung, breast, head-and-neck tumors, and hematologic malignancies, NF- κ B signaling promotes expression of anti-apoptotic proteins (e.g., BCL-2 family members, c-IAPs, XIAP), cell-cycle regulators such as cyclin D1, pro-inflammatory cytokines (TNF- α , IL-6, IL-8), adhesion molecules (e.g., ICAM-1, E-selectin), and angiogenic factor (VEGF), that support survival, proliferation, invasion, and angiogenesis (Mao et al., 2025; T. Zhang et al., 2021). At the same time, NF- κ B activity in stromal and immune cells within the tumor microenvironment shapes antitumor immunity by regulating the activation, differentiation, and effector functions of T cells, macrophages, and myeloid-derived suppressor cells (Capece et al., 2022; Mao et al., 2025) (Figure 1).

Figure 1: Role of NF- κ B in cancer (from Mao et al., 2025)

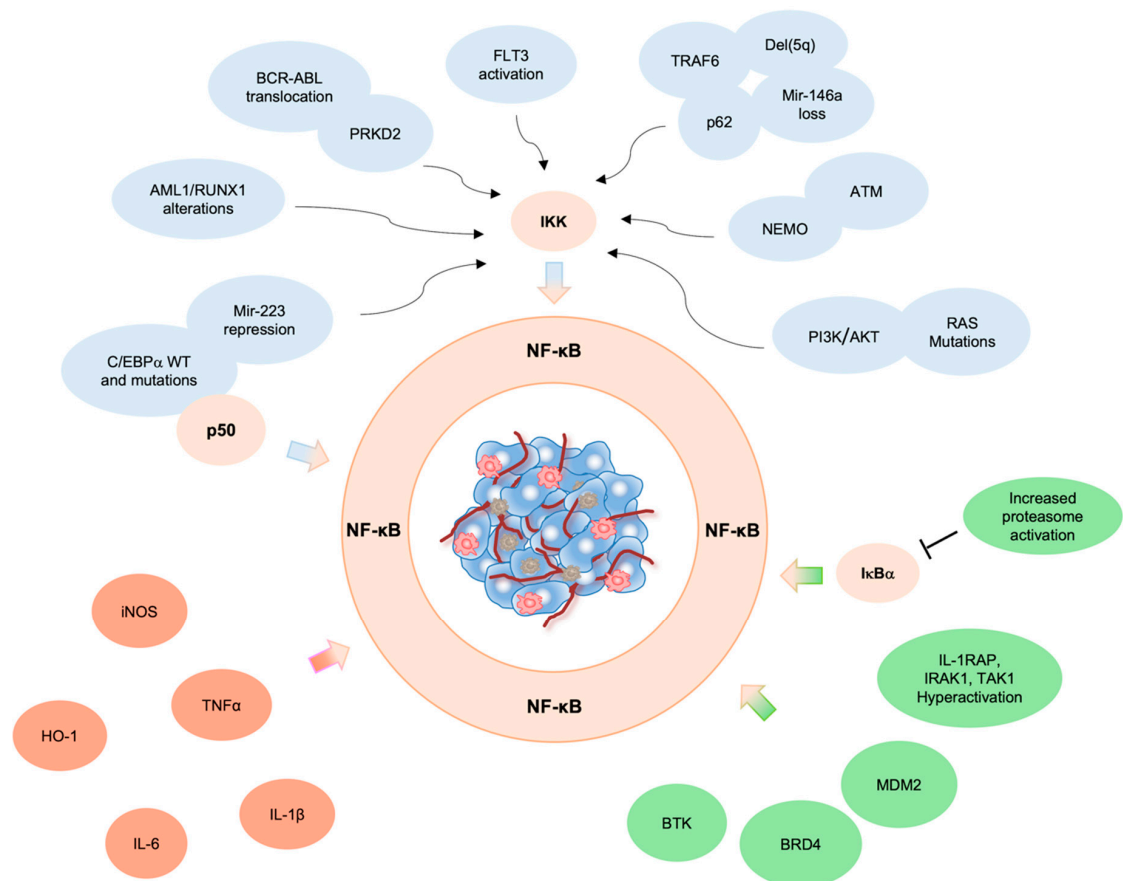


In AML, constitutive NF- κ B activation is a common feature, observed in approximately 40% of de novo cases, and is increasingly recognized as a driver of leukemogenesis, LSC maintenance, and treatment resistance (Di Francesco et al.,

2022). Early studies demonstrated that NF- κ B is persistently active in primary AML samples and is particularly enriched in the CD34⁺CD38⁻ compartment that contains LSCs, whereas normal CD34⁺ stem/progenitor cells display little or no basal NF- κ B activity (Guzman et al., 2001; Bosman et al., 2016).

In AML, NF- κ B activity is sustained by a network of leukemia-associated lesions, autocrine and paracrine signaling, and increased expression of components of the NF- κ B pathway, rather than by recurrent mutations in NF- κ B subunits themselves (Figure 2) (Di Francesco et al., 2022).

Figure 2: Mechanisms underlying constitutive NF- κ B activation in AML. (from Di Francesco et al., 2022)



At the genetic level, several canonical AML drivers converge on NF- κ B. It has been shown that loss-of-function RUNX1 mutations and RUNX1-ETO fusion proteins lose their ability to inhibit the IKK complex, resulting in abnormal activation of the NF- κ B pathway (Nakagawa et al., 2011). Similarly, CEBP α , another key myeloid

transcription factor frequently mutated in AML, physiologically cooperates with NF- κ B p50 to regulate myeloid differentiation genes. Still, both full-length and oncogenic CEBP α variants can bind p50 and activate a subset of NF- κ B target genes, including BCL-2, c-FLIP, and MCL-1, and through positive feedback, p50 induces CEBP α expression, and C/EBP α induces p50 (Paz-Priel et al., 2011). Constitutive activation of NF- κ B also occurs in BCR-ABL rearranged leukemias. The fusion protein interacts with and activates the protein kinase PKD2, which, in turn, activates IKK β (IKK2), leading to I κ B α degradation and nuclear accumulation of RelA (Mihailovic et al., 2004; Carrà et al., 2016). Moreover, overexpression of FLT3 and FLT3-ITD can trigger both the canonical and non-canonical NF- κ B pathways. Specifically, FLT3 may bind IKK2 to induce canonical signaling or enhance TAK1 activation to trigger non-canonical signaling (Bosman et al., 2016; Di Francesco et al., 2022). RAS mutations also activate NF- κ B. Birkenkamp et al. reported an essential association between constitutive NF- κ B activity and persistent Ras signaling in human AML blasts. Indeed, pharmacologic inhibition of Ras reduces NF- κ B activity and restores apoptosis in Ras-dependent cases (Birkenkamp et al., 2004).

Besides genetic lesions, aberrant NF- κ B activation can arise from continuous exposure of leukemic cells to inflammatory signals. AML blasts often establish autocrine feedback loops fueled by TNF- α , IL-1 β , IL-6, and other cytokines that engage TNF and IL-1 receptors or Toll-like receptors, leading to chronic IKK activation (Bosman et al., 2016). Several studies have also demonstrated that the crosstalk between AML cells and the bone marrow microenvironment contributes to disease progression by establishing paracrine signaling that enhances the NF- κ B activity in AML cells (Bosman et al., 2016).

Moreover, heightened proteasome activity, frequently observed in AML, also facilitates continuous I κ B degradation and sustains nuclear translocation of NF- κ B proteins. At the same time, increased expression of upstream regulators (IL-1RAP, IRAK1, TAK1, BTK, IKK subunits) amplifies responsiveness to microenvironmental cues and activates NF- κ B signaling (Bosman et al., 2016; Di Francesco et al., 2022). This aberrant activation of NF- κ B is crucial for promoting the proliferation and survival of AML blasts and LSC. Notably, NF- κ B also plays a direct role in maintaining stem-like properties in AML by upregulating the RNA-binding protein LIN28B. Pharmacologic or genetic inhibition of NF- κ B reduces LIN28B expression, impairs LSC self-renewal, and sensitizes AML cells to apoptosis (Zhou et al., 2018).

Beyond these functions, NF- κ B also contributes to therapy resistance in AML. Constitutive NF- κ B activity maintains high expression of anti-apoptotic protein BCL-2, and stress-response genes such as *HO-1*, thereby protecting leukemic blasts from apoptosis induced by anthracycline- and cytarabine-based chemotherapy (Mehta et al., 2013; Zhe et al., 2015). NF- κ B can further promote drug resistance by upregulating multidrug resistance transporters, including MDR1/P-glycoprotein (Notarbartolo et al., 2002). Moreover, in a recent study, Culver-Cochran et al. showed that NF- κ B-driven expression of the deubiquitinase TNFAIP3/A20 contributes to chemotherapy failure in AML by blocking necroptosis, underscoring the role of NF- κ B in shaping cell-death modalities under treatment (Culver-Cochran et al., 2024).

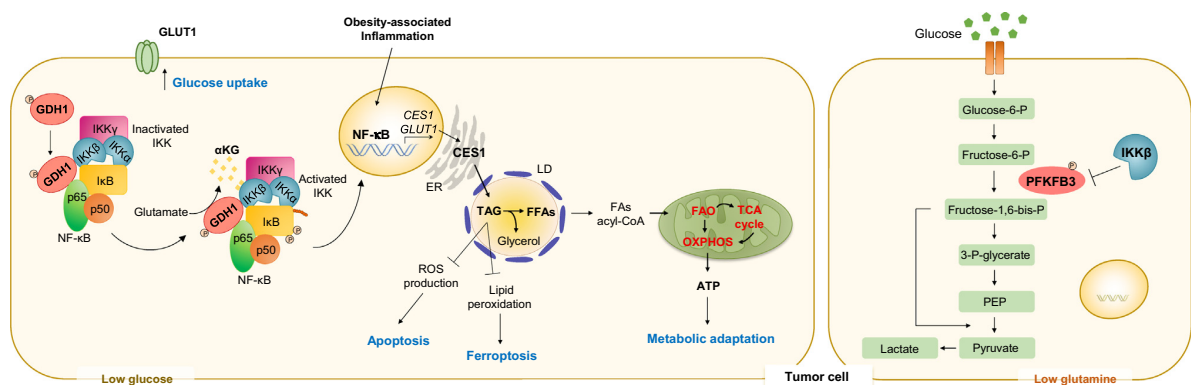
Beyond its canonical role as an inflammatory and survival transcription factor, NF- κ B is now recognized as an important regulator of cellular metabolism in both cancer and immune cells (Figure 1). NF- κ B signaling integrates cues from nutrient availability, reactive oxygen species (ROS), and oncogenic stress to reprogram glycolysis, glutaminolysis, oxidative phosphorylation (OXPHOS), and biosynthetic pathways (Capece et al., 2022; Mao et al., 2025).

Johnson et al. showed that NF- κ B directly promotes the Warburg effect, which is recognized as a hallmark of cancer. Specifically, in p53-deficient tumors, RelA translocates to mitochondria and interacts with mitochondrial transcription factors, suppressing electron transport chain function and enforcing a glycolytic phenotype (Johnson et al., 2011). Additionally, the IKK β /NF- κ B axis can upregulate the glucose transporter GLUT3, enhancing glucose uptake and glycolytic flux. In turn, glycolytic activity can further stimulate IKK/NF- κ B signaling, creating a feedback loop that helps sustain H-Ras-driven oncogenic transformation (Mao et al., 2025).

On the other hand, Mauro et al. demonstrated that NF- κ B can instead regulate the balance between the utilization of glycolysis and mitochondrial respiration for ATP production. Indeed, in cancer, NF- κ B mediates metabolic adaptation and survival under starved conditions by increasing OXPHOS through p53-dependent induction of mitochondrial factors such as SCO2 (synthesis of cytochrome c oxidase 2), a critical assembly factor of the cytochrome c oxidase (COX) (Complex IV) of the mitochondrial ETC. In contrast, RelA inhibition impairs OXPHOS, leading to a metabolic crisis and cell death (Mauro et al., 2011; Capece et al., 2022).

Together, these findings indicate that NF- κ B can sustain either glycolytic or oxidative programs depending on genetic background and nutrient availability. In the last few years, several studies have highlighted the role of the NF- κ B pathway in regulating metabolic programs in response to nutrient-depleted microenvironments (Figure 3) (Capece et al., 2022).

Figure 3: NF- κ B-driven metabolic reprogramming in nutrient-depleted microenvironments (from Capece et al., 2022)



Wang and colleagues showed that, when glucose is scarce, glutamate dehydrogenase 1 (GDH1) is phosphorylated and associates with RelA and IKK β . The α -ketoglutarate (α -KG) generated by GDH1 can bind directly to IKK β , activating it and the downstream NF- κ B signaling. This activation supports tumor cell survival under metabolic stress by increasing glucose uptake through upregulation of GLUT1, ultimately promoting and accelerating glioma development (Wang et al., 2019; Capece et al., 2022).

Another study showed that under glutamine deprivation, IKK β promotes the survival of fibrosarcoma HT1080 cells by phosphorylating and inhibiting 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3 (PFKFB3), which is involved in glycolysis. This mechanism, in turn, reduces glycolysis and lactate production when glutamine levels are low, redirecting glucose-derived carbons to the TCA cycle and the pentose phosphate pathway, thereby decreasing glutamine dependence for the generation of TCA cycle intermediates. Consistently, co-inhibition of IKK β and glutamine metabolism leads to synergistic killing of cancer cells, *in vitro* and *in vivo* (Reid et al., 2016; Capece et al., 2022).

Recently, the role of NF- κ B in coordinating lipid metabolism to adapt to nutrient scarcity has also been demonstrated. Specifically, in colorectal cancer (CRC), NF- κ B/RelA directly drives expression of carboxylesterase 1 (CES1), promoting the mobilization of free fatty acids from lipid droplets to fuel fatty acid oxidation (FAO) and OXPHOS during nutrient deprivation (Capece et al., 2021).

Evidence suggests that NF- κ B coordinates not only survival signals but also metabolic programs under energy stress, even in AML, supporting leukemic cell fitness, LSC maintenance, and resistance to therapy. Fabre et al. showed that NF- κ B inhibition sensitizes AML cells to starvation-induced cell death (Fabre et al., 2007). Importantly, recent studies have begun to connect lipid handling to NF- κ B in AML. Zhang and colleagues sharpened the connection between lipid handling and NF- κ B signaling by showing that CD40 promotes AML survival and drug resistance through non-canonical NF- κ B activation, accompanied by broad lipid metabolic rewiring, which directly affects AML survival and drug response (L. Zhang et al., 2025).

These observations raise the possibility that NF- κ B-driven lipid mobilization contributes to metabolic flexibility also in AML, providing a rationale to investigate lipid-hydrolyzing enzymes such as carboxylesterase 1 (CES1) in this disease.

1.3 Carboxylesterases (CESs)

Mammalian carboxylesterases constitute a multigene family of hydrolytic enzymes that has been highly conserved during evolution. In humans, six carboxylesterase genes have been identified: *CES1*, *CES2*, *CES3*, *CES4A*, *CES5A*, and a *CES1-like pseudogene*, *CES1P1*, all of which are localized on chromosome 16. In mice, 20 carboxylesterase genes have been identified, including one pseudogene, all of which are clustered on chromosome 8 (Lian et al., 2018). Mammalian carboxylesterase genes generally comprise 12-14 exons and encode proteins of about 60 kDa (Lian et al., 2018). Carboxylesterases have been reported to be present in several subcellular compartments. Most mammalian carboxylesterases are soluble proteins residing in the lumen of the endoplasmic reticulum (ER), and some carboxylesterases are secreted from cells. Soluble proteins that reside in the ER lumen are prevented from secretion thanks to the HXEL (e.g., HIEL in CES1) variations of the KDEL consensus ER retrieval sequence at their C-terminal end and

may associate with cytosolic or luminal lipid droplets (LDs). Conversely, enzymes lacking the ER retrieval motif can be released from their membrane-associated state and secreted out of the cell. The CESs are α/β -hydrolases composed of a highly conserved central catalytic domain containing the catalytic triad Ser, Glu, and His (Ser221, Glu354, and His468 in CES1), an adjacent regulatory domain, and an α/β domain that contributes to substrate binding and oligomerization (D. Wang et al., 2018).

1.3.1 Tissue distribution and function

Carboxylesterases are widely distributed across mammalian tissues, with specific isoforms and expression levels varying among tissues. In humans, the main carboxylesterase isoforms are CES1 and CES2. CES1 is highly expressed in the liver and is also expressed in adipose tissue, kidney, lung, heart, and monocytes/macrophages, whereas it is absent in the intestine (X. Wang et al., 2019). CES2 is mainly expressed in the intestine, with a lesser amount in liver, kidney, and heart. Rodent CESs show overlapping but not identical patterns of expression compared to humans. For example, mouse *Ces1d* (formerly known as triacylglycerol hydrolase, TGH, or *Ces3*) and its human ortholog CES1 have similar tissue expression profiles, except in macrophages, where *Ces1d* has little or no expression, whereas CES1 is highly expressed (Lian et al., 2018). Other carboxylesterase subfamily members display more restricted distributions. For instance, *Ces1g* has a more restricted distribution than *Ces1d* and is mainly expressed in the liver and intestine (Lian et al., 2018). The mouse *Ces2* gene family exhibits more specific and abundant expression in the intestine than in other organs (Lian et al., 2018).

Functionally, carboxylesterases are capable of cleaving esters, thioesters, and amide bonds in both xenobiotic and endogenous compounds. CES1 and CES2 have been extensively investigated for their role in drug detoxification and activation of prodrugs. They are responsible for the metabolism of a wide range of common drugs, including antithrombotics, ACE inhibitors, antivirals, anticancer prodrugs, narcotics, antihypertensives, analgesics, and anesthetics (Casey Laizure et al., 2013). They also contribute to the detoxification and metabolic clearance of many ester-containing toxicants, such as pyrethroid insecticides (Ross et al., 2010).

1.3.2 Carboxylesterases' role in lipid metabolism and in metabolic diseases

Beyond their well-known role in xenobiotic metabolism, carboxylesterases have been recognized for their contribution to lipid metabolism and systemic energy homeostasis. Thus, CES catalyze the hydrolysis of endogenous lipids, including triacylglycerols (TG), diacylglycerols (DG), cholesteryl esters (CE), retinyl esters (RE), and endocannabinoids (Lian et al., 2018). Over the years, several studies have focused on the lipase activity of carboxylesterases, revealing their key role in regulating lipid turnover in both physiological and pathological processes. It has been reported that carboxylesterases regulate the mobilization of neutral lipids, as TGs and CEs, from pre-existing storage pools and thereby influence LDs dynamics and lipoprotein production (Lian et al., 2018). LDs, which originate from the ER (Walther & Farese, 2009), are dynamic organelles that play crucial roles in various cellular processes, including lipid metabolism, energy storage, and cell signaling (Z. Zhang et al., 2024). The LDs are composed of a core of neutral lipids, mainly TGs and CEs, covered by a monolayer of phospholipids and LD-associated proteins. They are present in nearly all cells and tissues, but adipose tissue and liver represent the primary sites of LD accumulation, consistent with their central roles in lipid synthesis and storage (Lian et al., 2018).

Ces1d has been reported as the first ER luminal lipase capable of modulating the morphology and dynamics of cytosolic LDs (CLDs). Specifically, Wang et al. demonstrated that isolated hepatocytes from fasted TGH-deficient mice had decreased size but increased LD numbers. Ces1 ablation did not affect nascent-LD formation but altered the transfer of newly synthesized lipids into preformed LDs (H. Wang et al., 2010).

Ces1d/CES1 is also involved in the hydrolysis of preformed TG from luminal lipid droplets (LLDs) to provide substrates for very low-density lipoprotein (VLDL) assembly and secretion in hepatocytes, through a process of hydrolysis/re-esterification cycle (H. Wang et al., 2007). Multiple studies confirmed the role of Ces1d in this process, both in vitro and in vivo. Specifically, Ces1d overexpression enhances TG lipolysis and VLDL secretion, whereas Ces1d inhibition decreases VLDL secretion and lowers circulating TG, free cholesterol, and CE levels (Gilham et al., 2003; Wei et al., 2007). Interestingly, the loss of Ces1d doesn't lead to hepatic steatosis. Ces1d knockout mice exhibit decreased plasma lipids, decreased fatty acid flux from the adipose tissue to the liver, increased hepatic fatty acid oxidation

(FAO), and improved insulin sensitivity and glucose tolerance (Wei et al., 2010; Lian et al., 2012), highlighting that CES1-family lipases not only govern lipoprotein output but also influence whole-body fuel utilization.

The role of CES enzymes has direct implications in the development of metabolic disorders, including non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), dyslipidemia, atherosclerosis, and type 2 diabetes. Indeed, several studies investigated their role in metabolic diseases. NAFLD is a common cause of liver injury that goes through different stages, including steatosis, inflammation, fibrosis, and cirrhosis. Lian et al. demonstrated that inhibition of *Ces1d* has a protective effect in high-fat diet (HFD) mouse models (Lian et al., 2016).

The contribution of CES1/*Ces1d* to lipid metabolism is not restricted to the liver. In adipose tissue, *Ces1d* expression increases during adipocyte differentiation, and the enzyme localizes to LDs, where it helps mobilize neutral lipids. Adipose tissue-specific *Ces1d* knockout mice fed a high-fat diet gain more weight, store excess fat, and develop impaired glucose and lipid metabolism along with worsened hepatic steatosis (Li et al., 2022). Mechanistically, *Ces1d* deficiency in adipocytes leads to abnormally large LDs and decreases the ability of adipose tissue to handle lipid flux, resulting in ectopic TG deposition in the liver and other peripheral tissues (Li et al., 2022). Accordingly, human adipose CES1 expression correlates positively with clinical parameters of adiposity and circulating triglyceride levels (Marrades et al., 2010; Nagashima et al., 2011).

It has also been proposed that CES1 has a protective role in atherosclerosis. It catalyzes the hydrolysis of CE stored in macrophage CLDs and, in the liver, hydrolyzes high-density lipoprotein (HDL)-CE, and promotes cholesterol excretion and reverse cholesterol transport (Lian et al., 2018). While esterification of cholesterol into CE provides a protective mechanism against free cholesterol-induced cytotoxicity, excessive CE accumulation can lead to foam cell formation, a hallmark of early atherogenesis. CES1-mediated hydrolysis of CE is thus considered the rate-limiting step in cholesterol efflux. Zhao et al. found that CES1 overexpression in cultured THP1 macrophages increases the mobilization of CE stores. Furthermore, specific CES1 expression in the macrophages of *Ldlr*^{-/-} mice was found to reduce the atherosclerotic lesion area and the necrotic core formation, and to increase free cholesterol efflux (Zhao et al. 2005; Zhao et al. 2007). Consistent with these findings and supporting a global atheroprotective role of

CES1, Bie et al. demonstrated that liver-specific deletion of *Ces1* in *Ldlr*^{-/-} mice decreases cholesterol elimination from the body and exacerbates atherosclerosis (Bie et al., 2013). However, in a more recent study, Lian et al. demonstrated that *Ces1d* does not significantly contribute to hepatic CE hydrolysis, refining earlier models and emphasizing that its primary role in the liver is in TG metabolism and VLDL-related lipid flux rather than cholesterol ester catabolism (Lian et al., 2021). At the same time, other members of the CES family were studied for their role in lipid homeostasis. For example, deletion of *Ces1g* in mice resulted in obesity, steatosis, and hyperlipidemia due to increased de novo lipogenesis and lipoprotein secretion (Quiroga et al., 2012). Mechanistically, *Ces1g* deficiency impairs the release of polyunsaturated fatty acids (PUFA) from TG stores, leading to sustained activation of SREBP1c and exacerbated lipogenesis, whereas dietary ω -3 fatty acids can rescue this phenotype (Quiroga et al., 2012). Similarly, *Ces2* and human CES2 display TG and DG hydrolase activity. Decreased hepatic *Ces2c*/CES2 activity correlates with obesity, insulin resistance, and hepatic steatosis, and CES2 overexpression in mouse liver ameliorates steatosis and inflammation (Li et al., 2016; Ruby et al., 2017).

These findings highlight CES1-family enzymes as essential regulators of systemic lipid distribution and metabolic flexibility and underscore that the potential of targeting CESs in metabolic liver disease is context- and isoform-dependent.

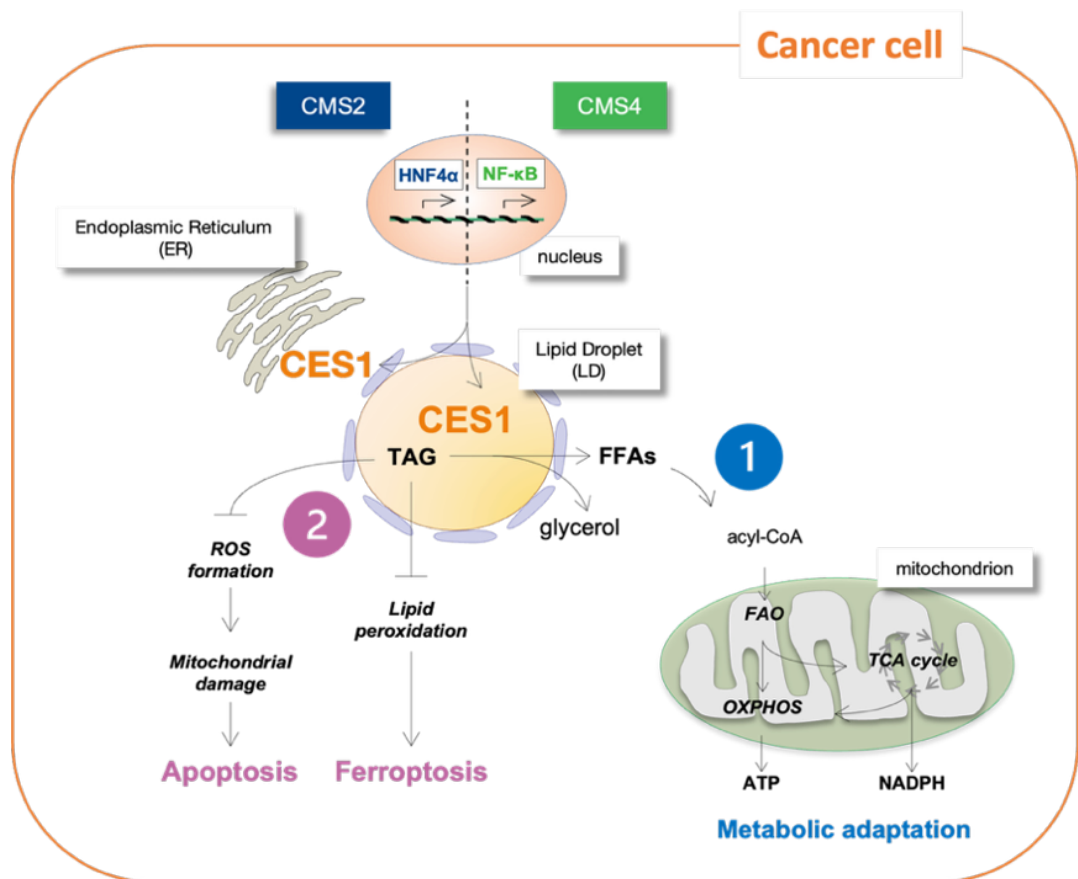
1.3.3 Carboxylesterase 1 as a driver of cancer progression

It is widely accepted that lipid disorders and lipid metabolism reprogramming are associated with cancer development and progression. Indeed, different enzymes involved in fatty acid metabolism are emerging as cancer biomarkers (Nagaoka et al., 2024). Recent research has identified CES1 as an enzyme important in cancer and has expanded our understanding of the role of human CES1 in cancer development.

The role of CES1 in cancer progression was first described in colorectal carcinoma (CRC) by Capece et al., who identified CES1 as an NF- κ B-regulated triacylglycerol lipase that links obesity-associated inflammation to metabolic adaptation in aggressive CRC (Capece et al., 2021). NF- κ B-driven induction of CES1 increased TG hydrolysis in lipid droplets, fueling mitochondrial FAO and preventing the toxic build-up of neutral lipids, enabling tumor cells to withstand nutrient limitation. CES1-

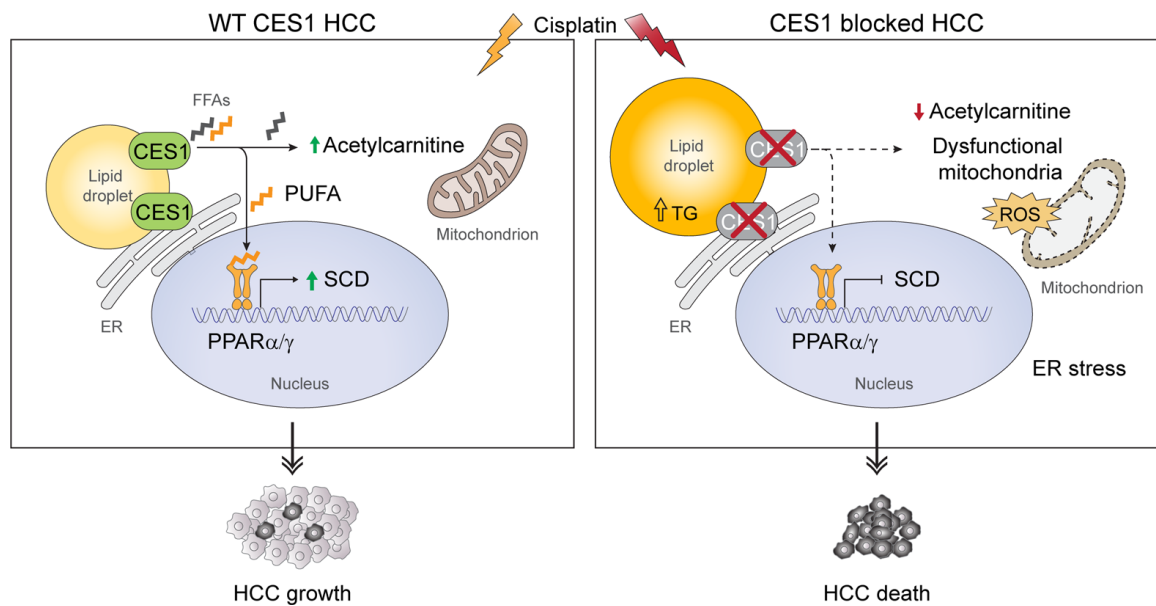
high tumors were enriched in obese patients, associated with advanced stage and shorter survival, and genetic or pharmacologic suppression of CES1 impaired FAO, increased lipid droplet accumulation, and reduced tumor growth *in vitro* and *in vivo* (Capece et al., 2021) (Figure 4).

Figure 4: CES1 function in CRC



In hepatocellular carcinoma (HCC) cells, Li et al. demonstrated that genetic deletion or pharmacological inhibition of CES1 alters LD morphology, decreases total TG and free fatty acid pools, impairs mitochondrial β -oxidation, and reduces levels of polyunsaturated fatty acids (PUFAs) that serve as ligands for PPAR α/γ (G. Li et al., 2023). These changes lead to decreased expression of stearoyl-CoA desaturase (SCD), increased oxidative stress, and heightened sensitivity to chemotherapeutic agents, such as cisplatin, indicating that CES1-generated fatty acids fuel both mitochondrial energy production and nuclear receptor-dependent transcriptional programs that support cancer cell survival (G. Li et al., 2023) (Figure 5).

Figure 5: CES1 function in HCC (from Li et al., 2023)



More recently, an integrated transcriptomic and clinical analysis in AML identified CES1 as a hub lipid metabolism-related gene associated with central nervous system (CNS) relapse (Bao et al., 2025). Hypercholesterolemia and elevated CES1 expression were independently associated with increased risk of CNS relapse and shorter overall survival. Functional experiments indicated that CES1 modulates the composition of tumor-infiltrating immune cells, particularly by promoting M2-like macrophage polarization, thereby shaping the leukemic microenvironment to facilitate CNS infiltration (Bao et al., 2025).

Collectively, these findings highlight CES1 as a lipolytic enzyme, originally studied in xenobiotic metabolism, which cancer cells can repurpose to enhance metabolic flexibility under stress.

2. AIM OF THE PROJECT

Despite significant advances in risk stratification and in the development of targeted therapies, long-term prognosis for most patients with AML remains unfavorable and disease relapse represents the leading cause of treatment failure in AML. A major barrier to durable responses is the profound clonal heterogeneity of AML, which limits the effectiveness of therapeutic strategies (Dembitz et al., 2025), suggesting the need to identify vulnerabilities which can be therapeutically targeted alone or in combination with existing therapies.

In this context, a growing body of evidence has highlighted metabolic reprogramming as a key hallmark of AML biology (Mishra et al., 2023). Notably, AML cells can persist under hostile microenvironmental conditions by rewiring bioenergetic and lipid metabolic pathways, thereby supporting survival and, potentially, resistance to therapy (Dembitz et al., 2025).

Constitutive NF- κ B activity is a common feature of AML and contributes to sustain survival signaling and therapy resistance (Di Francesco et al., 2022). However, how NF- κ B-driven biological programs could connect lipid mobilization and mitochondrial metabolism in AML remains insufficiently defined.

Since CES1 has been identified as an NF- κ B-regulated lipase driving metabolic adaptation of CRC cells (Capece et al., 2021), and recent evidence has demonstrated the association between CES1 expression with dyslipidemia and CNS relapse in AML (Bao et al., 2025), this study aimed to investigate the role of CES1 as a potential mediator of metabolic adaptation and druggable target in AML.

3. MATERIALS AND METHODS

3.1 Cell lines culture and treatment

Human AML cell lines THP1, CESS, and K562 were cultured in RPMI-1640 (Roswell Park Memorial Institute) medium enriched with 10% FBS (fetal bovine serum). OCI-AML3 cell line was cultured in alpha-MEM (Minimum Essential Medium) with 20% FBS. AML-193 cell line was cultured in IMDM (Iscove's Modified Dulbecco's Medium) with 20% FBS, 5ng/mL of GM-CSF (Gibco), and 0,005mg/mL of ITS (Insulin-Transferrin-Selenium) (Gibco). The HEK-293T cell line was cultured in high-glucose DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS. All media were supplemented with 50 U/mL Penicillin-Streptomycin (Euroclone) and 2 mM L-glutamine (Euroclone).

Cells were cultured in a humidified incubator in 5% CO₂ at 37°C. All cell lines were periodically tested for mycoplasma contamination by PCR assay using the Mycoplasma Detection Kit (Applied Biological Materials, abm).

For experiments under energy stress (ES), THP1, CESS, and AML193 were cultured in RPMI without glucose, supplemented with 2% FBS, antibiotics, 2 mM L-glutamine, and 0,5 mM D-glucose (Sigma-Aldrich); OCI-AML3 was cultured in RPMI without glucose, supplemented with 2% FBS, antibiotics, 2 mM L-glutamine, and 1 mM D-glucose; K562 was cultured in RPMI without glucose, supplemented with 2% FBS, antibiotics, 5 mM L-glutamine, and 1 mM D-glucose.

The following reagents were used for cell treatments: GR-148672X (10 µM) from Chemgood LLC, Tween-80 (2%) from Sigma-Aldrich, Venetoclax (ABT-199) (0.01-0.5 µM) from Selleckchem, and DMSO from Sigma-Aldrich.

3.2 Cell viability assay

Cell viability assays were performed using the CellTiter-Glo® 3D Cell Viability Assay reagent (Promega) according to the manufacturer's protocol. Cells were counted and plated in 96-well plates in 100 µL of culture medium. At the endpoint, cells were lysed with CellTiter-Glo® 3D reagent, and luminescence was measured using a TECAN Infinite® 200 PRO microplate reader (Tecan).

3.3 Immunoblotting

Cell samples were lysed in Lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) supplemented with Complete mini EDTA-free protease inhibitors (Roche Molecular Biochemicals), 1 mM Na₃VO₄, 50 mM NaF. Protein concentrations were quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Denatured proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Primary antibodies used were: CES1 (ab45957 Abcam), cleaved caspase-3 (Asp175) (9661 Cell Signaling), PARP (9542 Cell Signaling), GPX4 (ab125066 Abcam), β -actin (4967 Cell Signaling), and α -Actinin (3134 Cell Signaling). Western blot detection was performed using ECL (Thermo Fisher Scientific). β -actin and α -actinin were used as loading controls in the total cell lysates.

3.4 RT-PCR and qRT-PCR

Total RNA was extracted using the Quick-RNA MiniPrep kit (Zymo Research) according to the manufacturer's specifications. The isolated RNA was spectrophotometrically quantified, and equal amounts were used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). qRT-PCRs were carried out using TaqMan Universal Master Mix and an ABI 7500 Fast Real-time PCR system (Life Technologies) with the following predesigned TaqMan® Gene Expression Assays: *Actin* Human Hs01060665_g1 FAM and *CES1* Human Hs00275607_m1 FAM. Experimental Ct values were normalized to *β -actin*, and the relative mRNA expression was then calculated using a reference sample.

3.5 ATP production analyses

For bioenergetic profiling, AML cells were seeded onto Cell-Tak (22,4 μ g/mL; Corning) coated XFe96 cell culture microplates, in XF Assay Medium (Agilent Seahorse) containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. ATP production rates were measured using the XF Real-Time ATP Rate Assay Kit (Agilent Seahorse) following the injection of GR-148672X, where indicated, using an XF-96 Extracellular Flux Analyzer (Agilent Seahorse). The detected

mitochondrial OCR and glycolytic Extra-Cellular Acidification Rates (ECAR) were then transformed into mitochondrial (mito-ATP) and glycolytic (glyco-ATP) ATP production rates using validated algorithms in the Agilent Seahorse software and expressed as percentages. The measurements were performed following injection of 10 μ M GR-148672X or vehicle (DMSO) in combination with 50 mM 2-deoxy-D-glucose (Sigma Aldrich), where indicated; 1,5 μ M oligomycin A (Sigma Aldrich); 0,5 μ M rotenone (Sigma Aldrich); and 0.5 μ M antimycin A (Sigma Aldrich).

3.6 Lentiviral transduction

High-titre lentivirus production in HEK-293T cells was carried out using Lipofectamine 3000 Transfection Reagent (Thermo Fisher) according to the manufacturer's specifications. The DNA sequences of the shRNAs used for targeting the human *CES1* gene and the *non-specific (ns)* control shc003v in the pLKO.1 lentiviral vector were purchased from Sigma-Aldrich. The sequences were previously described and validated for specificity. Target cell lines (THP1 and AML-193) were transduced with lentiviruses in the presence of 0.8 μ g/ml polybrene, followed by spinfection to increase transduction efficiency.

3.7 Xenograft model

Mice were housed in the animal facilities at the University of L'Aquila in accordance with national and international laws and regulations. For the xenograft model of AML, 6-week-old immunocompromised mice were subcutaneously injected with 1×10^7 THP1 cells in 200 μ L of sterile PBS (1x) and Matrigel (1:1; Corning), using a 1-mL Luer-Lok™ Tip syringe (BD Biosciences) with a 25-gauge needle. When tumors reached 100 mm³, mice were randomized into treatment groups and then treated intraperitoneally (i.p.) with GR-148672X (50 mg/kg of body weight; n=6) or vehicle (n=6), twice a day for three weeks. For in vivo administration, GR-148672X was dissolved in DMSO at a final concentration of 100 mM and then diluted in 2% Tween 80/saline solution to a stock concentration of 5 mg/mL. Tumor volumes (mm³) were measured using a digital caliper and the following formula: volume = $A \times B^2/2$. At the experimental endpoint, mice were humanely euthanized in accordance with institutional animal care guidelines, and all tumors were weighed and photographed.

3.8 Synergy Analysis

The combinatorial index (CI) was calculated using SynergyFinder 2.0 based on the Bliss model. Bliss synergy scores denote antagonistic (< -10), additive (from -10 to 10), and synergistic (> 10) responses.

3.9 Statistical analyses

The data were shown as mean $\pm$ standard deviation (SD) or Standard Error of the Mean (SEM), as indicated in the figure legends. Two-tailed Student's t-test was used to compare the difference between two groups. Results were considered statistically significant if the p-value was < 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. RESULTS

4.1 Nutrient deprivation induces a metabolic switch in AML cells to OXPHOS and upregulates CES1 expression

AML cells display the capability to metabolically adapt in response to environmental cues (Kreitz et al., 2019). Among the pathways contributing to this metabolic flexibility, lipid metabolism has emerged as a key determinant (Dembitz et al., 2025). Moreover, CES1 has been described in CRC as a lipase that supports the metabolic adaptation of cancer cells to nutrient-depleted conditions (Capece et al., 2021).

To investigate the role of CES1 in AML, we tested a panel of cell lines showing distinct genetic and phenotypic features: THP1, OCI-AML3, AML-193, CESS, and K562.

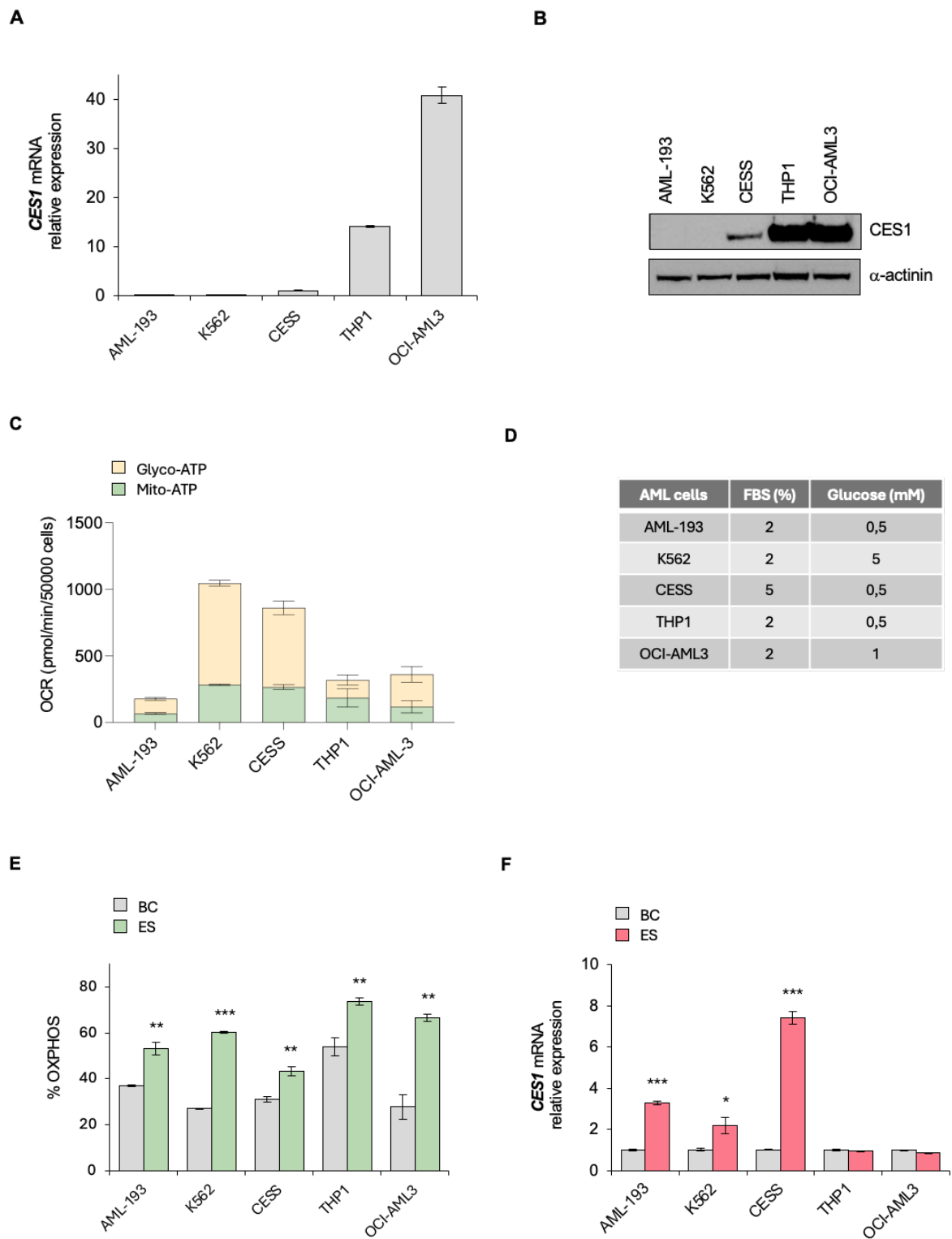
We first measured CES1 levels under basal culture conditions (BC), and we found that CES1 expression was highly heterogeneous across the myeloid cell lines analyzed (Figure 1A-1B), where OCI-AML3 and THP1 exhibited the highest levels of CES1 (Figure 1A-1B).

Then, assessing the ATP production rate of these cell lines, we found that all of them displayed a glycolytic phenotype when cultured under BC (Figure 1C). This indicates that, despite differences in CES1 expression, these cells primarily rely on glycolysis for energy production.

To induce energy stress (ES) and evaluate metabolic adaptation, each cell line was cultured for 72 hours under nutrient-depleted conditions by reducing fetal bovine serum (FBS) and glucose concentrations in the culture medium (Figure 1D). Under these conditions, we observed a metabolic shift from glycolysis to OXPHOS (Figure 1E).

To better understand how nutrient deprivation affects CES1 expression, we analyzed CES1 levels under BC and ES. After 72 hours of culture under ES, we observed that CES1 expression was significantly upregulated in AML-193, K562, and CESS compared to BC, as demonstrated by both mRNA and protein levels (Figure 1F-1G). In contrast, THP1 and OCI-AML3, which display high basal CES1 expression, did not show appreciable changes at the transcript level and only a slight increase in protein levels after 72 hours of ES (Figure 1F-1G). Overall, these data indicate that the ES-induced metabolic shift from a glycolytic to a more

oxidative phenotype is associated with the increasing expression of CES1, suggesting that, as in CRC, CES1 is induced in response to nutrient deprivation and could be a potential mediator of metabolic adaptation in AML.



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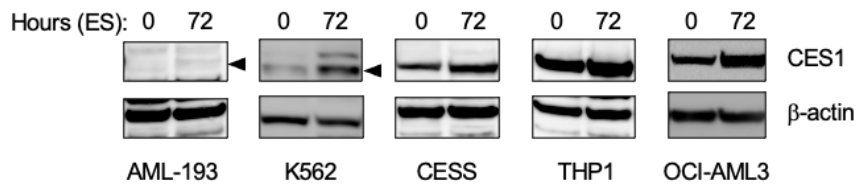


Figure 1: CES1 is induced during ES and promotes a metabolic switch toward OXPHOS in AML cells

A) qRT-PCR analysis showing the relative *CES1* mRNA levels in the indicated AML cell lines under BC. **B)** Western blot showing CES1 protein levels in the indicated AML cell lines under BC. Actinin is shown as a loading control. **C)** ATP production rate of AML cells cultured under BC, measured by Seahorse XFe96 Extracellular Flux Analyzer, showing the amount of mito-ATP and glyco-ATP. **D)** Table showing the selected culture conditions used to induce energetic stress (ES), reporting FBS (%) and glucose (mM) for each cell line. **E)** Percentage of OXPHOS after 72 hours of BC and ES in the panel of AML cells. **F)** qRT-PCR showing *CES1* mRNA levels at 0 hrs and 72 hrs under ES in the indicated AML cell lines. **G)** Western blots showing the CES1 protein levels under ES as in F. β-actin was used as a loading control. **C, E)** Data are shown as mean ± SEM. **A, F)** Values are shown as mean ± SD. * $p < 0,05$; *** $p < 0,001$.

4.2 CES1 inhibition impairs OXPHOS and AML cell survival under ES

To determine whether CES1 activity is functionally required for metabolic adaptation and survival of AML cells under ES, we pharmacologically and genetically inhibited CES1 in the panel of AML cell lines.

We first assessed the effect of the CES1 inhibitor GR-148672X on mitochondrial ATP production after 72 hours of ES. In all AML cell lines, GR-148672X reduced the percentage of OXPHOS used by the cells to adapt (Figure 2A), as measured by the Seahorse ATP Rate Assay. This result indicates that CES1 activity sustains oxidative metabolism when the cells survive under ES.

Accordingly, the treatment with GR-148672X caused a substantial decrease in cell survival across all AML cell lines compared to vehicle under ES (Figure 2B). In contrast, we didn't find a significant inhibitory effect on cell survival in BC (Figure 2C), suggesting that CES1 is particularly critical for AML cell viability in the context of ES.

To further confirm the role of CES1 in AML cell survival upon ES, we performed a genetic silencing experiment in AML-193 and THP1 cells using a specific CES1 targeting hairpin (*CES1* shRNA). As expected, knockdown of CES1 significantly reduced AML cell survival in ES (Figure 2D), underlining that AML cell lines depended on CES1 expression for survival under ES. The successful of CES1 knockdown was confirmed by Real Time PCR (Figure 2E).

It is known that the accumulation of ROS induces both ferroptosis and apoptosis in *Ces1d*-deficient CT-26 cells during glucose limitation (Capece et al., 2021). To explore the mechanisms by which CES1 inhibition triggers AML cell death during ES, we analyzed markers of apoptosis and ferroptosis in two selected AML cell lines, THP1 and AML-193, with high and low CES1 expression in BC, respectively. As shown in Figure 2F-G, the GR-148672X treatment in THP1 cells led to the accumulation of cleaved caspase-3 in ES compared to BC (Figure 2F). Conversely, the pharmacological inhibition of CES1 in AML-193 cells induced a reduction of glutathione peroxidase 4 (GPX4) protein levels, a hallmark of ferroptosis, in ES (Figure 2G).

Together, these data demonstrate that CES1 represents a key player during the metabolic adaptation in AML.

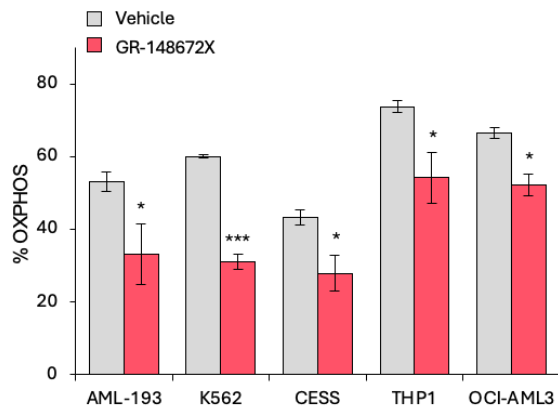
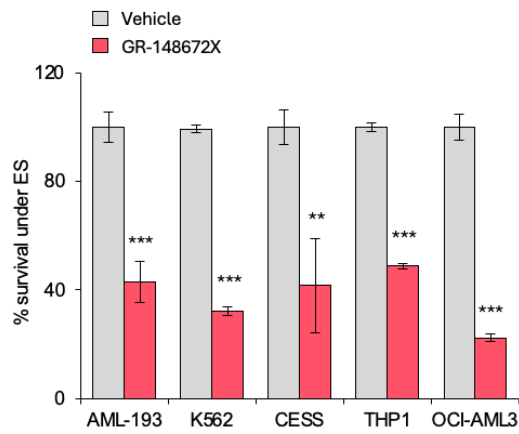
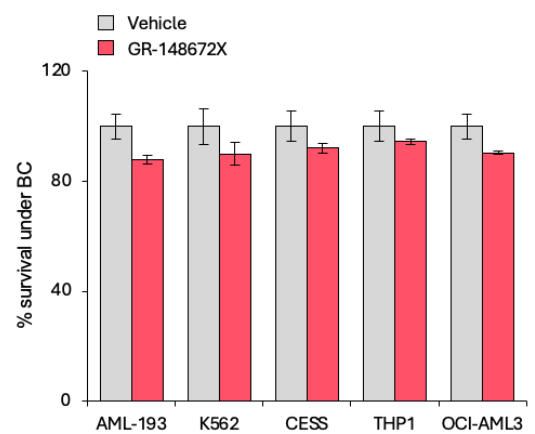
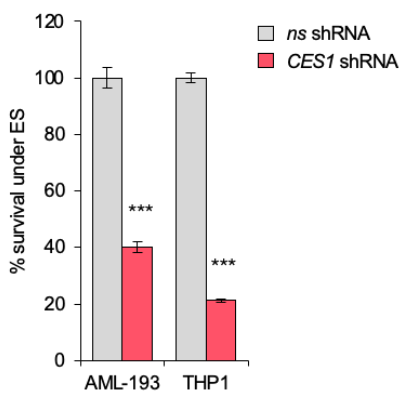
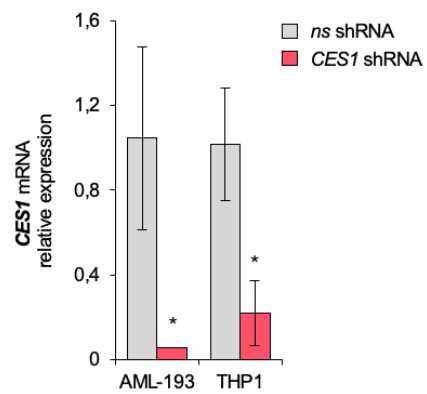
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Figure 2: CES1 mediates metabolic adaptation in AML cell lines

A) Percentage of OXPHOS used by AML cells treated with vehicle or GR-148672X after 72 hrs of BC and ES. **B)** CellTiter-Glo® viability assay showing the percentage of AML cell survival after 72 hrs of ES treated with vehicle or GR-148672X. **C)** CellTiter-Glo® viability assay showing the percentage of AML cell survival after 72 hrs of BC treated with vehicle or GR-148672X. **D)** CellTiter-Glo® viability assay showing the percentage of AML cell survival after 72 hrs of ES following lentiviral infection of *non-specific* (*ns*) and *CES1-specific* (*CES1*) shRNAs. **E)** qRT-PCR showing the *CES1* mRNA expression levels following lentiviral infection as in D. **F)** Western blot analysis showing cleaved caspase-3 in THP1 cells cultured under BC or ES after 72 hrs of treatment with vehicle or GR-148672X; β-actin was used as loading control. **G)** Western blot analysis showing GPX4 in AML-193 cells cultured under ES after treatment with vehicle or GR-148672X in the indicated time points; β-actin was used as loading control. **B-E)** Values are shown as mean ± SD. **A)** Values are shown as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.3 CES1 inhibition reduces tumor growth in vivo

Given the promising results obtained following CES1 inhibition in AML cells in vitro, we performed an AML xenograft model using the human cell line THP1 to evaluate the role of CES1 in mediating metabolic adaptation in vivo. We observed a reduction of tumor growth in mice treated with GR-148672X (Figure 3A-C). This result is consistent with a previous study conducted in CRC.

As shown in Figure A, tumors in the vehicle-treated group showed a progressive and pronounced increase in volume, whereas tumor growth was slower in GR-148672X-treated mice (Figure 3A). Consistently, the reduced tumor volume observed in treated mice was also confirmed ex vivo (Figure 3B-C), suggesting that CES1 inhibition limits leukemic mass expansion in vivo.

Overall, these findings indicate that treatment with the CES1 inhibitor exerts an antitumor effect in this murine AML model.

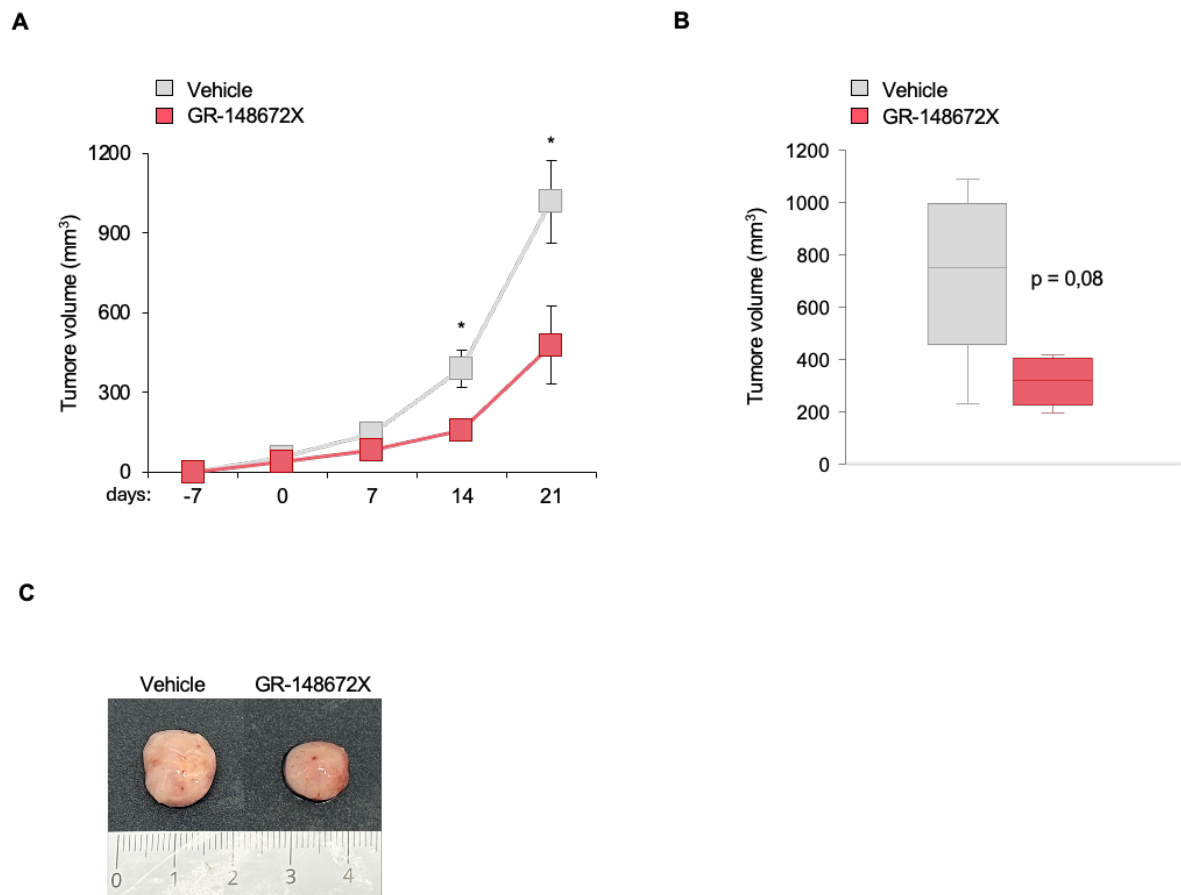


Figure 3: Pharmacologic CES1 inhibition impairs AML tumorigenesis in vivo

A) Volumes of subcutaneous THP1 AML xenograft in mice treated i.p. with vehicle or GR-148672X at the indicated time points. **B)** Boxplot showing the volumes of subcutaneous THP1 AML tumors from A measured ex vivo at day 21. **C)** Images of representative tumors isolated from mice at day 21. **A-B)** Values are shown as mean $\pm$ SEM. * $p < 0.05$.

4.4 The combination of GR-148672X and venetoclax exerts synergistic cytotoxicity in specific AML cell lines

The antileukemic activity of the BCL-2 inhibitor, venetoclax, in AML has been closely linked to its ability to disrupt mitochondrial respiration and selectively target leukemic stem cells (LSC), particularly when combined with hypomethylating agents such as azacitidine (Pollyea et al., 2018). Clinical and preclinical studies have shown that venetoclax-based regimens are most effective in AML subsets that are highly dependent on OXPHOS, whereas resistance is frequently associated with metabolic adaptations, including increased oxidative metabolism, fatty-acid oxidation, and rewiring of lipid handling pathways (Stevens et al., 2020). In several models, the combination of venetoclax with agents that impair mitochondrial function or fatty-acid utilization restores sensitivity and produces synergistic cytotoxicity (Stevens et al., 2020; Kawaguchi et al., 2025). Given the role of CES1 in FAO-dependent adaptation under energy stress (Capece et al., 2021), we hypothesized that pharmacological CES1 inhibition might modulate the response of AML cells to venetoclax by limiting their metabolic plasticity.

Notably, treatment with GR-148672X alone reduced cell viability of approximately 50-60% in AML cell lines during ES, indicating substantial but incomplete cytotoxicity (Figure 2B). The residual viability may reflect the persistence of therapy-tolerant subpopulations that could drive disease regrowth. This provided a rationale to explore whether the combination of venetoclax with GR-148672X could further reduce cell survival, enhance the efficacy of venetoclax, and potentially overcome resistance mechanisms in distinct leukemic subsets.

We performed dose-response experiments by treating the AML cells with increasing concentrations of venetoclax in the absence or presence of a fixed concentration of GR-148672X under ES and measuring cell viability after 72 hours.

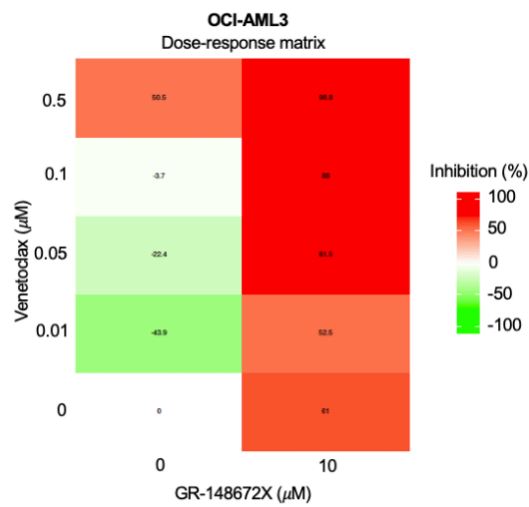
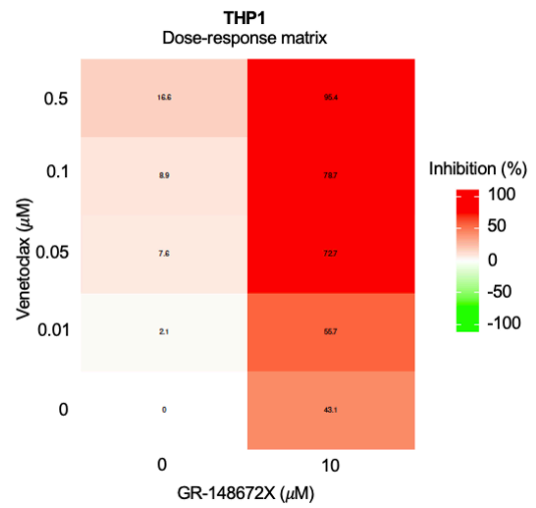
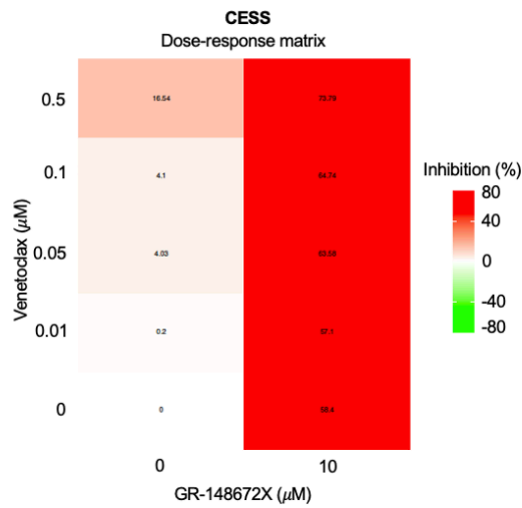
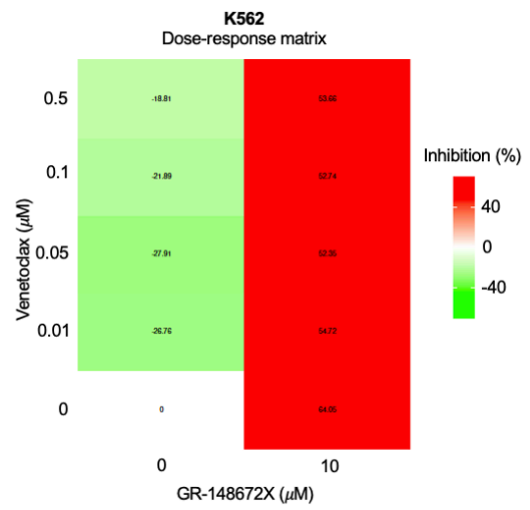
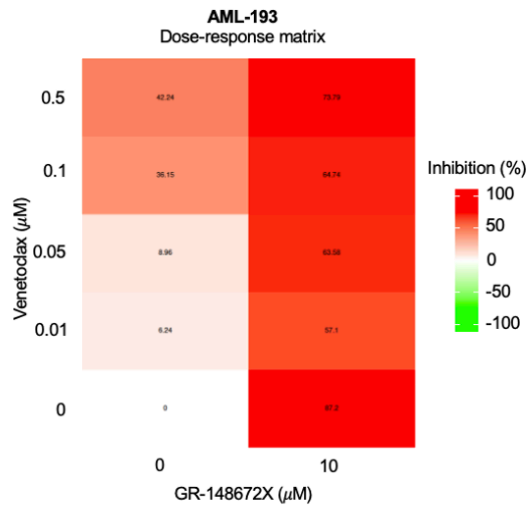
The resulting heatmaps show the percentage of growth inhibition relative to untreated controls (Figure 4A). As shown in Figure 4A, the treatment with venetoclax alone inhibits the proliferation of AML cell lines in a concentration- and cell-type-dependent manner. Specifically, in AML-193, venetoclax alone induced a reduction in cell viability, particularly at the highest doses. In contrast, K562, CESS, THP1, and OCI-AML3 seem to be resistant to venetoclax as monotherapy (Figure 4A). The

combination of GR-148672X and venetoclax decreased cell viability in all cell lines except for AML-193 (Figure 4A).

To quantitatively assess drug interaction, we calculated Bliss synergy scores (Figure 4B-C). Specifically, AML-193 showed a negative synergy score, with a mean Bliss score of -25.39, indicating an overall antagonistic interaction between GR-148672X and venetoclax. In K562 and CESS, mean synergy scores were close to zero (-2.11 and 3.82, respectively), consistent with predominantly additive effects of the two drugs. Interestingly, THP1 and OCI-AML3 displayed synergistic interactions, with mean Bliss scores of 27.52 and 21.1, respectively, indicating that CES1 inhibition potentiates venetoclax-induced cytotoxicity.

Overall, this heterogeneity suggests that the benefit of combining GR-148672X with venetoclax is likely influenced by the underlying molecular mechanisms of each AML cell line. The integration of genetic and metabolic profiling could be interesting for deeper clarify the therapeutic effects of this combination.

A



B

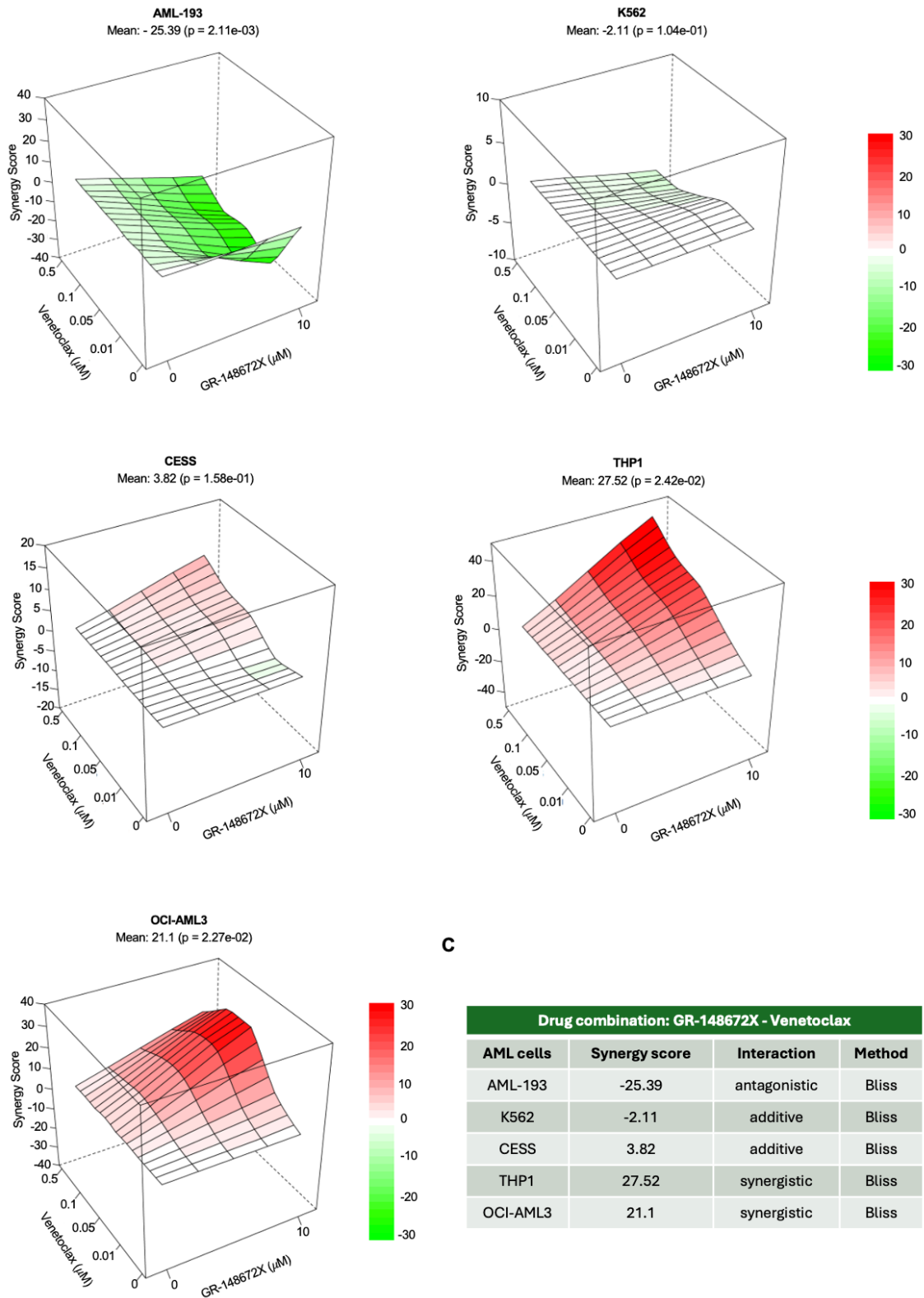


Figure 4: Cytotoxic effect of GR-148672X and venetoclax on THP1 and OCI-AML3 cell lines

A) Heatmaps showing the dose-response matrix (% inhibition) for venetoclax and GR-148672X in the AML cells after 72 hrs of treatment at the indicated concentrations. **B)** 3D synergy surface plot for AML cells treated as in A. **C)** Table showing the synergy score of venetoclax and GR-148672X for the experiment in A. **A-B)** Values are shown as mean $\pm$ SD.

5. DISCUSSION

In this study, we tested the hypothesis that AML cells are able to metabolically adapt to nutrient deprivation, and that CES1 constitutes a key node within this adaptive program. We found that AML cells exhibit metabolic plasticity by switching to OXPHOS and increasing CES1 expression when cultured under ES, indicating that CES1 can act as either a stress-responsive effector or an active metabolic vulnerability, depending on the AML cellular context.

Accordingly, CES1 pharmacological inhibition impaired the capability of AML cells to adapt in ES by reducing the OXPHOS-derived ATP production rate and inducing cell death. As expected, genetic silencing of *CES1* impaired cell survival, reinforcing the idea that CES1 is a mediator of metabolic adaptation of AML cells under ES. In vivo evidence revealed that the inhibition of CES1 reduces tumor growth, suggesting the potential antitumor effect of GR-148672X. Further experiments using AML-relevant systemic models, such as disseminated xenografts or patient-derived systems, which better mimic marrow nutrient distribution, adipocyte influences, and treatment pressures, are needed to better clarify the effects of CES1 inhibition on AML cancer cells and in other cell types, such as LSC.

These findings are in accordance with previous research that identifies CES1 as a stress-induced lipase that supports survival by enabling triacylglycerol catabolism to fuel fatty-acid oxidation (FAO) and sustain oxidative metabolism, while preventing toxic lipid accumulation in CRC (Capece et al., 2021).

It is known that anti-BCL2 strategies increase leukemic cell death, disrupt mitochondrial metabolism, and also act as immunometabolic modulators of NK function in AML (Lagadinou et al., 2013; Pollyea et al., 2018; Y. Wang et al., 2024). In the era of venetoclax-based treatment, recent studies demonstrated that increasing questions about clinical issues like dosing strategies and resistance are arising (Goulart et al., 2025). Chen and collaborators showed the possibility to overcome venetoclax resistance by targeting mitochondrial architecture (Chen et al., 2019). In accordance, Stevens and colleagues pointed out that resistance to venetoclax frequently emerges as a metabolic escape program in which FAO and lipid-fueled mitochondrial pathways become upregulated, restoring OXPHOS and survival (Stevens et al., 2020). Given the involvement of CES1 in metabolic adaptation of AML cells and its role in sustaining mitochondrial function (Capece et

al., 2021), we found that the combination of GR-148672X and venetoclax produces synergistic cytotoxicity in two AML cell lines, THP1 and OCI-AML3, suggesting that this combination therapy could be a new promising therapeutic approach in a specific subtype of AML to enhance the efficacy of venetoclax alone. The different responses observed across the panel of AML cells to GR-148672X and venetoclax are probably due to the AML biological and genetic heterogeneity. The characterization of the mutational landscape, together with the expression of CES1 in AML cells, could be useful for predicting therapeutic response.

Overall, our findings suggest that CES1 acts as a key metabolic hub to sustain metabolic adaptation and cell survival of AML cells. Therefore, targeting CES1 represents a promising new therapeutic strategy to treat AML both as monotherapy and in combination with conventional drugs. Our data underscore the importance of developing new pharmacological strategies to turn this metabolic vulnerability into a clinically actionable strategy.

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Finanziato dall'Unione europea-*NextGeneration EU*
Missione 4, Componente 1 – CUP E13C22001060006



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