Proteomic characterization of acute myeloid leukemia for precision medicine

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Abstract

Acute myeloid leukemia (AML) is a highly heterogeneous cancer of the hematopoietic system with no cure for most patients. In addition to chemotherapy, treatment options for AML include recently approved targeted therapies against proteins with roles in AML pathology, such as FLT3, BLC2 and IDH1/2. However, due to disease complexity, these therapies produce very diverse responses, and survival rates are still low, which highlights that, despite considerable advances, there remains a need for therapies that target different aspects of disease biology, and for associated biomarkers that define patient populations likely to respond to each available therapy. Indeed, drugs that target different AML vulnerabilities currently are in advanced stages of clinical development. Here, we review proteomics and phosphoproteomics studies that aimed to provide insights into AML biology and clinical disease heterogeneity not attainable with genomic approaches. To place the discussion in context, we first provide an overview of genetic and clinical aspects of the disease, followed by a summary of proteins targeted by compounds that have been approved or are under clinical trials for AML treatment and, if available, the biomarkers that predict responses. We then discuss proteomics and phosphoproteomics studies that provided insights into AML pathogenesis, from which potential biomarkers and drug targets were identified, and studies that aimed to rationalize the use of synergistic drug combinations. When considered as a whole, the evidence summarized here suggests that proteomics and phosphoproteomics approaches can play a crucial role in the development and implementation of precision medicine for AML patients.

Introduction to acute myeloid leukemia

Acute myeloid leukemia (AML) is a hematological cancer with an incidence of approximately 18,500 and 20,000 new cases in Europe and USA, respectively, making this the most common form of acute leukemia (1). Contrary to other forms of leukemia, for which targeted therapies have transformed their prognosis (2,3), curative treatments do not exists for most AML patients, and according to the American Cancer Society, AML will cause 11,500 deaths in the US in 2022. The risk of suffering AML at some point in life is 0.5 to 1%, and although it can occur at all ages, it is more frequent in adults with an average age at first diagnosis of 68 years. AML risks factors include exposure to chemicals (like benzene and chemotherapeutic agents) or radiation, suffering from myelodysplastic syndrome or myeloproliferative neoplasms, having germ line mutations in genes linked to familial AML (e.g., CEBPA, DDX4 or RUNX1) or somatic mutations in genes linked to clonal hematopoiesis (e.g., DNMT3A, TET2, or ASXL1) (4,5). Patients with inherited genetic syndromes such as Fanconi's anemia, Bloom syndrome, Down syndrome, and others also present an increased risk of AML (6-8).

AML originates from the malignant transformation of myeloid precursors, leading to the uncontrolled production of undifferentiated, immature and non-functional blasts, causing the displacement of hematopoietic stem cells (HSCs) and leading to bone marrow (BM) failure. This process was postulated to require genomic aberrations or mutations in epigenetic modifiers and/or transcription factors that impair hematopoietic differentiation alongside mutations in signaling pathways that sustain cell survival and proliferation (9). This "two-hit" model, although now thought to be over-simplistic, presents clinical implications for the design of combinational therapies (10).

Recent mutational studies show that frequent genetic alterations in AML include single gene mutations that affect proteins with multiple functions, together with large cytogenetic aberrations that involve chromosome gains, losses or rearrangements that generate fusion proteins with aberrant functions (Table 1). Another level of complexity in AML pathogenesis was provided by functional studies that showed that AML is initiated and maintained by leukemia stem cells (LSC, also known as leukemia initiating cells, LICs), from where the bulk of AML blast are derived (11,12). The LSCs are resistant to chemotherapy because they are quiescent and express anti-apoptotic molecules at high levels (13,14). Therefore, after the elimination of the bulk of leukemic cells by therapy, residual LSCs in BM have the potential to replenish the leukemic population, leading to the relapse of patients that initially responded to chemotherapy (15).

AML has been historically subdivided by the French-American-British (FAB) classification system (Table 2), which is based on morphological and immunophenotypic features linked to the differentiation and maturation stage of blasts (16). More recently, the World Health Organization (WHO) established an AML classification that incorporated genetic abnormalities and other clinicopathological features. In addition, the European LeukemiaNet (ELN) integrated genetic and

cytogenetic alterations into a risk classification system (favorable, intermediate and adverse) that prognosticate overall survival and response to standard chemotherapy (17-19) (Table 2).

Therapeutic options for AML

Until recently, essentially all AML patients were treated with an induction therapy regime that aims to achieve a complete morphologic remission and the restoration of normal hematopoiesis, followed by consolidation therapy to minimize the probability of relapse (1). The exception are patients in the acute promyelocytic leukemia (APL) subgroup, whose blast cells have an immature phenotype and who respond differentiating agents such as all-trans retinoic acid. For induction therapy, non-APL fit AML patients (generally <65 years) receive several cycles of cytarabine and daunorubucin, while unfit patients (usually >65 years) are treated with low dose cytarabine (LDAC), DNA hypomethylating agents (HMAs) like azacitidine and decitabine or palliative care. Consolidation therapy for patients who achieve complete remission includes different dosages of cytarabine and stem cell transplantation depending on the fitness of the patient and the prognosis of the leukemia provided by genetic and other biomarkers. Although most patients initially respond to this standard chemotherapeutic regime, most of them eventually relapse, leading to a 5-year survival of just 20%, and highlighting the need for more targeted approaches to treat AML (1).

The standard of care started to change gradually from 2017, when several targeted therapies were approved to treat AML subpopulations (Figure 1 and Table 3). These advances where spurred by the increasing understanding of AML genetic landscape as well as of its molecular biology at the proteomic level.

Activating mutations of the receptor tyrosine kinase FLT3 due to internal tandem duplications (ITD) in the juxtamembrane domain or point mutations in the tyrosine kinase domain (TKD) occurs in approximately 30% of patients, making this the most common genetic alteration in AML. Wild type FLT3 when bound to its ligand, promotes the generation of myeloid cells from precursors. However, mutant FLT3 ITD and TKD constitutively activate their downstream pathways, which support tumorigenesis in hematopoietic precursor cells (20). Midostaurin, a multi-targeted kinase inhibitor, was approved by the FDA in April 2017 for adult patients with newly diagnosed FLT3-mutated AML, and this was followed in November 2018 by the approval of gilteritinib for the treatment of adult patients with relapsed/refractory (R/R) AML with FLT3 mutations (21,22). Quizartinib, another FLT3 inhibitor was rejected by the FDA, but was approved in 2019 by the Japan Ministry of Health for the treatment of R/R AML with mutated FLT3 (15).

Other druggable recurrent genetic alterations in AML include gain of function mutations in isocitrate dehydrogenases (IDHs), which occur in about 20% of AML patients (23,24). IDH1 (cytoplasmic) and IDH2 (mitochondrial) catalyze the transformation of isocitrate into α-ketoglutarate (αKG) and generate NADPH. However, gain of function mutated forms or IDHs use NADPH as a co-

factor to generate the oncometabolite 2-hydroxyglutarate (2HG). Accumulation of 2HG together with the depletion of NADPH and αKG produce a series of metabolic and epigenetic alterations that favor the leukemogenic process (24). In August 2017 the IDH2 mutant inhibitor enasidenib and in July 2018 the IDH1 mutant inhibitor ivosidenib were approved for treatment of refractory or relapsed AML patients with gain of function mutations in IDH2 and IDH1, respectively (25,26).

Although the genetic mutational landscape has revealed opportunities for therapeutic intervention, not all new drugs derive from such new knowledge. A critical feature of cancer cells is their ability to evade pro-apoptotic signals that prevent normal cells to be transformed. BCL2, a key anti-apoptotic protein, has roles in leukemogenesis, and is overexpressed in leukemia stem and progenitor cells, as well as in AML blast when compared to normal hematopoietic cells (27,28). BCL2 uses its BH3 domain to bind and inhibit the pro-apoptotic proteins BIM, truncated BID and BAX that facilitate the translocation of cytochrome C form the mitochondria to the cytoplasm, which consequently activates caspases that execute the apoptotic process (29). As an anti-apoptotic protein, BCL2 has also been shown to play a role in resistance to chemotherapy of AML (29). Venetoclax is a "BH3 mimetic" small-molecule inhibitor that blocks BCL2 anti-apoptotic activity (30). In November 2018, the FDA approved the use of venetoclax in combination with HMAs or LDAC for the treatment of newly diagnosed AML patients aged ≥75 years or unfitted for intensive induction chemotherapy (31). In addition, venetoclax is currently being studied in numerous clinical trials as single-agent and in combination therapies (15).

The kinase Smoothened (SMO) inhibitor glasdegib is another drug derived from an increased mechanistic understanding of AML biology. The Hedgehog (HH) signaling pathway is important to normal hematopoiesis, promotes embryogenesis, maintain adult stem cells, and regulate cell proliferation and differentiation (32). In the absence of HH ligands (Sonic HH, Indian HH and Desert HH), SMO is inhibited by the Patched transmembrane proteins (PTCH-1 and PTCH-2). The union of the HH ligands to the Patched proteins triggers the activation of SMO and the phosphorylation of its downstream targets: the transcription factors GLI1 and GLI2, that regulate the expression of proteins involved in cell cycle, apoptosis and differentiation (33). HH signaling is aberrantly activated in AML and associated with poor clinical outcomes, and SMO activity plays a critical role for disease progression in several AML models (34). Glasdegib was approved by the FDA in November 2018 for use in combination with LDAC for the treatment of newly diagnosed AML patients aged ≥75 years or unfitted for intensive induction chemotherapy (35).

Another proteomic feature that has been exploited for drug development is the presence of cluster of differentiation (CD) proteins on the surface of blast cells. These CD markers have roles in cell adhesion, immune recognition and signaling, are indicative of the maturation state of the myeloid cell type from where AML derived and confer them with a unique immunophenotype. The CD33 protein antigen in particular is frequently expressed on the surface of AML blasts (36). Gemtuzumab is

an antibody drug-conjugate (ADC) directed at CD33, where a humanized monoclonal antibody covalently linked to the cytotoxic drug N-acetyl gamma calicheamicin binds to the CD33 antigens. Binding prompts internalization and subsequent calicheamicin release to the nucleus, leading to DNA damage and cell death (36). In September 2017 the FDA approved gemtuzumab for the treatment of adults with CD33 positive AML (37).

Candidate targets for new AML therapies

The drugs discussed above are gradually being incorporated into treatment protocols in healthcare systems as monotherapies or in combination. In addition, several other proteins are currently being tested as targets for the treatment of AML. Below, we provide a non-exhaustive summary of those that are currently (end of 2022) undergoing clinical trials (Figure 1 and Table 3).

In addition to FLT3, other RTKs that have a major impact on leukemia biology include KIT (also known as CD117 and SCF receptor), MERTK and AXL. KIT, a protein with important roles in self-renewal and differentiation of HSCs (38), is found mutated in about 17% of AML patients, and its expression is increased in 60 to 80% of AML samples when compared to normal hematopoietic cells (39). There are no specific KIT inhibitors, but multiple clinical trials are currently evaluating broad-spectrum RTK inhibitors that also target KIT including midostaurin (already approved for AML treatment), sorafenib (NCT05404516) and dasatinib (NCT02013648). Inhibitors of MERTK and AXL, which are also frequently overexpressed in leukemic cells, reduced the proliferation of AML cell lines and increase survival in animal models of AML (40). Clinical trials with the MERTK inhibitor MRX-2843 (NCT03510104) and the AXL inhibitor bemcentinib (NCT03824080) are ongoing. In addition, the approved FLT3 inhibitor gilteritinib also targets AXL.

Overactive RTKs promote cell proliferation by activating intracellular kinase-driven signaling cascades – including MEK/ERK, PI3K/AKT and JAK/STAT –, which in turn regulate cell cycle kinases. Although inhibitors of MEK and PI3K/AKT have not progressed in the clinic, considerable effort is currently underway to treat cell cycle kinases downstream of these signaling pathways (41,42). CDK9 is a cyclin-dependent kinase that forms part of the positive transcription elongation factor b (TEFb). This factor phosphorylates the CTD of RNA polymerase 2 and stimulates the elongation of the transcription of most protein coding genes, but in particular those regulated by super enhancers like MYC and MCL1 (43). In *in vitro* AML models, CDK9 inhibitors decreased the phosphorylation of RNA Pol II and the expression of MYC, MCL-1, XIAP and cyclin D1, induced apoptosis and reduced cell proliferation. In animal AML models, these compounds reduced tumor growth and prolonged survival (43). These results encouraged the initiation of clinical trials with the CDK9 inhibitors alvocidib (NCT03969420), dinaciclib (NCT03484520), AZD4573 (NCT03263637) and voruciclib (NCT03547115). Another cell cycle regulator that has been targeted in AML is CDK6, a kinase important for the leukemogenic processes triggered by FLT3-ITD and JAK-V617F mutations and

KMT2A-MLLT3 and RUNX1-ETO fusion proteins (44). Currently the CDK4/6 inhibitor palbociclib (NCT03844997) is in clinical trials for the treatment of AML.

In addition to promoting uncontrolled proliferation, overactive RTKs and downstream signaling pathways promote survival of cancer cells by suppressing apoptosis, a process that, in normal cells, regulate cellular homeostasis (15). Therefore, several small molecule inhibitors of anti-apoptotic proteins have been tested for AML treatment. Indeed, the success of venetoclax provides proof-of-concept for this inhibitor class. Targeting the MCL1 provides an approach for targeting apoptosis because this protein is an anti-apoptotic member of the BCL2 protein family that, similarly to BCL2 (the target of venetoclax), binds and inhibits the pro-apoptotic activity of BIM, truncated BID, BAK and BAX (29,45). MCL1 is highly expressed in patients with untreated AML, is necessary for survival of AML cells and for the development and persistence of the disease (46,47). Inhibitor screens (48) showed that AML cells were sensitive to MCL1 inhibition leading to the initiation of several phase I studies for the MCL1 inhibitors AZD5991 (NCT03218683), MIK665 (NCT02979366), AMG 176 (NCT02675452) and AMG 397 (NCT03465540) in AML.

Targeting the DNA damage response (DDR) provides another opportunity for interfering with the anti-apoptotic process in AML. DDR is a complex system that maintains genomic integrity through regulation of DNA damage repair, cell cycle progression and apoptosis. Deregulation of the DDR allows cancer cells to withstand DNA damage, which would normally trigger apoptosis, but this also generates certain vulnerabilities that can be exploited therapeutically. One of such targets is CHK1, a kinase that upon activation by DNA damage blocks cell cycle progression and triggers DNA damage repair (15,49,50). The CHK1 inhibitor prexasertib is being tested in combination with chemotherapy in a clinical trial for the treatment of R/R AML (NCT02649764). Similarly, PARP1, a key mediator of various forms of DNA damage repair, is used by AML cells to withstand replicative stress and/or deficiencies on the homologous repair system, features that sensitize cells to PARR1 inhibitors (51). Currently, the PARP1 inhibitors talazoparib (NCT02878785) and veliparib (NCT03289910) are in clinical trials for the treatment of AML. A further protein involved in DDR and apoptosis that is being targeted in AML is MDM2, which is an ubiquitin ligase that negatively regulates the levels and activity of TP53, a key regulator of DNA repair, cell cycle progression and apoptosis. Impairment of TP53 plays a pivotal role in the process of leukemogenesis, and, although 10% of AML cases present TP53 inactivating mutations, its impairment is more generally associated to the overexpression of MDM2 (52). This has led to the development of MDM2 inhibitors like idasanutlin, currently in clinical trials for the treatment of AML (NCT04029688).

Epigenetic regulation has also been targeted for the development of AML therapeutics because genes coding for epigenetic proteins are frequently mutated or otherwise deregulated in this disease. One of such protein is the histone deacetylases (HDACs) family of epigenetic erasers that remove acetylation marks from histones, leading to chromatin compaction and gene silencing. HDACs also act

on non-histone proteins to regulate their activities. HDAC inhibitors cause changes in the expression of multiple genes, including downregulation of the key oncogene MYC, and induce differentiation, cell cycle arrest, and apoptosis in AML cells (53). Belinostat (NCT03772925), entinostat (NCT01305499) and pacrinostat (NCT01912274) are currently in clinical trials for AML treatment.

Another epigenetic protein targeted in AML is the lysine methyltransferase KMT2A, which methylates histone H3K4. In 10% of AML cases, KMT2A fusion proteins generated by chromosomal rearrangements drive the leukemogenic process. In these fusion proteins, the catalytic domain of KMT2A is replaced by a region of the partner protein that facilitates recruitment of the histone H3K79 methyltransferase DOT1L to the KMT2A target genes, a process that requires the KMT2A-MENIN protein-protein interaction (54). Numerous small molecule inhibitors have been designed to target proteins necessary for the activity of KMT2A fusion proteins and currently, the KMT2A, MENIN and DOT1L inhibitors SNDX-5613, JNJ-75276617 and pinometostat, respectively are in clinical trials for the treatment of AML (NCT05326516, NCT04811560, NCT03724084).

The bromodomain and extraterminal domain-containing (BET) protein BRD4 is yet another promising epigenetic target for AML treatment. BRD4 is an reader that binds to acetylated histones and other proteins thus recruiting the TEFb complex that, as previously discussed, stimulate the elongation of the transcription of genes relevant for the leukemogenic process like MYC and MCL1 (55). Currently, the BET inhibitors mivebresib (NCT02391480), FT-1101 (NCT02543879) and birabresib (NCT02303782) have entered clinical trials for the treatment of AML.

Protein miss-localization is a frequent occurrence in cancer cells and a potential mechanism of ontogenesis. Indeed, several nuclear tumor suppressors cannot serve their functions in cancer cells because they are miss-located to the cytoplasm. TP53, BRCA1/2, NPM1, FOXO, RB1 and other tumor suppressors are substrates of XPO1, an export receptor responsible for the nuclear-cytoplasmic transport of hundreds of proteins and RNA species (56). In addition, XPO1 levels inversely correlate with the overall survival of AML patients (57). Encouraging preclinical studies showing that XPO1 inhibition promoted cell cycle arrest and apoptosis in *in vitro* and *in vivo* AML models (58), accelerated the initiation of multiple clinical trials with the XPO1 inhibitor selinexor in AML patients (NCT02835222, NCT02403310 or NCT04898894).

Like in other cancer types, targeting metabolic processes open avenues for therapeutic intervention in AML. The electron transport chain (ETC) powers the mitochondrial oxidative phosphorylation (OXPHOS), which has been proved as a main source of energy in AML LSCs and chemotherapy resistant cells (59). The ETC complex I inhibitor IACS-010759 is a potent anti-leukemic agent in *in vitro* and *in vivo* models and has entered clinical trials for the treatment of AML (NCT02882321) (60). Another way to target OXPHOS is with activators of the ATP-dependent mitochondrial caseinolytic protease P (CLPP), which regulates OXPHOS by controlling the degradation

of the respiratory chain components and triggering the mitochondrial unfolded protein response. ONC201 is an allosteric agonist that hyper activates CLPP umpiring the OXPHOS and triggering an atypical integrated stress response mediated by ATF4. This compound has also been effective as an anti-leukemic agent in *in vitro* and *in vivo* AML models and is in clinical trials for the treatment of AML (NCT02392572) (49).

In addition to the targeted agents discussed above, new immunotherapy approaches have been developed in AML with the aim to treat the disease using ADCs or immne cells directed against surface markers on LSCs or bulk cell populations. As noted above, gemtuzumab, an approved ADC against CD33 provides proof-of-concept for this approach. The receptor of interleukin 3 CD123 and the anti-phagocytic protein CD47 are highly expressed in LSCs (61,62). Reagents targeting CD123 in clinical trials for AML include MGN632 (NCT04086264), an anti-CD123 antibody linked to a genotoxic compound, and tagraxofusp (NCT04342962) an agent composed of human IL-3 fused to a portion of the diptheria toxin. In addition, the CD47 antibody magrolimab that facilitates the phagocytosis of LSC is also in clinical trials (NCT04435691). CAR-T cells are cytotoxic T cells with T cell receptors modified to target specific antigens (63). There are currently multiple clinical trials testing the treatment of AML with CAR-T cells design to target CD33 (NCT05445765), CD123 (NCT04318678), CLL-1 (NCT04219163), CD7 (NCT04033302), CD28 (NCT04850560), CD38 (NCT05239689), CD19 (NCT04257175), FLT3 (NCT03904069) and NKG2D (NCT04658004).

The need to prioritize therapies for AML patients

As outlined above, intense research is producing a large array of therapies that could potentially be used to treat AML. Oncologists are already facing the issue of having to select the drugs or drug combinations, out of the many available, more likely to be efficacious for a given patient, a dilemma that will become even more intricate as some of the new therapies discussed above reach the clinic. Current technologies for precision and personalized medicine are based on the analysis of genomic markers (64). Although these methods can enrich for potential responders, and are thus an improvement over therapies in unselected patient populations, results of clinical trials show that genetic screens often fail to accurately predict therapy outcome. For example, only ~20% of R/R AML patients positive for NRAS or KRAS mutations responded to the MEK inhibitor trametinib (65). Similarly, ~40% of FLT3-mutant positive newly diagnosed AML patients failed to respond to the FLT3 inhibitor midostaurin in combination with chemotherapy, (66) while, conversely, 41%–56% of FLT3-WT patients responded to FLT3 inhibitors (65,67,68).

Focusing only on the genomic and transcriptomic layers of cell function regulation leaves us blind to other important regulators of cell phenotypes and outcomes (64). It is well known that changes in gene expression do not always reflect changes in protein abundance (69) and protein amounts in cells may not predict how enzymatically active they are (70,71). Proteins are the major effectors of cell

functions and their regulation by allosteric and posttranslational modifications (PTMs), localization, interaction partners and abundance combine to regulate their enzymatic activity and proximity to their substrates, which together influence pathway fluxes and cell phenotypes. It is therefore critical to also consider proteomics, phosphoproteomics and other PTM-'omics' data sets to understand disease development and subtypes, as they can better capture the functional state and dynamic properties of a given cell or cell population (64). Therefore, the combination of genomics and transcriptomics with proteomics and phosphoproteomics could complement current approaches for disease classifications (including AML) by defining new pathological subtypes linked to specific therapeutic vulnerabilities.

Overview of proteomics and phosphoproteomics approaches

Proteomics and phosphoproteomics methods based on LC-MS/MS can accurately and simultaneously measure abundances for thousands of proteins and phosphorylation sites. Consequently, LC-MS/MS techniques are contributing to the identification of clinically relevant biomarkers and targets for disease diagnosis and prognosis in precision medicine (72-75). Quantitative proteomics and phosphoproteomic approaches for profiling cell lines and primary samples could be subdivided into label-based and label-free methods. Labelling approaches put a limit on the number of samples that can be assessed and makes it difficult to compare results of different experimental batches, but are reputed to offer greater analytical precision. Label-free and targeted proteomics methods, such as those based on data-independent analysis, solves many of these issues and renders them suitable for clinical assays (76,77). Detailed description of quantitative labelling and label-free mass spectrometry methods have been described elsewhere (78-83) and will not be discussed here.

Quantitative proteomics produces large volumes of data that requires specialized computational methods for their biological interpretation. Multiple bioinformatics tools originally designed for the analysis of gene expression, such as term overrepresentation analysis and gene set enrichment analysis (GSEA), are also useful for obtaining pathway level information from proteins differentially expressed or phosphorylated between groups of interest. Term and ontology enrichment can be computed against different databases (e.g., gene ontology, KEGG and NCBI) utilizing algorithms frequently used in genomics and transcriptomics (84). In addition, specialized software has been developed for the analysis of phosphoproteomics data. The algorithm kinase substrate enrichment analysis (KSEA) uses phosphoproteomics data to estimate kinase activities based on the phosphorylation of their substrates. The first version of the algorithm (85) used databases based on empirically demonstrated associations between kinases and substrates. An improved version of the algorithm uses databases defined by a chemical proteomics approach that codes information on kinase–kinase interactions that can allow the reconstruction of kinase network topologies (71). Several other methods for the inference of kinase network activity from phosphoproteomics data have been developed and discussed in detail elsewhere (86). One of such tool, named integrative inferred kinase activity (INKA), calculates kinase activity form phosphoproteomics data takes by measuring phosphorylation sites on the kinase and on its

activation loop, in addition to experimentally established substrates (as in KSEA) and on substrates predicted by NetworKin (87).

Studies that inferred kinase activity from phosphoproteomic data are starting to provide biological insights in the field of AML. For example, Van Alpen et al showed that INKA analysis of 16 AML cell lines using tyrosine targeted phosphoproteomics identified hyperphosphorylated, active kinases as candidates for targeted therapies. Validation drug response experiments showed that, in addition to driver kinases corresponding with activating mutations present in these cell lines, INKA analysis also pinpointed driver kinases undetected by standard molecular analyses. Furthermore, INKA detected hyperactivation of FLT3 in two clinical AML samples with a FLT3 internal tandem duplication (ITD) mutation (88). Another example is the application of KSEA to primary cells from a cohort of 20 patients (85), which identified PI3K, casein kinases, CDKs and PAKs as the most frequently activated kinases in AML cases compared to GMPBs from healthy donors. Furthermore, KSEA showed that substrates of ERK, CDC7 were more phosphorylated in primary cells resistant to a compound targeting the PI3K/mTOR pathway, while substrates of Abl, Lck, Src, and CDK1 were more phosphorylated in sensitive cells.

Limitation of AML cell lines as clinically relevant models for proteomics and phosphoproteomics studies

Cell lines have frequently been used as disease models in basic research. However, cell lines may not always resemble the biology of primary cells, and therefore are not useful to model all aspects of cancer biology. In the case of AML, although cytogenetic signatures based on gene expression have been found to be conserved, to some extend at least, between AML primary samples and cell lines, other reports highlight the limitations of cell lines as disease models (89). For example, analysis of the expression of 380 genes linked to multidrug resistance proteins (MDR) and up-regulated genes that facilitate cell survival showed that cell lines from different origins (including AML) were more similar between them than to the primary tumor cells that they were supposed to model (90). Regarding protein expression, Aasebo et al. found, in a dataset comprising five AML cell lines and twenty-seven primary AML samples, that about one third of the proteins quantified were differentially expressed between cell lines and primary cells, with proteins involved in translation overexpressed in cell lines and proteins associated to the mitochondria overexpressed in primary cells (91). These results imply that although cells lines can be used to investigate some aspects of tumor biology and for drug development, they do not fully recapitulate the biological complexity of AML (89). Therefore, the focus of this review is in studies that used primary AML cells as the focus of the analysis.

Proteomics and phosphoproteomics studies for target identification and drug response prediction

Access to large cohorts of primary AML clinical specimens and the availability of appropriate control samples still remains challenging (89). Nevertheless, multiple studies have used mass

spectrometry to identify proteins or phosphorylation sites differentially regulated between AML patients and healthy donors, to define subgroups of AML patients, to model drug responses and other features using machine learning (ML) algorithms and to identify determinants of responses to approved and experimental therapies. Differentially expressed features derived from these studies could potentially be used as new drug targets and/or response markers for precision medicine in AML. Here, we review, in a chronological order of publication, proteomics and phosphoproteomic studies of primary AML that aimed to identify drug targets or mechanisms of drug sensitivity.

Casado et al profiled the basal phosphoproteomes a cohort of 20 primary AML samples linked to preclinical responses to kinase inhibitors (85). A pathway activity signature of phosphosites that decreased by the treatment of cell lines with PI3K and mTOR inhibitors was then evaluated in primary AML cells for its ability of predict responses to kinase inhibitors in preclinical development. Linear regression models were trained using signatures derived from individual phosphopeptides as well as KSEA estimated kinase activities and values of phosphorylation motifs enrichment. Interestingly, it was found that the activity of the PI3K/mTOR pathway was not the only determinant of sensitivity to inhibitors of this pathway, and that the activity of pathways, such as ERK1/2 and PKC, that can compensate for PI3K target inhibition were elevated in resistant cells. Thus, accurate predictive models could be constructed by combining kinase activities in the target kinase pathways relative to those that act in parallel to compensate for target inhibition.

A follow up study from the same group integrated phosphoproteomics, proteomics, genomics and mass cytometry (immunophenotype) data with *ex vivo* responses to MEK, PAK, PKC/FLT3, CK2 and MAPK P38 inhibitors (92). The study showed evidence of molecular features associated to response to treatment and identified kinase and differentiation determinants as markers of sensitivity to kinase inhibitors in primary leukemic blasts. The study, performed in primary cells form 30 AML cases, revealed that protein phosphorylation positively correlated with the surface expression of differentiation makers (CDs) associated to myeloid differentiation. AML cases were subsequently separated into more and less differentiated cases (CDs+ and CDs- groups, respectively) using the surface expression of myeloid differentiation markers. More differentiated (CDs+) cases expressed higher levels of kinases and signal transduction regulators and increased the activity of kinases downstream of growth factors such as MAPK1, MAPK3, PAK2 and PKCδ. Consistently, CDs+ cases were more sensitive to the MEK, PAK and FLT3/PKC inhibitors in *ex-vivo* assays. These results established a linked between differentiation, kinase activity and sensitivity to kinase inhibitors.

In the same study, a more integrative and systematic analysis of mutational profiles and mass spectrometry and cytometry data showed that NRAS or BRAF mutations, high MAPK1 activity or the CDs+ phenotype were associated with sensitivity to trametinib, while FLT3-ITD mutations or high pSTAT5A phosphorylation were linked to resistance. These data suggested two distinct mechanisms of intrinsic resistance to MEK inhibition. In the first one, cells with low RAS/MEK/ERK pathway activity

were not addicted to the pro-survival actions of MEK. In the second, cells with highly active RAS/MEK/ERK, as a result of RAS/RAF mutations and/or expression of myeloid differentiation markers, but resistant to MEKi were found to activate FLT3/STAT5 axis, which may act in parallel to MEK/ERK to sustain cell survival. Interestingly, the response to midostaurin was not associated to FLT3 mutations; instead, the CDs+ phenotype and the phosphorylation of PKCδ, a known target of midostaurin, were associated to the response to this drug, suggesting that the mode of action of midostaurin involves inhibiting other kinases in addition to FLT3.

In a different study, Alanazi et al identified miss-expressed or miss-localized proteins to the nucleus that could regulate the malignant properties of AML blasts (93). The comparison of the nuclear proteomes of $CD34^+$ cord blood cells from 5 healthy donors and blasts from 15 cases with FAB-M2 AML led to the identification of 113 proteins, 11 of which were transcription factors frequently miss-localized in AML blasts. S100A4 was the highest differentially expressed protein in AML nuclei that was not previously implicated in AML. Relevantly, protein but not mRNA levels of S100A4 were over-expressed in the nucleus of a larger cohort of 24 patients. Functional experiments showed that knock down of S100A4 strongly impacted the survival of AML cell lines, but not the survival of normal hematopoietic stem progenitor cells, suggesting that S100A4 could be a new target for AML treatment.

In a follow up from (92), Hijazi et al analyzed drug response data by multivariate regression using kinase network edge activities (derived by KSEA) as input to predict response to trametinib, midostaurin, silmitasertib and the PAK4 inhibitor PF-3758309 (71). These models predicted drug response with an accuracy between 20 and 40% and highlighted that the activity of target and parallel pathways contribute to model performance.

Aasebo et al profiled the proteome and phosphoproteome of AML primary blasts at the time of diagnosis from 41 AML patients that reached complete remission (94), from where proteins differentially expressed or phosphorylated were identified in patients that relapsed within five years relative to those who did not. Relapsed cases presented increased expression of RNA processing proteins and increased phosphorylation of proteins liked to CDKs and CK2, while relapse free cases increased the expression of V-ATPase proteins. Therefore, this study suggested markers that could help to predict relapse in AML.

Assessing drug response in *ex vivo* models has provided highly valuable information related to targetable pathways in AML (95). However, *ex vivo* models are devoid of all signals from stromal components and clearly differ from human *in vivo* responses. Therefore, proteomics and phosphoproteomics studies in primary AML blast obtained at diagnosis form AML patients with known responses to treatment would provide extremely useful information. In line with this, Hernandez-Valladares et al performed a proteomics analysis in non-treated samples from 28 AML cases that were subsequently treated with all-trans retinoic acid (ATRA) and vaporic (a HDAC inhibitor) (96), a

treatment used alone or in combination with low dose chemotherapy for patients unfit for standard chemotherapy. Non-responders overexpressed the lysosomal protein ARSA, while responders overexpressed proteins linked to myeloid cells, neutrophil degranulation, lysosomes, carcinogenesis (including ANO6, CHI3L1, CTSG, ELANE and FGR) and M phase of the cell cycle (including CENPE, CENPK, CDK1, NCAPG; and several histones). Interestingly, the proteins differentially expressed between responders and non-responders presented a low overlap between mRNA and protein levels. Phosphoproteomics analysis showed that responders increased the phosphorylation of the apoptosis liked proteins SPTAN1 and ACIN1, as well as LIMKs and CDKs substrates. In addition, proteomics and phosphoproteomics analysis comparing patient samples before and after three-day treatment releveled an altered expression and phosphorylation of proteins involved in the regulation of transcription/translation/RNA metabolism.

Nguyen et al profiled the proteome of BM cells from 16 pediatric AML patients at diagnosis and found that 117 proteins that were differentially expressed in cases with fusion proteins involving components of core binding factor complex (CBF) (97). Patients with CBF rearrangements deregulate proteins involved in several metabolic pathways such as the TCA cycle and the ATP synthesis coupled to proton transport. In addition, CD34 protein expression was significantly increased in cases with CBF rearrangements, suggesting that CBF AMLs carry unique protein expressions that resemble $CD34^{+}$ progenitor cells. These data also imply that cases with or without CBF rearrangements could present a differential response to compounds that interfere with the electron transport chain.

Casado et al described a ML workflow aimed to predict *ex-vivo* patient response to trametinib, a specific inhibitor of MEK1 and MEK2, in primary AML using kinase activity (KSEA) as input (72). A model using the partial least squares algorithm (PLS) was used to rank and filter the most relevant kinase activities in the training set, which were subsequently used as input for a random forest regression model. The ML model predicted trametinib response with a root median square error (RMSE) of 0.131 in the validation dataset.

A proteogenomic study by Kumar-Jayavelu et al, in addition to providing insights into the pathogenesis of AML, also revealed altered molecular features that could influence response to therapy (98) and suggested that a group, termed C-Mito AML, with defined mitochondrial protein expression patterns, tended to respond to venetoclax and electron transport I complex inhibitors.

In a more integrative study, Kramer et al performed proteomics and phosphoproteomics in BM samples from 6 healthy donors and 44 patients for which data on DNA and RNA sequencing were available (99). The patient cohort covered all ELN cytogenetic risk groups and frequent single gene mutations. Protein-mRNA level correlation analysis showed no positive correlation (spearman<0) for more than a thousand proteins that were mainly linked to spliceosome, oxidative phosphorylation, and RNA polymerase processes. On the other hand, the 1,198 proteins that showed positive correlation

included differentiation markers and other proteins relevant for the AML physiopathology. The authors demonstrated that IDH1 or IDH2 mutations caused increased protein expression of the 2-HG dependent histone demethylases KDM4A, KDM4B and KDM4C in cases with IDH1 or IDH2 mutations. NPM1c mutations relocate the NPM1 to the cytoplasm and it was shown that cases with NPM1c mutations had increased levels of the nuclear importins KPNA4 and KPNA1 that are able to interact with mutated but not with normal forms of NPM1. Of interest, proteomics data guided the identification of the markers CD180 and CD206 in the plasma membrane of AML blast from some patients but not in the surface of healthy $CD34^{+}$ cells, suggesting that these markers could represent novel targets for immunotherapy. Clustering analysis using phosphoproteomics data also separated healthy donors from AML cases and segregated patients according to the presence of FLT3 mutations or PML-RARA fusions. When compared to healthy donors AML cases presented abnormal phosphorylation of specific residues in PTPN11, STAT3, AKT1, and PRKCD. In addition, FLT3-TKD mutated samples increased the phosphorylation of activating residues in the tyrosine kinases FGR and HCK and other signaling related proteins, while the PML-RARA initiated samples displayed a unique phosphorylation signature that included increased phosphorylation of JUN at S60 and STK26 at the activation loop. Finally, TP53-mutant samples showed abundant phosphorylation of TP53 at S183. This study shows that the link between mRNA and protein abundance in AML cells is relatively limited, especially for proteins linked to RNA maturation and mitochondrial function, highlighting the relevance of proteomics studies.

In agreement with this, a study by Caplan et al combining proteomics and transcriptomics in AML mouse models identified a set of 34 proteins upregulated in AML tumors at the protein, but not at the mRNA level that also showed an enrichment of mitochondrial and spliceosome proteins (100). Relevantly, proteins differentially expressed or phosphorylated were found associated to AML and specific mutations that could constitute new targets or markers for precision medicine. However, no link was stablished between protein expression or phosphorylation and drug response.

In another study that used ML from mass spectrometry data to derive models of therapeutic response and mechanistic inference in AML cases, Gosline et al performed proteomics and phosphoproteomics from 38 AML cases for which genomics and transcriptomics data were available. Identified signatures from different omic layers were evaluated for their ability to model *ex vivo* responses to 26 drugs (101). Genetic mutations were found to be relatively robust in modelling responses to targeted therapy (e.g. trametinib and quizartinib for NRAS and FLT3 activating mutations) but models based on protein features showed greater performance when assessed across all drugs. The proteins and phosphopeptides more relevant for quizartinib and trametinib predictive models clustered AML cell lines based on response to these drugs. In addition, these features were integrated to identify putative underlying molecular pathways and provide a biological interpretation of the signatures. The phosphatase SHP-1 (also known as INPP5D) was part of the signature used by LASSO and logistic regression models, and patients sensitive to quizartinib downregulated this phosphatase, a known

regulator of signaling downstream of FLT3 (102). An integration algorithm added the expression of SCM-1, a protein expressed in AML blasts, and the phosphorylation of SMC3, that synergize with FLT3 in AML, to the SHP-1 network. Patients highly resistant to trametinib expressed proteins associated to mRNA processing and catabolism. Network integration using mRNA and protein expression data highlighted BID, CASP1, GZMB and other proteins linked to apoptosis, thus suggesting that expression of apoptosis-related proteins and transcripts could predispose patients to trametinib sensitivity.

In another recent study, Casado et al, in collaboration with Jude Fitzgibbon (UK) and Caroline Heckman (Finland) groups, profiled 74 AML patients with poor risk karyotype using genomics, transcriptomics, proteomics and phosphoproteomics platforms, as well as *ex vivo* responses to 550 drugs (103). Integration of the data identified a phosphoproteomics signature that defined two biologically distinct groups of KMT2A rearranged leukemia, which were termed MLLGA and MLLGB. Increased DOT1L phosphorylation and HOXA gene expression indicated that MLLGA cases have higher activity of DOT1L and TEFb at KMT2A target genes when compared to MLLGB and no KMT2A rearrangement group. MLLGA cases also increased the activity of CDK1 and the phosphorylation of proteins involved in RNA metabolism, replication and DNA damage when compared to MLLGB and no KMT2A. Compared to other groups, MLLGA was particularly sensitive to 15 compounds including genotoxic drugs and inhibitors of mitotic kinases and inosine-5-monosphosphate dehydrogenase (IMPDH). Further experiments suggested that the ability of IMPDH inhibitors to interfere with the nucleolar biology is, at least partially, responsible for the higher efficiency of these compounds in MLLGA. This study identifies a signature that classifies AML patients with KMT2A rearrangements into two biologically and functionally different groups provided a rationale for the potential testing of IMPDH inhibitors and potentially mitotic and genotoxic compounds in KMT2A patients positive for the MLLGA signature.

In summary, multiple strategies using phosphoproteomics and proteomics data have been implemented to identify potential new targets and response markers in AML (Figure 2). The wealth of data that is now generated using proteomic approaches require data science strategies to mine such rich datasets for biological and clinical insights. A frequently used strategy consists of classifying AML cases into groups using previously known labels (e.g., responder vs non-responder groups to a given drug) for the identification of proteins differentially phosphorylated or expressed between groups using classical statistics (Figure 2). Differentially expressed or modified features may reveal candidate drug response markers and drug targets specific for each patient population. Groups have been defined based on patients vs healthy donors (85), relapsed vs no relapsed patients (94), patients with presence vs no presence of chromosome rearrangements (97) or mutations (99) or responders vs non-responders to a treatment (96).

Another option is to define new labels using proteomics, phosphoproteomics or other omics data as input for unsupervised ML algorithms like hierarchical clustering or PCA to generate new labels, which may be used to classify cases and identify potential response markers and drug targets as described above (Figure 2). This strategy has been used to define the CDs groups and C-Mito cluster (92,98). The rationalization of response markers and drug targets can also be used to refine the definition of the labels used to classify the patients (92,93). Other approaches used supervised ML to generate regression or classification models that model and predict drug response or other relevant features (71,104). While these models may in the future be useful to clinicians when deciding on therapeutic options, it is clear that these will not be used in isolation and will instead complement the information derived from the assessment of classical clinicopathological and genomics features, which together may provide more accurate drug response predictions. A limitation of this approach is that it produces black box models from which is it difficult to derive mechanistic insight, although considerable work to solve this issue is being carried out (105). In some instances (e.g., when explainability is an essential requirement, or when sample numbers are limited), it may be preferable to use ML methods based on linear regression or random forest (106), which although simpler and less powerful than deep learning (105), allow inferring feature importance with relative ease (106).

Rationalization of drug synergy in AML cells using proteomics and phosphoproteomics

Tumors depend on a limited number of molecular mechanisms for their survival and proliferation. Therefore, drug combination therapies that simultaneously target several of these crucial mechanisms would produce a synergistic effect that is greater than the sum of the effect of the single treatments. Although attractive, precision medicine for cancer treatment using combinations of drugs with a synergistic effect proves to be highly challenging (107). Proteomics and phosphoproteomics approaches may contribute to the rationalization of drug combinations, and below we summarize illustrative studies to that aim to do so in AML.

Cucchi et al used an *ex vivo* drug response analysis in 19 AML cases to identify sensitive and resistant patients to the FLT3 inhibitors gilteritinib and midostaurin (67). FLT3-ITD mutated samples were more responsive towards gilteritinib and midostaurin, compared with FLT3 wild type (Wt) cases, but the presence of FLT3-ITD mutations could not fully explain these responses. To address this, phosphoproteomics was used to investigate associations between response and phosphorylation. Gilteritinib-resistant samples increased the phosphorylation and activity of MAPK1/MAPK3, EGFR1 and KIT. While no difference in responses were found between FLT3 mutant and WT cases, a small fraction of peptides differentially phosphorylated between sensitive and resistant cases to gilteritinib and midostauring were also differentially phosphorylated between ITD and Wt FLT3 cases, suggesting that most of the differences in phosphorylation between resistant and sensitive cells come from parallel pathways to FLT3 signaling that support survival in the absence of FLT3 activity. Consequently, their targeting could sensitize cells to FLT3 inhibition, an hypothesis that was confirmed with the

combination of gilteritinib or midostaurin with the MEK inhibitor trametinib, which showed synergy, although this was only observed in a NRAS mutated sample. This is in line with studies reporting resistance to midostaurin in NRAS mutated cases (92).

Murray et al profiled the phosphoproteome of primary AML blasts from seven patients with Wt or mutant FLT3 (108). A set of 143 peptides were found to be highly phosphorylated in FLT3 mutant patients, and these presented an enrichment of proteins linked to the Non Homologous End Joining (NHEJ) DNA repair pathway, while the 90 peptides down phosphorylated in FLT3 mutant cases showed an enrichment of proteins associated to the Base Excision Repair (BER) pathway. Mutant cases increased the auto-phosphorylation DNA protein kinase (PRKDC) at S2612 and the phosphorylation of other key proteins in the NHEJ pathway like XRCC4, XRCC5 and 53BP1. PRKDC phosphorylation at S2612 was sensitive to FLT3 inhibitors. Furthermore, the PRKDC inhibitor M3814, when combined with the FLT3 inhibitor sorafenib, showed a synergistic effect in reducing the survival of primary blast with mutated FLT3 but not of blast with Wt FLT3. Finally, a xerograph mice model of the FLT3-ITD mutant AML cell line MV4-11 survived longer when treated with the combination of M3814 and sorafenib than when subjected to single agent therapy. In summary, phosphoproteome profiling showed that primary AML blast with mutated FLT3 over-activated the PRKDC pathway, and this rationalized a synergistic FLT3 and PRKDC inhibitor combination in FLT3 mutant positive AML cells.

Zhu et al carried out a phosphoproteome profiling of primary cells at diagnosis from 8 AML patients that did (remission) or did not (refractory) respond to standard chemotherapy. This study showed that refractory patients increased the phosphorylation of proteins linked to ATM, FLT3 and MAPK/ERK signaling (109). Kinase activity inference using NetworKIN showed that refractory samples had an increase in the phosphorylation of putative substrates of CK2 and CDKs. Consistent with these observations, the CK2 inhibitor silmitasertib increased cell death induced by cytarabine in primary AML cases from refractory cases. The CK2 substrate HMGA1 was highly phosphorylated in refractory cases; removal of this protein in AML cell lines reduced their proliferation, and conversely, transduction of a phosphomimetic mutant for HMGA1 at CK2 sites increased the colony formation of a MA9/ITD murine model of AML. HMGA1 and the transcription factor SP1 regulate the expression of BRIC5, relevant anti-apoptotic protein in AML. A functional study showed that only phosphosphorylable forms of HMGA1 at CK2 sites bind SP1 at the BRIRC5 promoter. This work revealed a potential mechanism by which HMGA1 phosphorylation at CK2 sites promotes intrinsic resistance cytarabine-based chemotherapy and how CK2 inhibitors could sensitize chemo-resistant cells.

In another elegant study, Emdal et al performed a phosphoproteomic analysis of 20 primary AML samples sensitive or resistant to *ex vivo* treatment with selinexor for 6h (110). In patient samples and cell lines sensitive to the drug, selinexor increased the phosphorylation of TP53 at S15, a marker of its transcriptional activity. However, in patients resistant to selinexor treatment, the drug increased

the phosphorylation of FOXO3A at S253, an AKT site that sequesters FOXO3A in the cytoplasm and inhibit its pro-apoptotic transcriptional activity. In AML cell lines with Wt TP53 and sensitive to selinexor, nutlin-3 (an MDM2 inhibitor that increase TP53 transcriptional activity) and selinexor treatment produced a synergistic effect in which nutlin-3 decreased the degradation of TP53, a key protein for the cell death induced by selinexor. In addition, in AML cell lines resistant to selinexor, the combination of the AKT inhibitor MK-220 with selinexor showed a synergistic effect together with an increase in the nuclear localization of FOXO3A where it executes it pro apoptotic transcriptional activity.

Inspired by previous studies showing that differentiation is closely linked to kinase signaling and response to kinase inhibitors (92), Pedicona et al tested the idea that inducing differentiation in AML would reshape kinase networks into topologies that confer sensitivity to kinase targeted drugs (111). Phosphoproteomics and proteomics showed that inhibitors of the lysine-specific demethylase 1 (LSD1) rewired kinase signaling in AML cells in a way that increased the activity of the kinase MEK and broadly suppressed the activity of other kinases and feedback loops. Consequently, AML cell lines and about half of the 17 primary AML samples tested were primed for sensitivity to the MEK inhibitor trametinib. Phosphoproteomics analysis showed that cases that responded to sequential treatment with LSD1 inhibitors and trametinib presented KRAS mutations and high MEK activity, whereas those with NRAS mutations and high mTOR activity were poor responders. This study revealed the MEK pathway as a mechanism of resistance to LSD1 inhibitors in AML, and more importantly, it provided a rationale to modulate kinase network circuitry to potentially overcome therapeutic resistance to kinase inhibitors. This approach targets both epigenetics and signaling processes, which are key for the "two hit" theory of leukemogenesis (10).

Another study used proteomics and phosphoproteomics to identify and rationalize the synergistic effect between FLT3 and autophagy inhibitors in AML cells positive for FLT3 ITD mutations. Koschade et al used quantification of translation changes by proteomics to reveal global attenuation of translation, but an increase in the transcription and synthesis of proteins involved in autophagy, upon FLT3 inhibition (112). Phosphoproteomics analysis showed an increase in the phosphorylation of proteins related to autophagy and mTOR signaling after pharmacological inhibition of FLT3. Functional studies demonstrated that FLT3 inhibitors induced autophagy in cell lines with FLT3-ITD mutations, but not in cells with wild type FLT3 though a pathway that involves inhibition of mTOR and activation of ULK1. Autophagy is a cytoprotective mechanism that increases survival. Genetic or chemical inhibition of the autophagy induced by FLT3 inhibitors in cell lines with FLT3-ITD increased the sensitivity of these cells to FLT3 inhibitors. Consistently, the combination of FLT3 inhibitors and autophagy inhibitors synergistically reduced the viability of FLT3-ITD primary blast form AML patients *ex vivo* and reduced the tumor burthen in FLT3-ITD cell line and PDX models.

Conclusions and outlook

Proteomics and phosphoproteomics provide biological information that cannot always be extracted from genomics and transcriptomics data. Protein expression rarely overlap with mRNA expression, especially for mitochondrial proteins, an organelle targeted by new AML treatments. Similarly, protein expression may not correlate with their extent of activation. Protein phosphorylation is closer to protein function and can be used to infer the activities of kinases, which are enzymes that participate in the regulation of essentially all biological processes in normal and cancer cells (113). Therefore, proteomics and phosphoproteomics approaches – alone or integrated with genomics and transcriptomics – provide new mechanistic insights into disease pathogenesis from which to identify drug targets and biomarkers of response, to be used as input of predictive ML models, and to rationalize drug combinations that synergistically induce AML cell death. Application of these concepts more broadly will advance precision medicine and may also be used for analyzing and interpreting single cell proteomics data, now that instruments with the required sensitivity are becoming available (114), thus contributing to the deconvolution of the intra-tumor complexity that characterizes AML. Proteomics and phosphoproteomics approaches are therefore likely to play a pivotal role in the design and implementation of precision medicine in AML in the near future.

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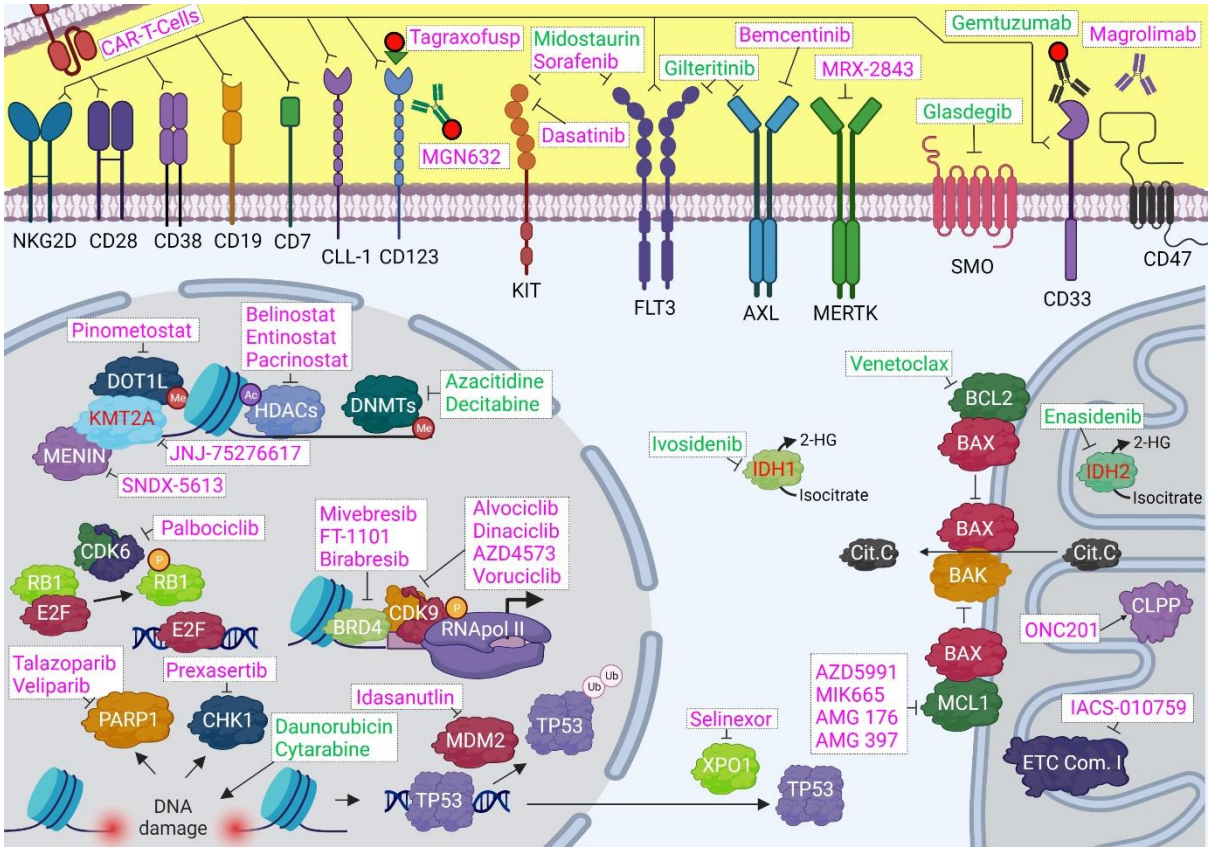

Figure 1. Compounds approved or under clinical trials for the treatment of AML and their intended targets. Names in green denote drugs that have been approved by the FDA for the treatment of AML, while those in magenta are compounds currently under clinical trials. Proteins that, when mutated or with a fusion partner produce 2HG or interact with DOT1L, are shown in red fonts.

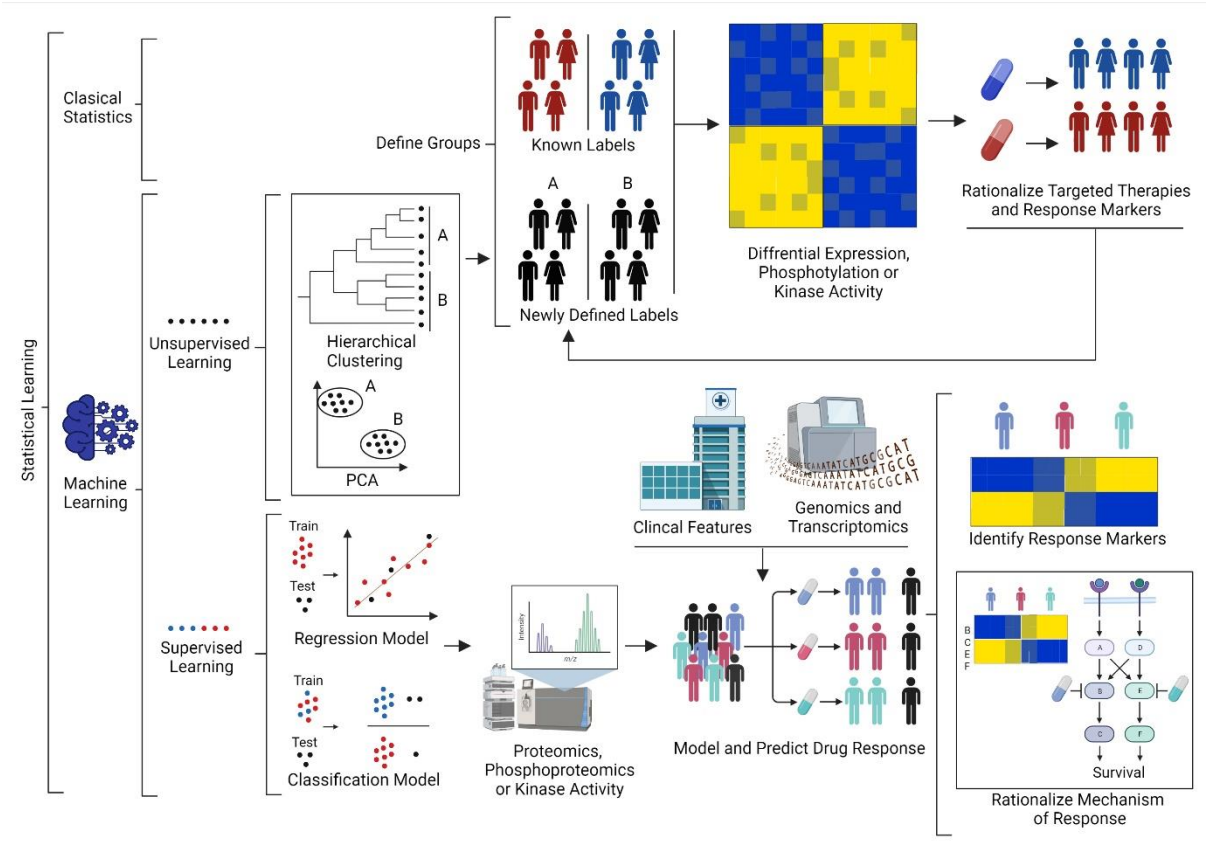

Figure 2. Data science strategies for the identification of new drug targets and response markers for the treatment of AML using proteomics or phosphoproteomics data. Individuals are classified based on known labels (e.g., resistant or sensitive to a given treatment). Differences in protein phosphorylation and expression between groups are determined using classical statistics and utilized to rationalize mechanisms, from which potential drug targets and response markers may be identified. Unsupervised ML methods, like hierarchical clustering or PCA, may use proteomics, phosphoproteomics or other omics data to define new labels and patient groups. Differences between such groups may again reveal potential treatments for these patient subpopulations, and response markers may in turn be used to refine labels further. Supervised ML is used to generate regression or classification models of drug responses from proteomics and phosphoproteomics data (or values derived thereof). Features (proteins, phosphopeptides, etc.) that define the models may be give insights into drug response mechanism.

Table 1. Frequent genetic alterations in AML

Cytogenetic alterations

Balanced	**Unbalanced**
KMT2A rearranged	Complex Karyotype
DEK-NUP	-5/del(5q)
RUNX1-RUNX1T1	-7/del(7q)
PML-RARA	-17/del(17p)
CBFB-MYH11	+8/8q
MECOM rearranged	

Single gene mutations

Signaling and kinase pathway	*FLT3, KRAS, NRAS, KIT, PTPN11, NF1, JAK2, CBL*
DNA Methylation	*DNMT3A, IDH1, IDH2, TET2,*
Chromatin-Remodeling	*ASXL1, EZH2, KMT2A, BCOR, BCORL1*
Transcription factor	*CEBPA, RUNX1, GATA2, SETBP1*
Tumor suppressor	*TP53, WT1, PH6*
spliceosome–complex	*SRSF2, U2AF1, SF3B1, ZRSR2*
Cohesin	*RAD21, STAG1, STAG2, SMC1A, SMC3*
Nucleophosmin	*NPM1*

Table 2. Classification of AML cases based on FAB, WHO and ENL

FAB Classification

FAB-M0	Minimally differentiated AML
FAB-M1	AML without maturation
FAB-M2	AML with maturation
FAB-M3	Acute promyelocytic leukaemia
FAB-M4	Acute myelomonocytic leukaemia
FAB-M4Eo	Acute myelomonocytic leukaemia with eosinophils
FAB-M5a	Acute monoblastic leukaemia
FAB-M5b	Acute monocytic leukaemia
FAB-M6	Acute erytroleukemia
FAB-M7	Acute megakaryoblastic leukaemia

WHO Classification

1-AML with recurrent genetic abnormalities	3-Therapy-related myeloid neoplasms
RUNX1-RUNX1T1 fusion	4-AML, not otherwise specified (NOS)
CBFB-MYH11 fusion	AML with minimal differentiation
PML-RARA fusion	AML without maturation
KMT2A-MLLT3 fusion	AML with maturation
DEK-NUP214 fusion	Acute myelomonocytic leukemia
GATA2(promotor)-*MECOM*(coding) fusion	Acute monoblastic and monocytic leukemia
RBM15-MKL1 fusion	Pure erythroid leukemia
BCR-ABL1 fusion (Provisional)	Acute megakaryoblastic leukemia
Mutated *NPM1*	Acute basophilic leukemia
Biallelic mutation of *CEBPA*	Acute panmyelosis with myelofibrosis
Mutated *RUNX1* (Provisional)	5-Myeloid sarcoma
2- AML with myelodysplasia (MSD)	6- Myeloid proliferations associated with Down syndrome (DS)
Phenotipic changes associated to MSD	Transient abnormal myelopoiesis associated with DS
Cytogenetic alterations associated to MSD	Myeloid leukemia associated with DS
Unbalanced: -7/del(7q), del(5q)/t(5q), i(17q)/t(17p), -13/del(13q), del(11q), del(12p)/t(12p) and idic(X)(q13)	7-Blastic plasmacytoid dendritic cell neoplasm
	Acute leukaemias of ambiguous lineage
	Acute undifferentiated leukaemia

Balanced: t(11;16)(q23.3;p13.3),	Mixed phenotype acute leukaemia with; BCR-ABL1 fusion
t(3;21)(q26.2;q22.1),	Mixed phenotype acute leukaemia with rearranged KMT2A
t(1;3)(p36.3;q21.2),	Mixed phenotype acute leukaemia, B/myeloid, NOS
t(2;11)(p21;q23.3), t(5;12)(q32;p13.2)	Mixed phenotype acute leukaemia, T/myeloid, NOS

ELN Classification

Favourable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD low
	Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD high
	Wild-type NPM1 without FLT3-ITD or with FLT3-ITD low (and no adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	Cytogenetic abnormalities not classified as favourable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214
	t(v;11q23.3); KMT2A rearranged
	t(9;22)(q34.1;q11.2); BCR-ABL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
	t (3q26.2;v); MECOM (EVI1)-rearranged
	–5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type NPM1 and FLT3-ITD high
	Mutated ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1 or ZRSR2
	Mutated TP53

Table 3. Compounds approved or under clinical trials for the treatment of AML and their intended targets

Target	Compound	Compound Class	Clinical Trial
Topoisomerase II complex	Daunorubicin	DNA intercalator	Approved
DNA polymerase complex	Cytarabine	Antimetabolite	Approved
DNMTs	Azacitidine	Pyrimidine nucleoside analogue	Approved
	Decitabine	Pyrimidine nucleoside analogue	Approved
FLT3	Midostaurin	Small molecule inhibitor	Approved
	Gilteritinib	Small molecule inhibitor	Approved
BCL2	Venetoclax	Small molecule inhibitor	Approved
Mutated IDH1	Ibosidenib	Small molecule inhibitor	Approved
Mutated IDH2	Enasidenib	Small molecule inhibitor	Approved
SMO	Glasdegib	Small molecule inhibitor	Approved
CD33	Gemtuzumab	Conjugated antibody	Approved
KIT	Sorafenib	Small molecule inhibitor	NCT05404516
	Dasatinib	Small molecule inhibitor	NCT02013648
MERTK	MRX-2843	Small molecule inhibitor	NCT03510104
AXL	Bemcentinib	Small molecule inhibitor	NCT03824080
XPO1	Selinexor	Small molecule inhibitor	NCT02835222, NCT02403310, NCT04898894
MCL1	AZD5991	Small molecule inhibitor	NCT03218683
	MIK665	Small molecule inhibitor	NCT02979366
	AMG 176	Small molecule inhibitor	NCT02675452
	AMG 397	Small molecule inhibitor	NCT03465540
CDK9	Alvocidib	Small molecule inhibitor	NCT03969420
	Dinaciclib	Small molecule inhibitor	NCT03484520
	AZD4573	Small molecule inhibitor	NCT03263637
	Voruciclib	Small molecule inhibitor	NCT03547115
BRD4	Mivebresib	Small molecule inhibitor	NCT02391480
	FT-1101	Small molecule inhibitor	NCT02543879
	Birabresib	Small molecule inhibitor	NCT02303782
ETC complex I	IACS-010759	Small molecule inhibitor	NCT02882321

CLPP	ONC201	Allosteric agonist	NCT02392572
CHK1	Prexasertib	Small molecule inhibitor	NCT02649764
PARP1	Talazoparib	Small molecule inhibitor	NCT02878785
	Veliparib	Small molecule inhibitor	NCT03289910
MDM2	Idasanutlin	Small molecule inhibitor	NCT04029688
HDAC	Belinostat	Small molecule inhibitor	NCT03772925
	Entinostat	Small molecule inhibitor	NCT01305499
	Pacrinostat	Small molecule inhibitor	NCT01912274
CDK6	Palbociclib	Small molecule inhibitor	NCT03844997
KMT2A	SNDX-5613	Small molecule inhibitor	NCT05326516
MENIN	JNJ-75276617	Small molecule inhibitor	NCT04811560
DOT1L	Pinometostat	Small molecule inhibitor	NCT03724084
CD123	MGN632	Conjugated antibody	NCT04086264
	Tagraxofusp	Conjugated interleukin	NCT04342962
CD47	Magrolimab	Naked antibody	NCT04435691
CD33	Anti-CD33-CART	CAR-T Cell	NCT05445765
CD123	Anti-CD123-CART	CAR-T Cell	NCT04318678
CLL-1	Anti-CLL-1-CART	CAR-T Cell	NCT04219163
CD7	Anti-CD7-CART	CAR-T Cell	NCT04033302
CD28	Anti-CD28-CART	CAR-T Cell	NCT04850560
CD38	Anti-CD38-CART	CAR-T Cell	NCT05239689
CD19	Anti-CD19-CART	CAR-T Cell	NCT04257175
FLT3	Anti-FLT3-CART	CAR-T Cell	NCT03904069
NKG2D	Anti-NKG2D	CAR-T Cell	NCT04658004