

***Precision Treatment of Acute Myeloid Leukemia: Predictive
Response Biomarkers for BET inhibitor JQ1***

Master's Thesis
University of Turku
MSc Degree Program in
Drug Discovery and Development
August 2018

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The originality of this thesis has been verified in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

UNIVERSITY OF TURKU
Institute of Biomedicine, Faculty of Medicine

SAAD, JOSEPH – Precision Treatment of Acute Myeloid Leukemia: Predictive Response Biomarkers to JQ1
Master's Thesis (59 pages, 4 appendices)
Pharmacology, Drug Development, and Therapeutics
Completion time (August 2018)

Abstract

Acute myeloid leukemia (AML) is a hematological malignancy characterized by the accumulation of immature and abnormally proliferating blast cells in the patient's bone marrow and peripheral blood. Despite the mounting knowledge, there remains a lack of targeted treatment options. A novel class of inhibitors targeting bromodomain and extra terminal (BET) proteins has recently demonstrated promising therapeutic activity in AML. This study sought to identify biomarkers predictive of sensitivity or resistance to BET inhibitors, using JQ1 as the representative compound. Samples from AML patients (n = 170) were analyzed for their clinical characteristics, molecular profiles, and *ex vivo* JQ1 responses. Associations between the different molecular features and the drug responses were investigated using statistical and bioinformatic approaches. As a result, a mutation in the *NCOR2* gene was identified as a predictor of sensitivity. On the other hand, mutually exclusive mutations in a set of genes (*IDH1*, *IDH2*, *TET2*, and *WT1*) appeared to confer JQ1 resistance. Incorporation of copy number variation data from the patients was in line with both hypotheses, which were also successfully validated using publicly available cell line data. The results suggest a novel strategy to identify AML patients for treatment using BET inhibition therapy. This study also demonstrates the feasibility of using the bioinformatics pipeline to identify treatment biomarkers for personalized medicine. This work supports the implementation of personalized medicine in the clinical setting, allowing the attainment of better disease management techniques and clinical outcomes.

Keywords. acute myeloid leukemia, JQ1, biomarkers, personalized medicine

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1. Introduction

1.1 Scientific Background and Study Motivation

Despite the advent of unprecedented advancements and breakthroughs in the entirety of the modern scientific domains, and of the continuous revolution that the fields of medicine and therapeutics have been thus far witnessing, human malignancies indisputably remain a major global burden, known for its largely unsatisfactory management practices. According to the World Health Organization's (WHO) 2018 fact sheets, cancer remains the world's second leading cause of death, accounting on its own for 8.8 million cases in 2015 (Cancer, 2018). This is notwithstanding the striking progress achieved in the molecular and genetic characterization of a wide variety of cancers to date. However, there appears to exist a considerable gap between the mounting knowledge in cancer biology on one side, and the actual translatability of this insight to clinical practice and therapeutic development on the other, which is depicted by the consistent increase in the reported cases of cancer worldwide, associated deaths, and the insufficiency of available treatment options for patients, who often progress to the development of more resistant malignancies. In brief, due to a series of complications associated with neoplasms, including but not limited to cell plasticity, patient/tumor intra- and inter-heterogeneity, and oncogenic compensatory signals, a distressing unmet medical need persists. Current research must therefore redirect its primary focus onto alleviating the many obstacles faced when attempting to translate the acquired molecular science of tumors to effective treatment approaches implementable at the clinical level.

1.2 Acute Myeloid Leukemia (AML)

AML is best defined as being a hematologic malignancy (HM) affecting blood progenitor cells committed to the myeloid lineage. The disease is characterized by a collection of abnormal processes involving rapid proliferation, escape from apoptosis, and aberrant differentiation patterns of the white blood cells, brought upon by the accumulation of acquired somatic mutations and chromosomal rearrangements. The aforesaid poorly or abnormally differentiated cells transform into leukemic blasts, which overpopulate the patient's bone marrow (BM), peripheral blood (PB), and occasionally other organs. This

ultimately leads to the disruption of normal hematopoiesis and to bone-marrow failure, resulting in numerous debilitating and life-threatening clinical conditions (De Kouchkovsky and Abdul-Hay, 2016; Pemovska et al., 2013).

1.2.1 Incidence

Statistics reveal that AML is the most common type of acute leukemia in adults, accounting for approximately 80 percent of the documented cases (De Kouchkovsky and Abdul-Hay, 2016). Slightly more prevalent in males than in females (ratio of 1.5:1), new AML cases are diagnosed annually in around 20,000 individuals in the United States, and account for 10,000 deaths, numbers of which have remained stable over the past years (De Kouchkovsky and Abdul-Hay, 2016; L. et al., 2016). The malignancy's risk of incidence has been set to 0.5% for any individual, a risk that is very infrequent before age 45, and which is seen to robustly increase with time, with a mean age of onset of around 67 years for both sexes (Key Statistics for Acute Myeloid Leukemia, 2018).

1.2.2 Diagnosis and Classification

Traditionally, AML was diagnosed and labeled according to the French-American-British classification system (also known as *FAB*), first published in 1976. The diagnosis criteria required that BM aspirates contain more than 30% myeloblasts (Döhner et al., 2017), and subsequently, the patient was allocated to one of seven classes, denoted as M0-M7 (as described in (Tenen, 2003)), which are defined solely based on recurrent morphologic and cytochemical characteristics (Abdou, 2016; Walter et al., 2013).

However, as the understanding of the biology of AML grew and insight was gained into its heterogeneity and clonal diversity, WHO devised a newer classification system to replace the French-American British classification scheme, which integrated genetic, immunophenotypic, biological, and clinical features for an enhanced definition of the disease. The system was first presented in 2001, and has since undergone two revisions, in 2008 and 2016 (Walter et al., 2013). Warranting an AML diagnosis now requires the detection of more than 20% myeloblasts in the patients' BM samples, or the detection of documented chromosomal anomalies associated with AML, irrespective of the blast count (De Kouchkovsky and Abdul-Hay, 2016). According to the most recent revision which

imposed several modifications to AML criteria, patient classification nowadays follows a list of 7 classes and 25 subclasses (Arber et al., 2016; Döhner et al., 2017).

1.2.3 Signs and Symptoms

In terms of signs and symptoms, an AML patient generally presents to the clinic suffering from fatigue, weight loss, and/or anorexia, combined with signs of leukocytosis and BM failure, notably anemia and thrombocytopenia. All occurring symptoms are the direct result of the accumulation of the immature blast cells and their harmful effects on normal hematopoiesis. Ultimately, the patient succumbs to the disease either due to the infiltration of blast cells into the central nervous system and/or the lungs (or other vital organs), or due to complete BM failure, infection and bleeding (De Kouchkovsky and Abdul-Hay, 2016; Elihu, 2014).

1.2.4 Genetic Complexity and Risk Stratification

Upon presentation to the clinic, a patient is stratified into a risk group, primarily determined by two main factors: the probability of treatment-related mortality, and more importantly, that of resistance to standard therapy. These factors are determined by the patient's mutational and cytogenetic landscapes (Elihu, 2014), and efforts have been continuously made for more accurate characterizations. To illustrate, in a major attempt to unveil the genomic heterogeneity of AML, the Cancer Genome Atlas (TCGA) Research Network profoundly analyzed in 2013 two hundred *de novo* adult cases of AML (The Cancer Genome Atlas Research Network, 2013). The study confirmed that, despite the fewer mutations detectable in AML genomes compared to other cancers, AML entails a wide genetic diversity based on a variety of potential driver mutations (typical nature of myeloid leukemogenesis), and on a complex pattern of mutational co-occurrences and exclusivities which hint to existent molecular synergies and redundancies whose biological relevance remains to be elucidated. The analysis reported a set of recurrently mutated genes, classified into 9 distinct functional categories (The Cancer Genome Atlas Research Network, 2013; Grove and Vassiliou, 2014).

Thanks to the enhancement of the disease's molecular background, the European Leukemia Net (ELN) allows, since its most recent revision in 2017, the incorporation of

information retained from the patient's blasts' karyotype and specific molecular markers into an updated risk stratification scheme. In brief, the classification was simplified to include three categories (favorable, intermediate and adverse prognosis), which are defined based on gene mutation statuses (*NPM1*, biallelic *CEBPA*, *RUNX1*, *ASXL1*, *TP53*, and *FLT3-ITD*), specific chromosomal aberrations (translocations, deletions), karyotype aberrations (complex, monosomal), and harbored allelic burden (notably of *FLT3-ITD*) (Döhner et al., 2017; Kontro, 2017). While evidence concerning the involvement of other notable genes (*DNMT3A*, *IDH1/2*, genes in the chromatin/spliceosome group besides *ASXL1* and *RUNX1*) in the prediction of prognosis has been mounting, the ELN awaits further in-depth studies to be performed before their integration into its risk stratification table (Döhner et al., 2017).

1.2.5 Current Treatment Regimens and Outcomes

The standard treatment regimen in AML is largely based on cytotoxic chemotherapy. In particular, treatment consists of a combination of cytarabine with an anthracycline (an antileukemic drug belonging to the antitumor antibiotics class acting as topoisomerase II inhibitors, such as idarubicin, daunorubicin and doxorubicin). The goal of treatment is achieving a complete remission (CR) state, defined as a blast count lower than 5% in the patient's BM. Treatment can be therefore divided into 2 main phases. The first phase, referred to as remission induction, aims at achieving the CR condition described above. This is followed by a second phase known as post-remission, which starts with consolidation (maintenance of the remission state using chemotherapy after the patient has recovered from the first, aggressive treatment stage, in the aim of eradicating the remaining blast cells) and then proceeds to maintenance treatment (use of low dose chemotherapy to prevent relapse). Chemotherapeutic treatment can also be accompanied by hematopoietic stem cell transplantation for patients with high risk of relapse. However, allogeneic transplantation remains a risky procedure with several undesirable long-term complications and is largely dependent upon the availability of a matched donor, the patient's age, and her/his specific disease characteristics (Mohty and Mohty, 2011). Although the characterization of AML has improved over the past several decades, standard therapy has remained unchanged for over 40 years. However, the efficacy of

conventional therapy has reached a plateau, as it has been reported that the current treatment strategy effectively cures less than 40% of patients below 60 years of age, but only as little as 10-15% of the more elderly population (Döhner et al., 2015). Furthermore, an estimated average of 30% of patients will never achieve CR, and as many as 70% of those who do will progress to a relapsed and more aggressive disease with very poor prognosis within 3 years from the first CR (Cheng et al., 2014). The most recent estimate of the 5-year survival rate for AML patients according to the National Institute of Health is as low as 27% percent (Cancer Stat Facts: Leukemia - Acute Myeloid Leukemia (AML), 2018).

Finally, the overall disease-free survival and remission rates are significantly worse for both older patients and for those assigned an elevated risk at diagnosis (for example those patients suffering co-morbidities such as myelodysplastic syndrome (MDS) or developing treatment-related or secondary AML due to previous exposure to chemotherapies), compared to the rest of the AML population (Dombret and Gardin, 2016).

Given the extremely dismal outcomes (especially for patients judged unfit for the described intensive treatment approaches), and the almost complete lack of successful innovation in targeted AML treatment (except for ATRA used in the case of acute promyelocytic leukemia (APL), and for midostaurin, recently approved for the treatment of AML with *FLT3* mutations), there exists an urgent need for novel treatment options. Those should ideally be able to effectively utilize the mounting molecular and cellular knowledge in the field, as well as the patient-stratification processes that have been devised, thus allowing the establishment of “genetically-driven” personalized treatment regimens. Out of the numerous molecularly-targeted options that are currently being investigated (with so far limited success in terms of clinical translatability), one class of molecules, referred to as the Bromodomain and Extra-Terminal (BET) inhibitors, has sparked particular interest over the past few years in cancer treatment in general, and in HMs in particular, and will constitute the major focus of this work.

1.3 Bromodomains & Bromodomain and Extra Terminal Family Proteins

1.3.1 Epigenetics and the Family of Bromodomain (BRD)-Containing Proteins

The term *epigenetics* defines the entirety of gene expression regulatory mechanisms that occur in the cellular genome without involving modifications to its DNA sequence. Histone post-translational modifications (PTMs) constitute one of the most immediate contributors to epigenetic regulation by affecting the chromatin architecture through a variety of mechanisms, involving among others the modulation of DNA affinity to histones, histone interactions among each other and with other proteins such as chaperones, and the creation of “docking sites” to other complexes, which themselves affect nucleosome packing, gene transcription, and DNA repair and replication mechanisms. It has been established that PTM-affecting enzymes in the cell can be classified into one of three distinct classes: PTM writers, which add a modification to the histone amino acids (such as histone methyltransferases and histone acetyltransferases (HATs)), PTM erasers, which remove such modifications (including histone deacetylases (HDACs) and lysine demethylases) and PTM readers, which recognize specific PTMs and mediate downstream effects (those include bromodomain- and chromodomain-containing proteins, such as the BET proteins) (Ferri et al., 2016; Falkenberg and Johnstone, 2014). These epigenetic agents are known to play a key role in disease development, either directly (when they are mutated and functionally aberrant) or indirectly (when they drive erroneous gene transcription regulation patterns due to dysfunctional upstream drivers). In both cases, those enzymes can serve as valuable drug targets for renormalizing transcriptional activity, with the notable example of the previous success achieved in HDAC inhibition with several approved drugs (e.g. vorinostat, belinostat, panobinostat and romidepsin).

The family of BRD-containing proteins has been recently characterized in the human proteome: it is composed of 46 members with a total of 61 well-defined BRDs, distributed over 8 major clades, based on sequence and structure homology (Ferri et al., 2016). All members contain one (or more) BRD(s), and all are involved in *epigenetic processes* including chromatin remodeling and transcriptional activity. The term *bromodomain* refers to the primary amino acid sequence contained by all members of the family, first

discovered as a chromatin-modifying factor in the *Drosophila brahma* in 1994 by Tamkun et al (V. et al., 2010). Roughly 110 amino acids-long, a BRD is an epigenetic reader that specifically recognizes ϵ -N-acetylated lysine residues (Kac) (namely H2AK15Ac, H2AK36Ac, H3K14Ac and H4K5Ac). This results in the euchromatinization of DNA and the initiation of transcriptional activity in the cell via the recruitment of positive transcription factors by BRD modules. Different BRDs recognize Kac in different settings leading to different downstream effects, which largely depend on the possible structural interactions that form between the ligand and the proteins' binding sites (Ferri et al., 2016; Wang and Filippakopoulos, 2015). A detailed structural description is available in Ferri et al.'s *Bromodomains: Structure, function and pharmacology of inhibition (2016)* (Ferri et al., 2016).

1.3.2 Bromodomain and Extra Terminal Family Proteins (BETs)

To date, most of the attention for BRD-containing proteins has been centered around a subclade of family II, a group composed of four members (BRD2, BRD3, BRD4 and the testis-specific BRDT), collectively referred to as the BETs (Ferri et al., 2016). Overall, the 4 members depict a conserved modular architecture, composed, from the N to the C terminus, of the following shared features: a first N-terminal BRD effector module BD1, a conserved motif A, a second N-terminal BRD effector module BD2, a conserved motif B, an extra-terminal recruitment domain ET, and a conserved motif SEED. BRD4 and BRDT share the additional particularity of having a conserved C-terminal motif (Wang and Filippakopoulos, 2015). It has been found that each feature in the described architecture of BET proteins is essential for the formation of key interactions, which are crucial for the assembly of the transcriptional machinery that will exert the downstream effects of those proteins' activation, i.e. transcription initiation and elongation. As expected, BD1/2 bind mostly to histones, but also to non-histone proteins (e.g. NF κ B, GATA1, TWIST...), at single Kac (or occasionally at paired Kac which confers additional stability, as is the case in histone H4). When present (i.e. in the BRD4 and BRDT members), the C-terminal motif has been reported to promote the formation of the active form of the positive transcription elongation factor b (P-TEFb), by dissociation of the inhibitory subunit in the complex, HEX1M1, which in turn leads to the phosphorylation

and the activation of RNA Polymerase II. As for the extra-terminal recruitment domain, it has been suggested to initiate crucial protein-protein complexes with downstream positive transcriptional effectors (such as with the methyltransferase NSD3) independently of P-TEFb. Finally, motif A is thought to act as a nuclear localization signal for the proteins, while motif B is crucial for their homo- and/or hetero- dimerization (Wang and Filippakopoulos, 2015).

BET proteins have been recognized as essential adaptors that play a delicate role in controlling the tethering of specific transcriptional complexes to chromatin in a tissue- and environment- specific fashion, leading to the activation of relevant transcriptional programs. It thus becomes obvious that even slight deregulations of this activity are sufficient for disease development, including inter alia tumorigenesis, inflammation, and viral infections. Two cancer-related illustrations are hereby reported. It has recently been found, in lung adenocarcinoma, that BRD2 greatly contributes to the disease by forming a complex with RUNX3, which activates the expression of p21 (a cell-cycle inhibitor), promoting lack of differentiation in a KRAS-dependent (oncogenic) fashion. Similarly, a BRD4-NUT oncogenic fusion detected in NUT midline carcinomas (NMCs) was shown to produce an oncogenic product that blocked differentiation and maintained constitutive tumor growth by the creation of transcriptionally-inactive, hyperacetylated foci, through the constitutive recruitment and sequestration of p300. This in turn leads to the acetylation, and therefore, the inhibition of p53. p300 and other HATs' sequestration by the NUT moiety also leads to a local sequestration of BRD4 and other transcription machinery components at those foci, creating a nearly complete functional repression of the BRD4, and depleting its capability of inducing the expression of other genes critical for differentiation (e.g. *FOS*). Finally, it has also been hypothesized that the fusion product favored, through colocalizing BRD4 at the loci, the transcription of the proliferative and anti-differentiation gene *MYC*. Attempts to knockdown the fusion or to incapacitate binding of BRD4 were shown to effectively restore normal functions, namely through squamous cell differentiation (restored *FOS* expression) and arrest of the cell-cycle (decreased *MYC* expression) at G1 (Wang and Filippakopoulos, 2015; Reynoird et al., 2010; Chaidos et al., 2015).

1.3.3 BET Inhibitors (BETi)

Epigenetic modifiers continue to spark interest in the field of targeted drug discovery, and BET proteins have recently been recognized as very promising druggable targets in a variety of disorders, including inflammation, viral infection, and importantly, cancer (Wang and Filippakopoulos, 2015). This is the result of previously reported genetic and molecular events that have established clear associations between BET proteins and tumorigenesis, notably but not exclusively, in HMs (Chaidos et al., 2015). For instance, it was observed that the overexpression of BRD2 in mouse lymphocytes leads to the development of B cell lymphomas. On the other hand, BRD4 inhibition has produced promising antileukemic effects in AML. Similarly, BET inhibition has been shown to decrease *MYC* expression and to restore normal cellular functions in a variety of cancers, including HMs (AML, mixed lineage leukemia, T-cell acute lymphocytic leukemia) and solid tumors (prostate and ovarian cancers, lung adenocarcinomas, skin malignancies) (Wang and Filippakopoulos, 2015; Chaidos et al., 2015).

The first potent and selective probe investigated is the thieno-tiazolo-1,4-diazepine, known as the positive enantiomer (+) of JQ1 (denoted henceforth as JQ1 for brevity). Competitively binding at the BRD pocket of BETs, JQ1 effectively displaces those proteins from acetylated chromatin at the micromolar range, with a notable affinity to BRD4. This has shown remarkable preclinical efficacy in NMC (described in Section 1.3.2 and in detail by *Filippakoulous et al., 2010*) (Filippakopoulos et al., 2010), and offered a first validation of the rationale of inhibiting BET proteins as a novel oncologic therapeutic approach. Since then, several studies have focused on verifying the extendibility of the observed efficacy to other cancers including leukemia. For example, the study done by Baltz et al. in 2016 successfully demonstrated the usefulness of the molecule in selected myeloid leukemia cell lines, in terms of the inhibition of proliferation and colony formation, at subtoxic concentrations (Baltz et al., 2016). The second study, very relevant to *MYC* overexpression also detected in AML and previously described in Section 1.3.2, was presented by Brondfield et al. in 2015 and showed that JQ1 exhibited in vitro and in vivo efficacy against numerous *MYC*-overexpressing AML models by constitutively lowering the expression of that protein in all sensitive cells (irrespective of the genetic abnormalities underlying *MYC* deregulation) (Brondfield et al., 2015).

Owing to those promising preclinical results, numerous BETi have been investigated, and several of them are already being tested in as many as 20 ongoing clinical trials (CT). For instance, I-BET762 (also known as GSK525762) has previously reported binding profiles and affinities to BET proteins similar to those seen with JQ1 and has proven effective in downregulating the expression of several inflammatory genes in cell studies. It is currently being investigated in several ongoing CTs for solid tumors (NCT03266159), for relapsed, refractory HMs (NCT01943851) and in NMC (NCT01587703). Similarly, OTX015 (also known as MK-8628) has been used in several studies, notably for NMC and lung and prostate cancers, and is about to be used in a dose-escalation study recruiting both de novo and secondary MDS-to-AML patients (NCT02698189). Other molecules currently in CTs targeting AML patients include FT-1101 (NCT02543879) and CPI-0610 (NCT02158858).

1.4 Individualized Medicine in AML Using BETi

1.4.1 Need for Individualized Therapies

Given the presented limitations still faced by applying AML therapies, the need for an approach that would bridge knowledge to predictable therapeutic benefit becomes more essential than ever. The plethora of genetic and molecular information nowadays made available for patients shall allow for a more informed and meticulous understanding of the mechanisms (and targets) underlying their disease subtypes and possibly their responsiveness and resistance to treating agents.

Such an approach can be referred to as *personalized, individualized or precision medicine*, and operates at the individual, rather than the population, level. This is expected to be of immense relevance for the treatment of different cancers (particularly AML), owing to the high degrees of intra- and inter-heterogeneity they harbor at the molecular level. By rendering therapeutic interventions chiefly tailored to a subject's underlying genetic makeup, individualized medicine promises a better management of patients by optimizing drug benefits, and cutting off undesirable costs and side effects, while remaining optimally safe and tolerable.

It is strongly believed that, for the sake of this work, this strategy will ultimately guide successful selection of AML patient subgroups who are expected to benefit the most from treatment by BET inhibition.

1.4.2 Individualized Systems Medicine (ISM)

As a direct implementation of the personalized medicine strategy described above, ISM, initiated at the Institute for Molecular Medicine Finland (FIMM) and first described in the research article “*Individualized Systems Medicine Strategy to Tailor Treatments for Patients with Chemorefractory Acute Myeloid Leukemia*” (2013), is introduced as a systematic analysis workflow (*Annex I*). Specifically, ISM is defined as a “comprehensive functional strategy” that can effectively detect cancer cell dependencies to specific agents based on functional assessment using an *ex vivo* technique referred to as *Drug Sensitivity and Resistance Testing (DSRT)* (Pemovska et al., 2013). In brief, ISM starts by exposing patient-derived tumor and healthy samples to a panel of drugs consisting of a wide range of cancer therapeutics as well as other investigational molecules. It then proceeds to quantify the observed differential responses to each drug, using a novel scoring technique, and expressed either as a *Drug Sensitivity Score (DSS)* or alternatively as a *differential Drug Sensitivity Score (dDSS)*. The *DSS* is seen as a direct measure of a sample’s responsiveness (toxicity/viability) to a drug, taking into account multiple characteristic features from the dose-response curves generated (Yadav et al., 2014). It is believed to accurately reveal how effective a drug is in treating a particular cell sample, in such a way that a higher *DSS* reflects a higher sensitivity. Similarly, a cancer sample’s *dDSS* is a normalized value of the *DSS*, obtained by subtracting the healthy cells’ *DSS* for the same treatment agent (Yadav, 2017). The drug sensitivity and resistance profiles can be also integrated with the genomic and transcriptomic profiling done for the samples from the patients, elucidating the molecular mechanisms of the progression of the disease and its response to treatment attempts. Therefore, ISM allows the identification of targeted and actionable drugs for AML patients in a personalized fashion, which can eventually translate to clinically relevant (and low-risk) treatment options.

1.5 Research Question, Hypothesis, and Goals

The aim of the study was to identify genomic biomarkers that confer sensitivity or resistance to BET inhibition in AML patients. It was hypothesized that BETi effectiveness in AML treatment is dependent on specific underlying biomarkers harbored by respondent samples, which can be detected and validated using ex-vivo drug screening and molecular profiling. Through the implementation of a bioinformatics pipeline capable of performing a systematic investigation of clinical and genomic data collected for AML patient samples having been exposed to the BETi JQ1, this project sought to unveil specific patient characteristics, precisely clinical and/or mutational patterns, which appear to be significantly predictive of AML sample sensitivities to JQ1.

The significance of the study lies in its contribution to the implementation of personalized medicine into the conducted research in the molecular therapeutics' settings, in order to render findings more informative and realistically-linked to the complex molecular profiles of patients.

1.6 Summary

It is indisputable that AML remains until today a challenging cancer, where therapy options are still largely suboptimal. Despite the mounting high-quality knowledge concerning the disease's classes and subtypes at the molecular and genetic levels, targeted therapies are still far from being effectively implemented in the clinics. The persistent unmet medical need has to be urgently addressed, considering the devastating effects that are imposed on patients. Among the abundant novel targeted molecules investigated for their potential in treating AML, BET proteins are particularly promising targets for attenuation, and have constituted over the past few years, a major field of focus in AML drug discovery. It is unlikely that a single drug can be suitable for treating all AML patients due to the genetic and molecular heterogeneity. Therefore, it is believed that the class of BETi should be investigated more closely in a patient-specific context, in the aim of identifying specific characteristics and/or genomic signatures that can explain and ultimately predict the potency and efficacy of such molecules in AML tumors. This study thus seeks to rationalize the implementation of personalized therapeutics for cancers in routine clinical environments, in order to better guide the selection of drugs/drug

combinations as more-informed and effective treatment options for patients, promoting proper disease management and general well-being.

2. Results

2.1 Dose-Response Curves

From our entire cohort, the ten most resistant samples to JQ1, with DSS (the definition of which can be found in Section 4.2) scores comprised between 0 and 4.84, and the ten most sensitive ones, with their respective scores ranging from 22.48 to 26.23 generate 20 four-parameter log-logistic dose-response curves as shown in Figure 1.

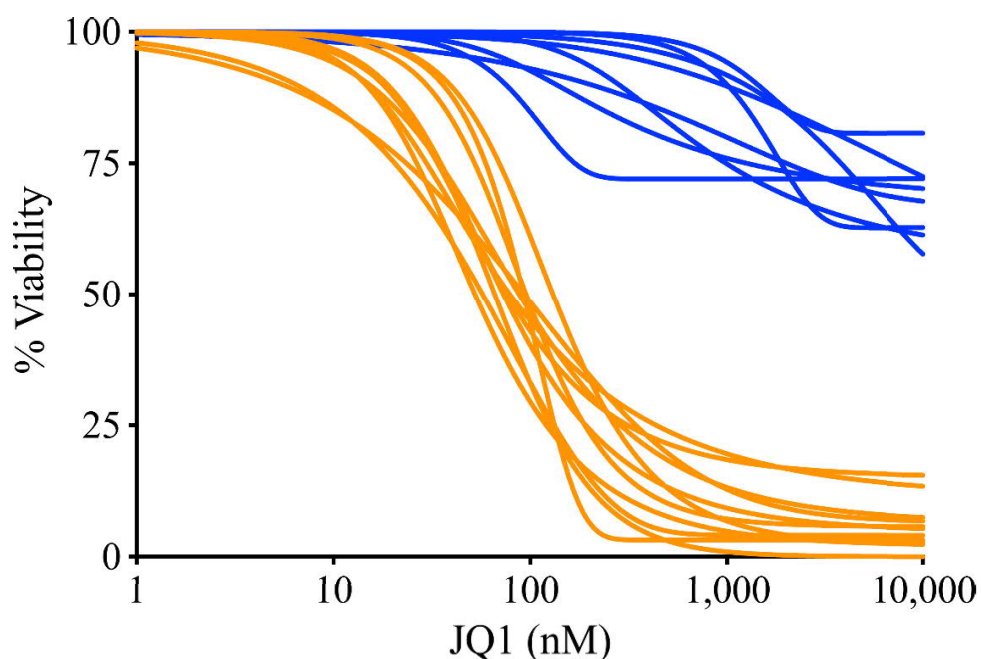


Figure 1. Dose-response curves of the 10 most-sensitive (orange) and the 10 most-resistant (blue) AML samples to JQ1 treatment.

The dose-response curves illustrated two separate clusters: the non-responsive samples (on average low DSS values, high area under the dose-response curve (AUC) values, and high absolute half maximal inhibitory concentration (IC_{50}) values) demonstrated limited reductions in viability (a maximum of 40% reduction under the highest JQ1 dose),

whereas the sensitive samples (on average high DSS values, low AUC values, and low absolute IC_{50} values) showed large reductions in viability (reaching 100% inhibition for certain samples well before the highest JQ1 dose was used).

2.2 Patient and Sample Characteristics

As is often the case in statistical analysis, the selection of suitable statistical tests to use depended on the assumptions made concerning the distribution of the data in the samples (in our case, the JQ1 DSS readout). Though DSS values by nature are percentages, due to the method of their calculation (bounded between 0 and 100) (Yadav, 2017), the 170 JQ1 DSS values in our cohort demonstrated a normal distribution, as shown in Figure 2, passing the classical Shapiro-Wilk test. Nevertheless, when comparisons concerned groups composed of such small sample sizes where the normality assumption could no longer be made, non-parametric equivalents were selected to ensure the quality of the consequent conclusions.

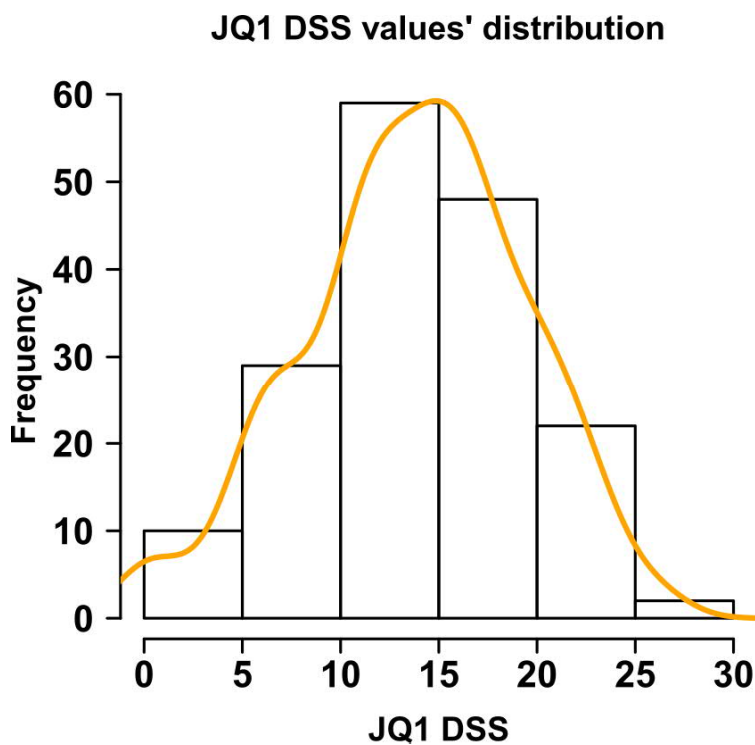


Figure 2. The bell-shaped curve of JQ1 histogram suggests a normal distribution (Shapiro-Wilk normality test p-value = 0.21).

2.2.1 Blast Content

Out of the total cohort of 170 AML samples, a clinically-recorded blast content was made available for a subset of 135 samples. In those samples, the association between the blast percentage value and the observed JQ1 response was evaluated using two statistical tests. Based on a simple linear regression: $JQ1_DSS \sim \beta_0 + \beta_b * blast_content + \varepsilon$, the blast showed zero association with the JQ1 response, shown by the regression coefficient close to 0 ($\beta_b = 0.029$, p-value = 0.102). Results from a Pearson correlation test yielded a similar conclusion with an insignificant correlation ($r = 0.141$, p-value = 0.102).

After filtering out those samples having a blast percentage lower than 20% (value determined by the WHO guidelines for a primary AML diagnosis – see Section 1.2.2), the same tests were repeated on the remaining 118 samples. The same conclusion was attained, with a linear regression coefficient β_b still estimated at 0.029, but with a larger p-value of 0.22, and a Pearson correlation coefficient $r = 0.114$ lacking statistical significance (p-value = 0.22). The results were summarized in Figure 3.

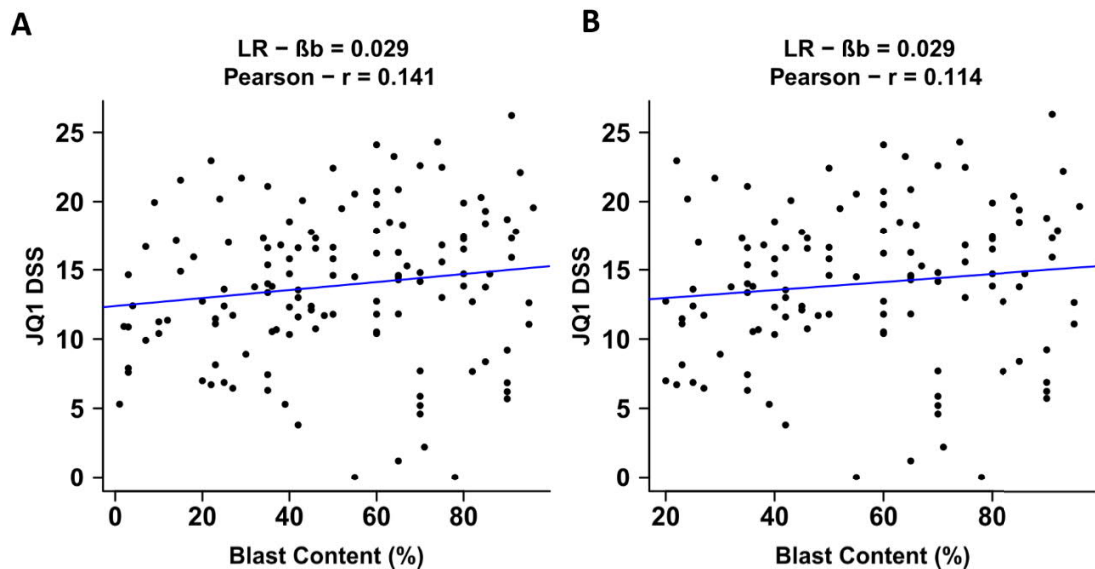


Figure 3. Linear regression of JQ1 DSS with Blast percentage for A) all AML samples having a blast content (N=135) and B) AML samples with a blast content $\geq 20\%$ (N=118).

2.2.2 Culture Media

To detect a possible effect that the culture media might have on the drug-treatment readouts, 17 samples tested with JQ1 in both conditioned medium (CM) and mononuclear cell medium (MCM) cultures (definition of which can be found in Section 4.3) were compared for their paired sensitivity values using non-parametric statistical tests (due to the small sample sizes and the distribution of their DSS values): a Spearman correlation and the Wilcoxon rank sum test. As for the remaining samples tested in either of the two media, a parametric unpaired 2-sample t-test was performed to evaluate whether a statistically significant difference existed between the CM-cultured (105) and MCM-cultured (46) samples. The conducted tests are summarized in Figure 4.

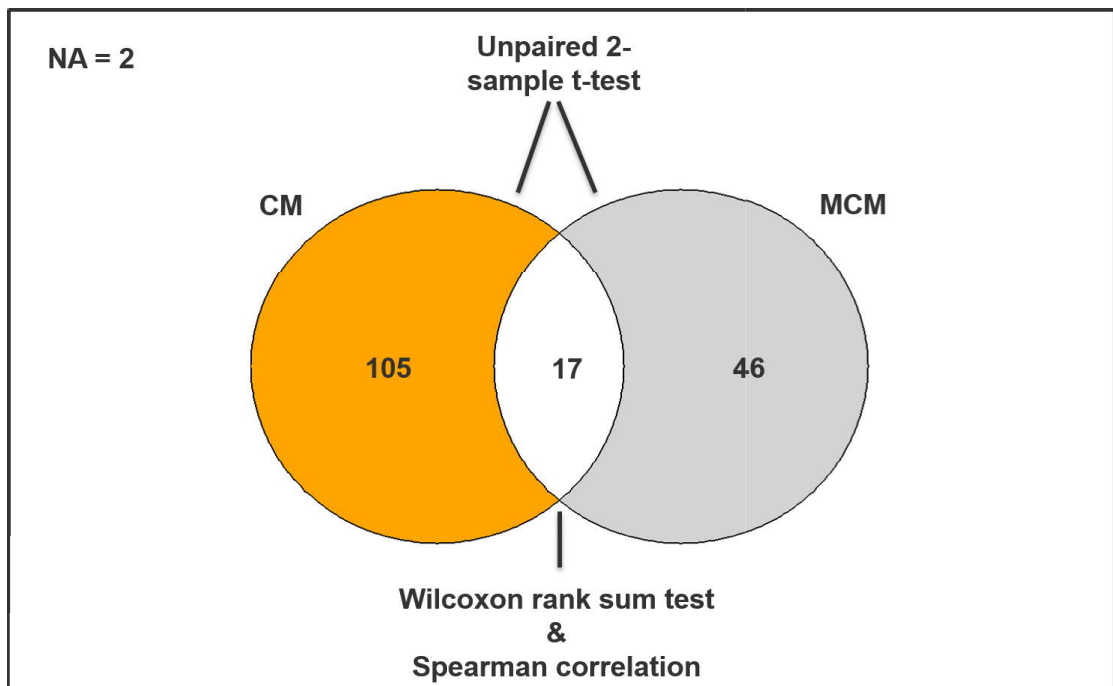


Figure 4. Statistical evaluation of the culture media effect on JQ1 response. NA: Not available.

For the 17 samples cultured in CM and MCM, statistical test results reveal a true and robust correlation existing between the scores obtained from both media with a Spearman correlation of 0.83 (p-value = 4.29e-05), as is shown in Figure 5A. Similarly, the Wilcoxon test performed on the matched scores failed to reject the null hypothesis,

implying no true difference between the two media (p-value = 0.683). In agreement with those results, the unpaired 2-sample t-test run for those samples tested in either one of the two media (105 in CM; 46 in MCM) also failed to prove a significant difference between the means: $t = -1.695$, 95% CI [-3.605, 0.288], p-value = 0.094 (Figure 5B).

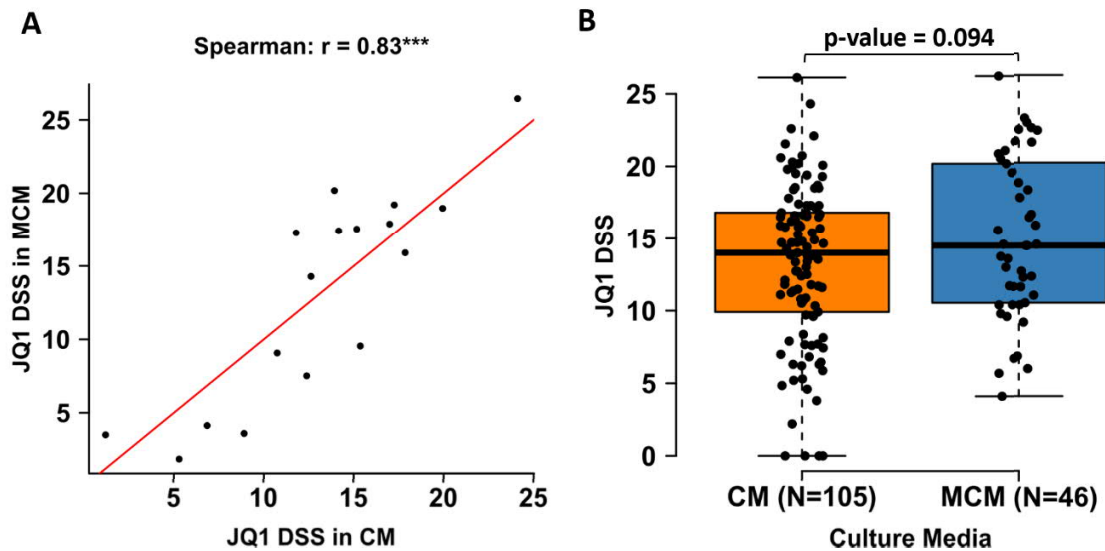


Figure 5. Cell culture medias have no significant effect on the obtained JQ1 responses, as demonstrated by the consistency of the readouts obtained for the same samples tested in two media (A), and by the lack of significant difference between the median readouts of the responses from the two cultures (B).

2.2.3 Sample Clinical Characteristics

To assess whether the age of the patient at the time when the sample is obtained is predictive of the sensitivity to JQ1, a simple linear regression was built of formula: $JQ1_DSS \sim \beta_0 + \beta_a * age + \varepsilon$ and fit using the samples for which an age could be retrieved (N = 145). The model returned a predictor coefficient that is slightly smaller than 0 ($\beta_a = -0.034$), implying a weak trend of inverse association between JQ1 DSS and age, which is nonetheless insignificant (p-value = 0.277). Similarly, the Pearson correlation test yielded a small negative coefficient of -0.091 lacking significance (p-value = 0.277) (Figure 6).

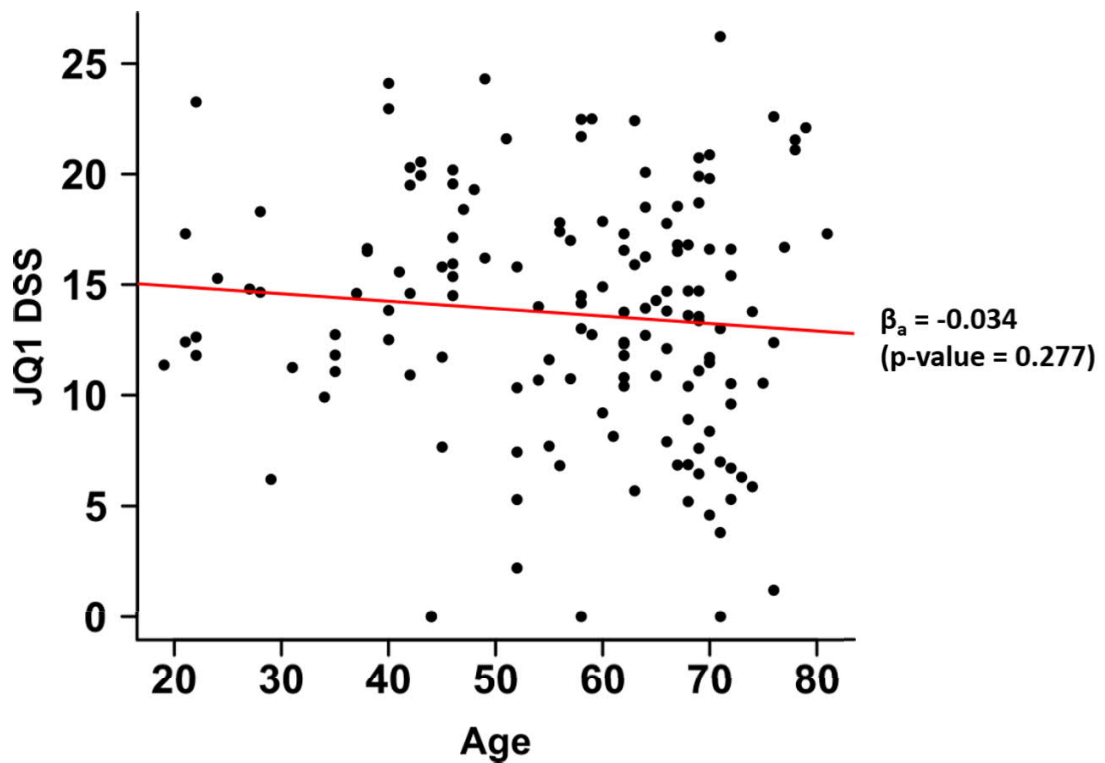


Figure 6. The age of the patient upon sample collection is not significantly associated with the response of the sample to JQ1.

Categorical variables reflecting the sample’s status (Diagnostic D vs Relapsed R) and the ELN risk assigned to the patient at the time of diagnosis also fail to show predictive potential for JQ1 sensitivity (Figures 7 and 8).

Using an unpaired t-test, the sample status analysis reports a slight decrease in the mean score of relapsed samples with respect to diagnostic ones ($t = -1.431$), while lacking significance (95% CI = [-2.904, 0.464], $p\text{-value} = 0.154$) as shown in Figure 7.

Figure 8 shows the results of the ANOVA test for the multiple group comparison of JQ1 DSS scores for 110 samples, stratified according to the risk assigned to the donors: once again, the score appeared to be consistent across the groups, and thus, independent of the risk ($p\text{-value} = 0.748$).

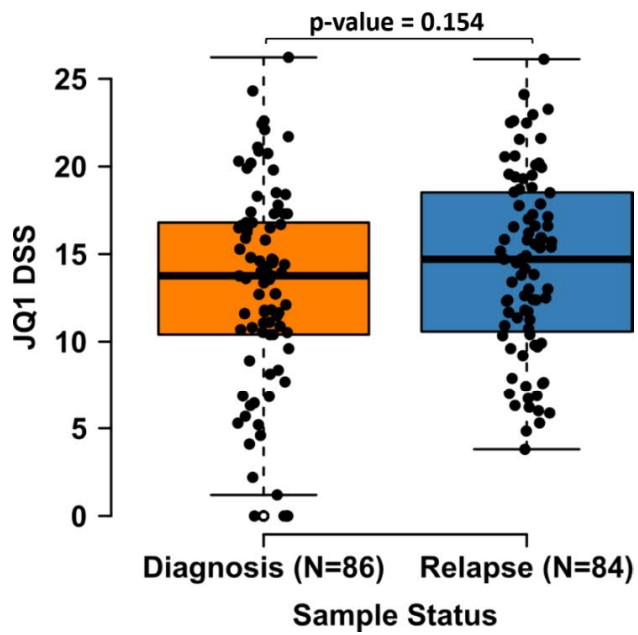


Figure 7. The JQ1 response is independent of the sample status.

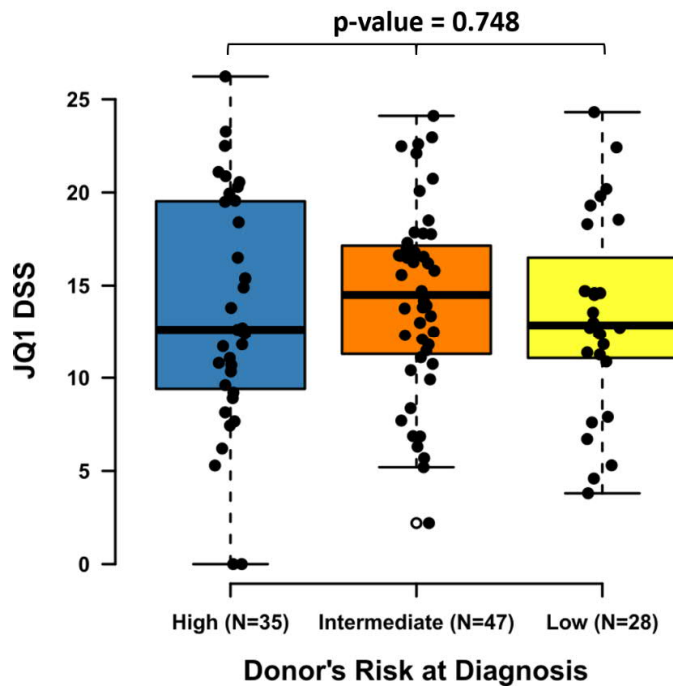


Figure 8. The JQ1 response is independent of the sample's donor's ELN risk.

2.3 Genomic Analysis

2.3.1 Mutation Burden

For 139 out of 170 samples, genomic data was available. The number of mutated genes in those samples varied with respect to the chosen p-value for the inclusion of a somatic mutation: this resulted in 4425 mutated genes for a softer threshold of 0.05, but only 2813 genes for a more stringent cutoff, set at 0.01. Before examination of individual associations between particular genes and sample sensitivity, a general investigation of the effect of a sample's mutation burden (definition of which can be found in Section 4.6) on its JQ1 response was investigated. However, given the high deviation in the mutation burden value across samples, this measure was log-transformed to approximate a normal distribution for improved visualization purposes. Afterwards, a simple linear model of the form $JQ1_DSS \sim \beta_0 + \beta_m * \log_2(\text{mutation burden}) + \varepsilon$ was calculated. Using either the larger or smaller set of mutations included, the linear regression returned coefficients whose magnitudes were small and p-values very large, implying no predictive potential by the mutation burden on JQ1 sensitivity (Figure 9A: the bigger cohort, $\beta_m = -0.142$ and p-value = 0.713; Figure 9B: the smaller cohort, $\beta_m = 0.118$ and p-value = 0.609). Similarly, Pearson correlations lead to the same conclusions, with coefficients of -0.032 (p-value = 0.713) and 0.044 (p-value = 0.609) for the larger and smaller samples, respectively.

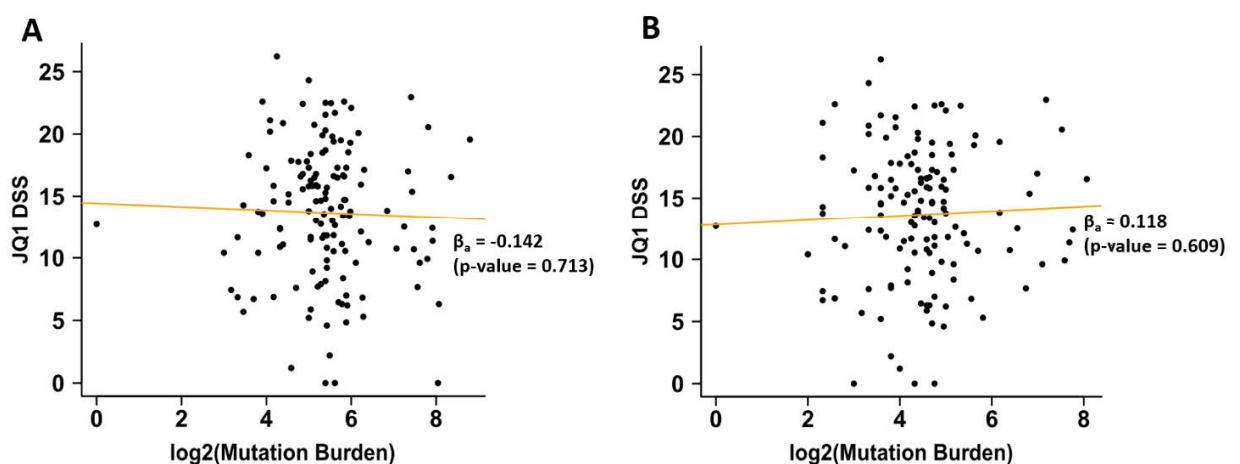


Figure 9. The mutation burden of a sample (taken in the \log_2 scale) is non-predictive of the sample's sensitivity to JQ1. This concerns mutations with a p-value inferior to A: 0.05 and B: 0.01.

2.3.2 Mutational Analysis

Using the larger cohort of mutations (p-value ≤ 0.05), each of the 4425 genes was used to fit a simple logistic regression model of formula: $\log \left[\frac{P(\text{mutation})}{1-P(\text{mutation})} \right] = \beta_0 + \beta_J * JQ1_DSS + \epsilon$, where $P(\text{mutation})$ represented the probability of the gene to be mutated in a sample; the log ratio, or log odds on the left hand side of the formula takes the values from minus to plus infinity, which can be regressed by the JQ1 response. The regression coefficient β_J thus represented the association between a gene's mutation status and the JQ1 response. Each model was fitted using all 139 samples having mutation data available, resulting in 46 out of the 4425 genes with statistical significance (i.e. with p-values ≤ 0.05). Out of these 46 genes, 11 were predicted with a positive association between existence of the mutation and JQ1 response, while the remaining 35 were predicted with an inverse relationship (Supplementary Table 1). Based on relevant sample sizes, on previous literature reports, and on prior biological knowledge, a subset of those genes was taken forth for more detailed analysis in sections 2.3.2.1 and 2.3.2.2.

2.3.2.1 Evaluation of literature-reported biomarkers

Mutations to two genes, namely *NPM1* and *FLT3*, have previously been shown to confer enhanced preclinical sensitivity to BET inhibition in AML (Abedin et al., 2016; Dawson et al., 2013). An investigation of the extendibility of such claims to our ex vivo testing of primary patient samples was performed for the sake of verification.

Contrary to expectations, *NPM1* mutated samples (N=26) did not appear to be consistently more sensitive to JQ1 than wild-type *NPM1* samples. With a large stretch of DSS scores in *NPM1*-mutated samples (ranging from very resistant to very sensitive) and an insignificant gene coefficient ($\beta_J = -0.039$; p-value = 0.326), the comparison concerning this AML subpopulation was, according to our cohort and as is shown in Figure 10, largely inconclusive.

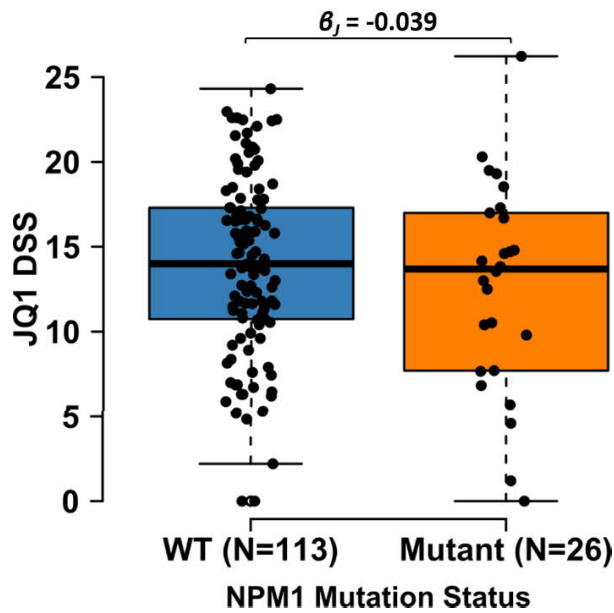


Figure 10. No statistically significant difference between samples which harbor an *NPM1* mutation and those who do not, in terms of response to JQ1-treatment.

In contrast, samples harboring a *FLT3* mutation (N=43) did display, as expected, a statistically significant increase in sensitivity to JQ1, with respect to the wild-type samples. This was illustrated in the regression model by a *FLT3* coefficient, slightly, yet robustly, greater than 0: $\beta_j = 0.084$ (p-value = 0.023) (Figure 11A). Afterwards, the *FLT3* mutants were split into either those harboring an internal tandem repeat (ITD; N=29) or those having a point mutation in their tyrosine kinase domain (TKD; N=15). Notice that one sample (4361_2) had both an ITD and a TKD, and thus belonged to both groups. Each of those subgroups were compared to the remaining samples in the cohort: coefficients of both subgroups remained slightly larger than zero ($\beta_j = 0.059$ for *FLT3*-ITD and $\beta_j = 0.083$ for *FLT3*-TKD), however, the statistical significance of both models was lost (p-value = 0.146 for *FLT3*-ITD and p-value = 0.128 for *FLT3*-TKD). The results are shown in Figure 11B and Figure 11C.

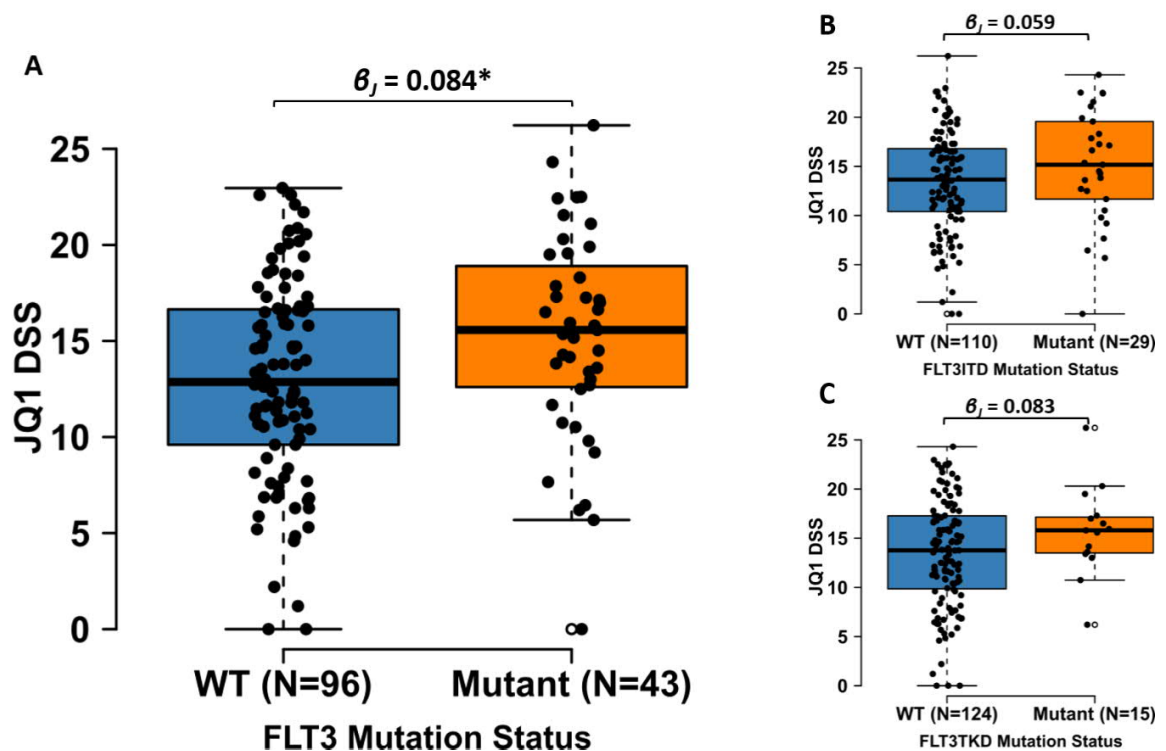


Figure 11. *FLT3*-mutated samples were slightly, yet significantly, more sensitive than wild-type to JQ1 treatment (panel A). While the statistical significance was lost, this trend was still observed even when the mutants' group was further split into sub-groups depending on the gene's mutation type: *FLT3*-ITD (panel B) or *FLT3*-TKD (panel C).

2.3.2.2 Novel suggestive biomarkers

2.3.2.2.1 *NCOR2* – a predictor of sensitivity

A biomarker significantly predictive for positive JQ1 response detected in our AML cohort was identified as a mutation harbored in the *NCOR2* gene (Nuclear receptor Co-Repressor 2). Despite the relatively small number of samples in which it was found (N=5), an *NCOR2* mutation was robustly and significantly associated with constitutive sensitivity to JQ1: $\beta_j = 0.257$ and p-value = 0.027 (Figure 12A).

To evaluate this finding further, gene copy number alteration data for *NCOR2* was incorporated in the comparison. Three new samples were identified with non-diploid statuses for the *NCOR2* gene, including one that had a homozygous deletion, another that had a heterozygous deletion, and the third one that had a gain in the copy number (4325_2,

4697_2, 2791_2). The sample having a homozygous deletion demonstrated remarkable sensitivity to JQ1 (DSS = 20.2) similar to those of the mutated samples, whereas the two other samples, which only have a wild-type copy of *NCOR2*, had scores that clustered closer to those of the wild-type samples (DSS = 15.3 and 11.1). Re-fitting the model by considering the activity status of *NCOR2* slightly enhanced both the gene effect ($\beta_J = 0.273$) and its significance (p-value = 0.013). The results are shown in the Figure 12B.

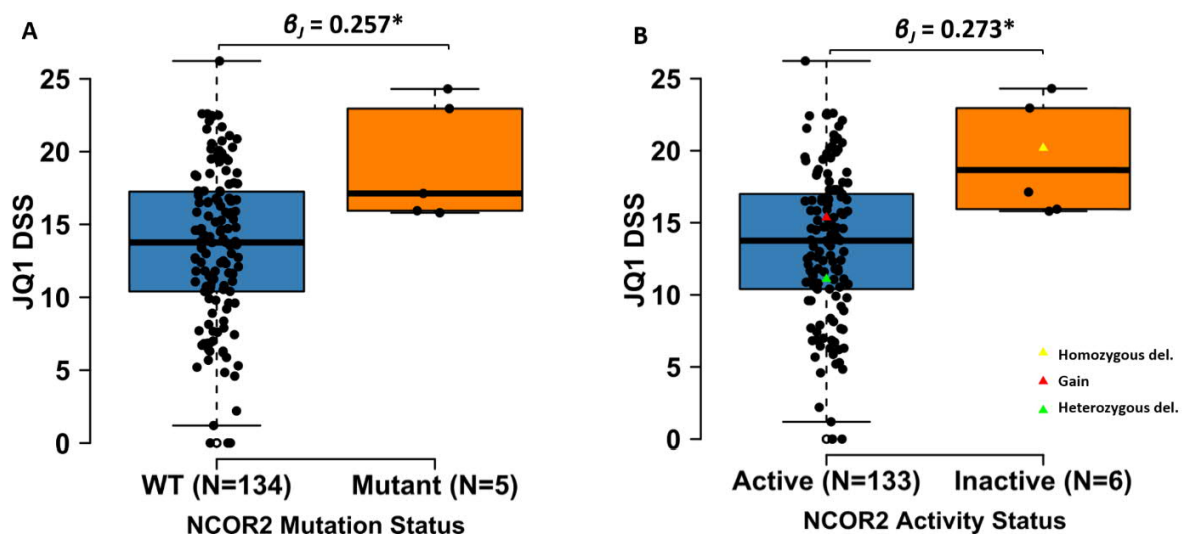


Figure 12. Mutation in the *NCOR2* gene appeared to confer pronounced and significant sensitivity to JQ1 treatment with respect to wild-type *NCOR2* (A). The effect was further confirmed upon incorporation of copy number alteration data (B), suggesting that *NCOR2* inactivity is the main JQ1-sensitivity predictor.

2.3.2.2.2 The “CPD” system – a predictor of resistance

Four separate genes, all known to be recurrently mutated in AML, were returned by our models as predictors of resistance to JQ1 in samples where they were mutated, with varying associated effect sizes and significance levels. Those genes were *IDH1* (N = 11; $\beta_J = -0.112$; p-value = 0.053), *IDH2* (N = 24; $\beta_J = -0.083$; p-value = 0.048), *WT1* (N = 8; $\beta_J = -0.005$; p-value = 0.937), and *TET2* (N = 13; $\beta_J = -0.019$; p-value = 0.720). Individual gene results are displayed in Figure 13.

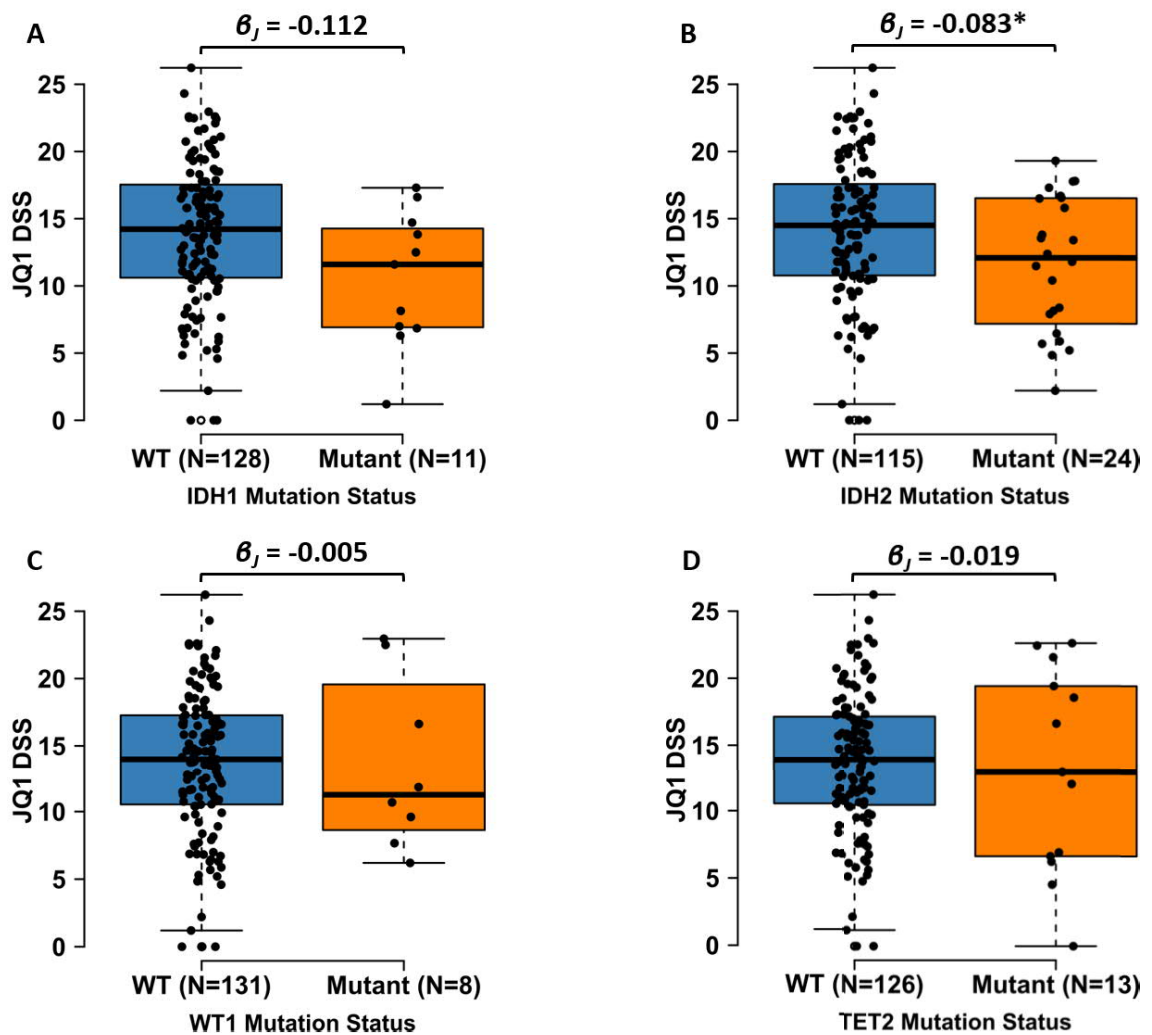


Figure 13. Four genes displaying negative associations upon mutation with JQ1 response, with varying effect sizes and significance levels. A: *IDH1*, B: *IDH2*, C: *WT1*, D: *TET2*.

According to a recent publication (Scourzic et al., 2015), the genes shown in Figure 13 all belong to one common biochemical pathway in the cell, hereby referred to as the “CPD” system (for Cytosine Passive Demethylation – See Section 3.6). Mutations disrupting the activity of any of those genes lead to a common downstream effect. Specifically, in AML, those genes have been reported to be mutually exclusive in a cell, an observation validated in both the TCGA (Figure 14A) and the FIMM (Figure 14B) study cohorts.



Figure 14. The “CPD” system genes display a mutual exclusivity in AML, an observation largely validated in the AML cohorts of both TCGA (A) and FIMM (B).

To optimize the results returned from this group of genes and based on the notion that having a mutation in one is equivalent to having a mutation in any, all samples harboring an *IDH1*, *IDH2*, *TET2*, or *WT1* mutation were grouped together (N=53), as representing the mutants of the “CPD” system, and those were compared to the remaining members of the cohort (“CPD”-wild types) for JQ1 response. The statistical test revealed that this component represented the most significantly predictive factor in the entire gene cohort, with an effect size $\beta_j = -0.087$ and a p-value = 0.01. After verifying that mutations included for *IDH1* and *IDH2* were actually occurring at the known oncogenic hotspots (R132 for *IDH1* and R140/R172 for *IDH2*), and after having incorporated the available relevant copy number alterations for those genes (*1399_2*: *TET2* heterozygous deletion and *3443_6*: *WT1* heterozygous deletion), the statistical test slightly improves for the filtered sample set (N=55), with $\beta_j = -0.091$ and a p-value = 0.0076, suggesting that the disruption of the normal activity of cytosine passive demethylation members is a predictor of resistance against JQ1 treatment in AML. The final results are reported in Figure 15.

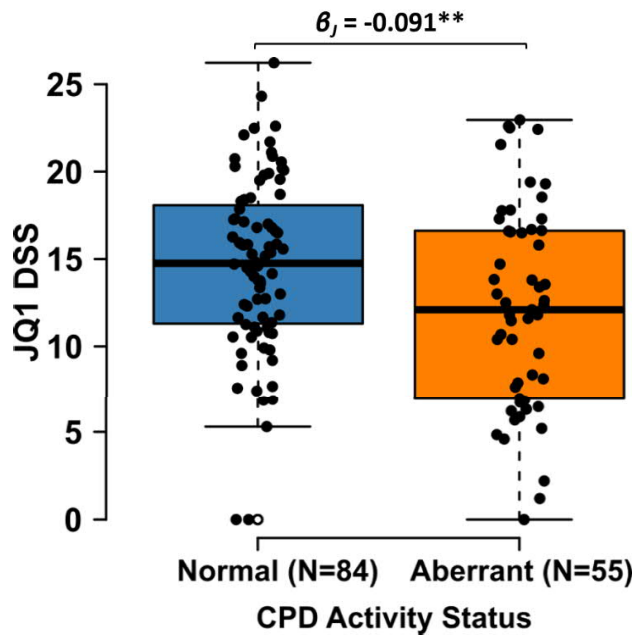


Figure 15. Members of the AML subgroup harboring aberrations in CPD genes display significant resistance to JQ1 treatment with respect to wild-type samples.

2.4 Validation of *NCOR2* mutations

All *NCOR2*-mutated samples in the FIMM cohort harbored an identical mutation occurring on the genomic reverse strand: a codon insertion at genomic positions 124402513-124402511. The resulting effect was a glutamine amino acid insertion after proline 511 on the *NCOR2* protein (P511QP). The aberration was observed to happen in a region of poly-Q repeats, leading to borderline significance in terms of certainty, which necessitated its validation through another sequencing method to eliminate the risk of it being a next-generation sequencing (NGS) artifact. The sequencing method of choice was Sanger sequencing: being a targeted technique which allows site-specific reading of relatively long genomic sequences, Sanger sequencing is considered as a well-established method that can be used to validate NGS signals.

Chromosome 12: 124,324,415-124,567,589



Figure 16. The genomic location of the *NCOR2* gene (enclosed in the red box) on the reverse strand of chromosome 12. Source: *ensemble.org*.

As such, 20bp primers (forward: 124402312-124402331; reverse: 124402677-124402658) were utilized in the PCR experiment in preparation for Sanger sequencing. Once the readouts were returned, the mutation was indeed observed to be present in all samples, at the expected genomic position: samples harboring the mutation had two signals emitted in Sanger readouts from the position of the insertion and onwards, coming from the normal and mutated *NCOR2* genes contained (heterozygous samples). Figure 17 represents an example Sanger sequencing sample output file validating the presence of the mutation.

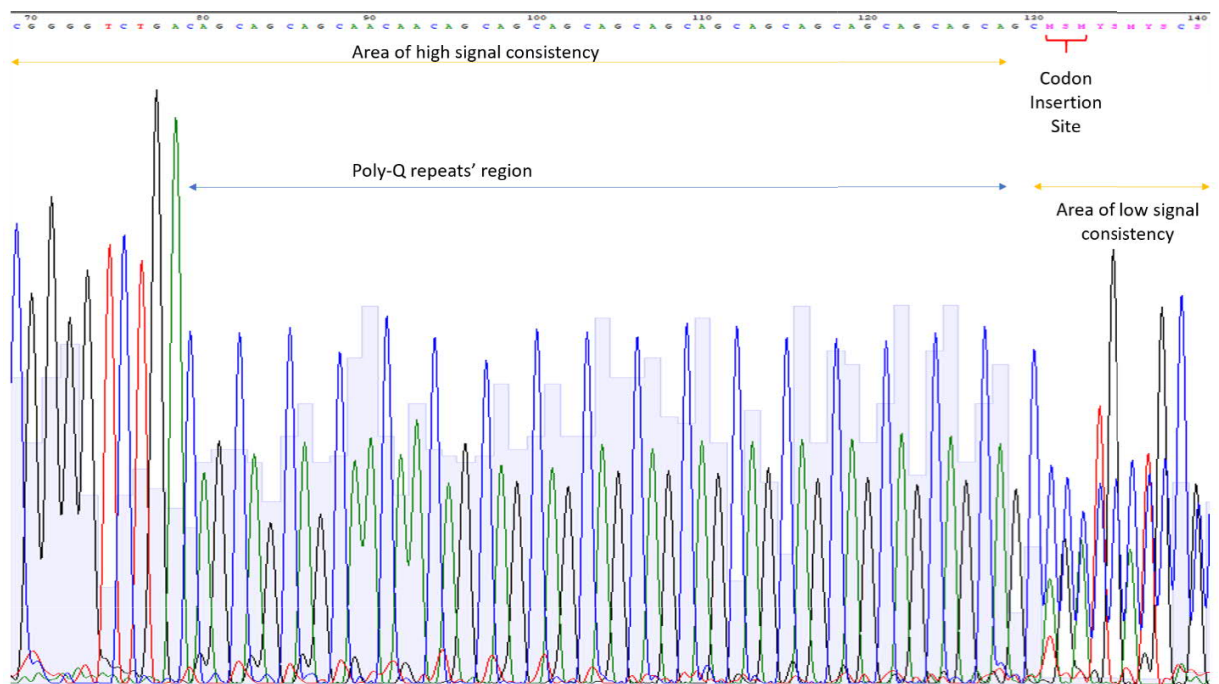


Figure 17. Example of a Sanger sequencing output file (Sample 743_5) as visualized in ApE (*A plasmid Editor*). Following a stretch of consistent signals spanning the region of poly-Q repeats, the codon insertion disrupts the signal at the expected genomic position, and the reads become afterwards inconsistent, coming from two separate DNA strands: the normal and the mutated *NCOR2* alleles.

2.5 Biomarker Validation in cell lines

29 AML cell lines from the Cancer Cell Line Encyclopedia (CCLE) database and 26 AML cell lines from the Catalogue of Somatic Mutations in Cancer (COSMIC) database were combined in the analysis. For each cell line, the mutation statuses for *NCOR2* and for the “CPD” system were retrieved. Similarly, drug sensitivity data of the selected cell lines to JQ1 treatment was collected from the Cancer Therapeutics Response Portal (CTRP) and the Genomics of Drug Sensitivity in Cancer (GDSC) database, respectively. Noting that the response data were reported with different metrics in each source (for instance, the AUC data is normalized to a 0-to-1 range by GDSC, but not by CTRP, and IC50 data is log transformed by GDSC, but not by CTRP), values per measure and per source were standardized before integration, for the sake of comparability. The standardization was performed as follows: subtracting the cohort’s mean value (\bar{x}) from every measurement from that cohort (CTRP or GDSC), and further dividing the obtained difference by the cohort’s standard deviation (σ): $[(x - \bar{x}) / \sigma]$. Consequently, a total of 55 cell lines, each reporting a standard AUC and IC50 value for JQ1 treatment, as well as characterized for its mutation status for our detected biomarkers (in binary), was constructed. Consequently, for each sensitivity measure, a simple logistic model of formula $\log \left[\frac{P(\text{mutation})}{1-P(\text{mutation})} \right] = \beta_0 + \beta_J * JQ1_sensitivity_measure + \varepsilon$ (as previously described) was fit, using all observations.

Using the standardized AUC data, *NCOR2* mutated cell lines are effectively predicted to be more sensitive to JQ1, displaying an inverse relationship between the mutation presence and the AUC values, with an effect size $\beta_J = -0.677$ and a borderline significance (p-value = 0.065). Switching to the standard IC50 values yields results in-line with AUC’s, with $\beta_J = -0.728$ (p-value = 0.15).

Similarly, AUC data successfully validate our “CPD”-system hypothesis, showing that mutants for genes in this system show on average higher AUC values for JQ1 treatment. The returned effect size β_J was equal to 0.713, again with a marginal significance (p-value = 0.088). IC50 data was also in accordance with the reported results, with $\beta_J = 0.6523$ and p-value = 0.052.

The results are summarized in Figure 18.

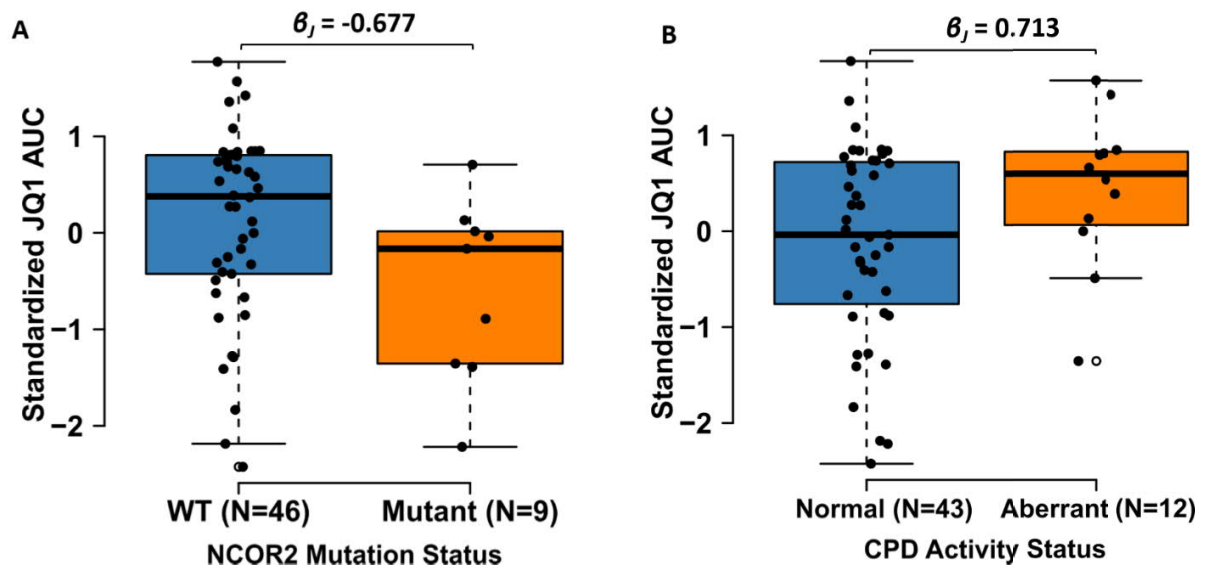


Figure 18. AML cell line sensitivity to JQ1 validates the returned hypotheses: *NCOR2* mutated cell lines display on average a higher sensitivity, reflected by low AUC values (A), whereas cell lines where the CPD system of genes is aberrant returns higher than average AUC scores, implying acquired resistance (B).

3. Discussion

3.1 A generalizable bioinformatic pipeline

Over the past decades, the fields of molecular biology and medicine have undergone a remarkable influence by the rapid advancement in relevant technologies, in particular those of high-throughput testing and sequencing facilities. The profile of molecular studies has thus acquired a novel “big-data” component, which has set the stage for systems biology approaches involving bioinformatics and biostatistics. Such disciplines have nowadays become essential in order to handle the bulk of data being routinely generated, with the purpose of extracting and/or summarizing the meaningful insights they encompass.

This work is an appropriate demonstration of the above: 170 samples are included, each of which is characterized by thousands of clinical and molecular descriptors to be evaluated (singly or in combinations) for association with recorded drug responses.

Clearly, achieving this goal required the development of a comprehensive bioinformatics pipeline allowing the realization a wide variety of tasks such as data collection, computation, evaluation, storage, and visualization, among others. Specifically, the pipeline requires as input a cohort of patients and their associated descriptors. The data is then preprocessed to fit statistical models for the downstream analysis to take place. For instance, certain clinical descriptors are categorized for patient stratification (such as sample types and risk allocations). The molecular data is systematically processed: for example, mutations have been binarized per gene, and copy number aberration scores were converted to discrete categories (amplification, normal, or deletion) in order to create sample groups that are sufficiently large for comparisons. Using suitably selected statistical tests, evaluations and model fitting per descriptor are then automatically performed, and only those statistically significant hits are returned to the user for further validation. The wide variety of used tests comprises 2-sample comparisons (paired t-test, unpaired t-test, Wilcoxon test) for two-class categorical variables (such as sample type and culture media), ANOVA tests for multiple-class categorical variables (such as ELN risk class), linear regression models for continuous data types (e.g. patient age and mutation burden), logistic regression models for binarized data types (i.e. mutational data analysis), and parametric (Pearson) and non-parametric (Spearman) correlations where applicable. Consequently, relevant data visualization (including density distribution, scatterplots, and boxplots) and long-term data storage are also ensured by the design of the pipeline. Finally, in the aim of validating suggestive biomarkers detected in the cohort, similar data made publicly-available from cell-line repositories (in this case, those maintained by the Broad and Sanger institutes) are collected, standardized (as described in Section 2.5) and evaluated in a similar fashion, as a check of reproducibility.

With the progress of personalized medicine and biomarker discovery attempts, it becomes evident that the tasks undertaken in this work are not uncommon, and that prospective project designs will closely resemble the one depicted in this study. Accordingly, the value of the aforementioned pipeline lies not only in the insights it unveiled for this specific research question, but also in its extendibility and potential usefulness for other prospective research projects with similar objectives of detecting sample biomarkers

robustly predictive of drug response, independently of the pathologic condition or the drug being investigated.

Evidently, several improvements and additions can be proposed to be incorporated into the pipeline. For instance, one option involves the use of additional data, notably transcriptomic, signaling pathway, and epigenetic data, known to contain particularly valuable molecular information, especially in the cancer setting (Aben et al., 2016; Flavahan et al., 2017). Applying additional bioinformatics techniques including differential gene expression, gene set enrichment analysis and network modeling would render the elucidation of the mechanisms of action underlying the detected associations feasible. Moreover, the ways of modeling the used data could also be altered (for instance, somatic mutations could be annotated and classified based on type or on functional effect, rather than binarized per gene). Finally, the pipeline may eventually be rendered available for public use as a web interface or as an R package (for example, see the *PAA* R package for biomarker discovery using proteomic data (Turewicz et al., 2016)).

3.2 JQ1 responsiveness illustrates sample dependency

It has been previously suggested that responsiveness of AML to BET inhibition is variable and largely dependent on the underlying characteristics of the sample/subject at hand. To illustrate, specific genome aberrations recurrent in different hematological malignancies were reported as predictors of response to BET inhibition (Abedin et al., 2016). In the case of AML, several sensitivity biomarkers (including *NPM1*-mutated AML, *MLL*-translocated AML, and *FLT3*-ITD AML) were presented, with the validating proof for each being obtained mostly from previously performed preclinical experiments using selected cell-lines and immunodeficient mouse models.

As is clearly demonstrated in the FIMM cohort (Figure 1), the AML samples' responsiveness to BET inhibition is indeed heterogeneous, and covers a wide spectrum ranging from high sensitivity to high resistance. The observation is therefore in-line with the previous studies where diverging responses were dependent on the samples' particular profiles. This result consequently stresses the need for the detection of suggestive biomarkers directly from patient-derived, diagnostic and relapsed, samples, ultimately

allowing for the conception of enhanced stratification protocols and the individualization of BET-inhibition based therapeutics in AML.

3.3 Unpredictability of response from clinical sample characteristics

Based on the FIMM AML cohort, the *ex vivo* response reported for JQ1 is not predictable neither by the malignant cell content of the sample at hand, nor by the media in which the said sample was cultured for DSRT.

Even though a sample's blast content is often essential to warrant an accurate AML diagnosis and prognosis (Döhner et al., 2017; Paul et al., 2014), current chemotherapeutic treatment regimens implemented in the clinics are not modified in practice based on that value, implying its unpredictability of the expected response. This is to be expected: the premise of chemotherapy consists of the eradication of rapidly-proliferating cells, with no distinction being made between healthy and malignant cells: this explains the appearance of the myriad of side effects typically-associated with chemotherapy, such as thrombocytopenia, hair loss, and gastrointestinal disturbances, all of which are the result of the destruction of healthy, yet mitotically very active cell types, making them “off-targets” of chemotherapy. One advantage of substituting traditional chemotherapeutics with agents specifically targeted to malignant cells hence lies in the possibility of sparing unwanted off-target activity, minimizing the adverse reactions to the treatment. Based on the above, it may appear safe to assume that the targeted therapies' activities are expected to steadily increase with an increasing proportion of malignant cells (with respect to healthy ones) in a sample; this is nevertheless, a logical fallacy. While targeted therapies are expected to have virtually no effect over healthy cells, their activity is not necessarily more marked in *any* malignant cell, since that will typically require the blast cell to have a profile fitting the “target” that those therapies depend on for activity. In simpler terms, targeted therapies are active in some, but not in all cancer cells, depending on the characteristics of the cancer population, rather than its frequency. This distinction becomes crucial to make in the case of AML which is known to entail an exceptional degree of internal heterogeneity in BM and PB samples, in terms of clonal architecture and molecular diversity. Therefore, the random association of blast counts and JQ1 responses in samples plays in favor of the above, further explaining why a sample with

few blasts can still harbor the profile needed for JQ1 to be effective and may as such return a high DSS (even though this may also result from unwanted JQ1 effects on normal cells in that sample), with the opposite being also true for a blast-rich sample, with a however unfavorable clonal composition. As a future step, a subpopulation analysis permitting the characterization of JQ1's effects on specific cell populations in the sample (the blast cells only for example) would be of interest. This may be realized via the implementation of a flow cytometry-based drug sensitivity assay.

Another factor further strengthening the hypothesis that a sample's sensitivity will depend primarily on its direct molecular profile (rather than on experimental variables) is the fact that the two different culture media (CM and MCM) do not contribute to the explanation of any logical trend in the responses. Even though the CM medium is enriched in cytokines and growth factors and is thus expected to mimic more closely the *in vivo* BM environment, it does not confer to the samples a distinguishable sensitizing or protective effect upon BET inhibition as is demonstrated in Figure 5. This is also in-line with a recently-published study which aimed to systematically investigate the effect of culture media on AML sample responses to varying drug classes, and which reported no effect on the activity of the different BETi included in the analysis (Karjalainen et al., 2017).

Specific patient and disease characteristics, herein referred to as clinical characteristics, included the patients' age, whether the stage of the disease is at diagnosis or relapse, and the risk class they are classified into according to the ELN scheme of 2017 (Döhner et al., 2017)). It is inarguable that the said characteristics are useful parameters that have been used as companion diagnostics for general treatment approaches and for decisions concerning the initiation of cytotoxic chemotherapy, the possibility of undergoing human stem cell transplantations, and/or the use of non-FDA (Food and Drug Administration) approved, yet promising, treatments in ongoing clinical trials (Almeida and Ramos, 2016; Bose et al., 2017). Nevertheless, reported studies have not detected a definitive response-predictive potential solely based on those parameters (notably for the recently approved or envisaged targeted therapies which are of greater relevance to us here), either because such a potential is inexistent on its own, or because of how limited the number of AML cases treated with targeted therapies still is at the moment (due to their very recent approval). Rather, treatment decisions have reportedly been based jointly on those factors

with specific molecular data (cytogenetic, mutational) (Bose et al., 2017). It therefore comes as no surprise that those factors did not seem to be singularly associated with the JQ1 responses of samples in our cohort.

Taken together, our cohort results indicate that AML sample sensitivity to BET inhibition is effectively subject to stratification and personalization, and that the molecular characteristics, rather than the clinical parameters, are instrumental for the classification.

3.4 Patient material does not fully recapitulate preclinical findings

The fact that no significant association between the number of somatic mutations harbored in an AML sample and its response to JQ1 exposure further indicates that the activity of BET inhibition depends on specific genetic biomarkers. The purpose thus becomes the identification of such sensitivity or resistance-conferring genetic biomarkers, either singularly or in combinations, for more accurate predictions of the response. Several such biomarkers have already been pinpointed in the literature, based on previous studies largely conducted at the cell line or animal level.

The first sensitivity biomarker is a set of specific mutations which occur in the nuclear localization sequence of the *NPM1* gene (containing two key tryptophan residues), referred to as the NPM1c mutations. Disrupting the normal folding of the C-terminus and hence the shuttling mechanism that the protein utilizes for alternation between the nucleus and the cytoplasm of the cell, mutated NPM1 proteins are faultily sequestered in the cytoplasm. The repressive role of BRD4-dependent transcription that NPM1 plays is thus lost, leading to an overactive BRD4 protein driving constitutive transcription of several oncoproteins, including c-MYC and BCL2, essential for disease progression. BET inhibition of BRD4 in such a scenario comes as a corrective measure for the lost NPM1 activity, and it has shown to effectively impair tumor growth, inhibit proliferation and induce apoptosis in *NPM1*-mutant cell lines, in ex vivo primary AML patient samples, and in in vivo immunodeficient mouse models grafted with *NPM1*-mutated cells (Abedin et al., 2016; Dawson et al., 2013). However, the data from our FIMM cohort could not verify the expected trend of increased sensitivity of *NPM1* mutants towards BET inhibition (Figure 10). The *NPM1* mutants among our samples display a high variability in their DSS scores, which are not significantly different from those of the wild-type

samples. We raise several hypotheses that may explain this seemingly-contradictory observation. The first concerns the variant allele frequency of the mutations, a factor not taken into account in the described mutational analysis procedure: the existence of an *NPM1* mutation (binarized in our technique) does not guarantee the occurrence of this mutation in all the cells of our heterogeneous samples. Consequently, it might occur that *NPM1*-mutants appearing to be insensitive to JQ1 might simply have the *NPM1* mutation in a minor clone, which does not considerably contribute to the sample readout, perhaps mostly coming from cells harboring wild-type copies of *NPM1* instead. Nevertheless, a correlation test between the *NPM1* variant allele frequencies in the mutated samples and the associated JQ1 DSSs returned an inconclusive and statistically insignificant association (data not presented). The second plausible explanation lies in the complexity and heterogeneity of patient-derived samples (and notably those from more progressed and relapsed patients): in such samples, the co-occurrence of different mutations raises the likelihood of genetic interactions, and hence the emergence of potential compensatory mechanisms enhancing the samples' resistance. The capture and confirmation of such potential interactions will require more advanced complex statistical modeling approaches to be successfully implemented.

A second sensitivity biomarker is the common *FLT3*-ITD mutation which usually confers a poor prognosis to AML patients, notably because of the multiple survival and proliferation pathways that a constitutively active *FLT3* kinase confers to the AML cell. The review reports that *FLT3*-ITD⁺ cell lines and primary AML patient samples respond better to a combination of *FLT3* and BET inhibitors, compared to the former, used as a single agent. Similarly, *FLT3*-ITD⁺ cells lines resistant to *FLT3* inhibitors are also observed to be sensitive to BET inhibition, which effectively reduces the aberrantly high level of expression of several oncogenic proteins in those cell lines, including *c-MYC*, *BCL2* and *EZH2*. Conversely, the activation of the Wnt/ β -catenin pathway (possibly by *PRC2* suppression) and the TGF- β pathway in cell lines proved sufficient to restore high levels of *c-MYC* expression and to confer significant resistance to BETi. Therefore, knocking down those pathways restored in vitro sensitivity to the drug (Abedin et al., 2016). In our cohort, *FLT3*-mutated patient samples do on average entail a significantly increased sensitivity towards JQ1, which is in line with the reported literature findings.

One important detail to denote here is the pooling of all *FLT3* mutants in our analysis into one cohort, including mainly the ITD and the TKD point mutations. Comparing each of those two sub-groups separately with the wild-type group still showed higher sensitivity among the mutants of each of the groups, despite compromised statistical significances due to the reduced sample sizes. The conservation of the trend comes as no surprise: it has been earlier shown that ITDs and point mutations in the TKD of *FLT3* both lead to the same erroneous downstream effect by the constitutive activation of the kinase (Patnaik, 2017). Taken together, those results not only successfully reproduce the observation that *FLT3*-ITD is a sensitivity-conferring biomarker towards BET inhibition in AML, but also rationally suggest that patients with activating mutations in the kinase domain of the protein are equally sensitive, and hence appropriate for BET inhibition treatment.

Another sensitivity biomarker is the recurrent *MLL* translocations seen in AML (but also other HMs such as acute lymphoblastic leukemia *ALL*), which often creates a fusion between the *MLL* histone methyltransferase product and SEC, the super elongation complex, of which BRD3 and BRD4 are crucial components. The fusion oncoprotein deregulates transcription, which drives c-MYC and BCL2 dependent leukemogenesis. It has been shown that BET inhibition is effective in *MLL*-translocation cell lines and that it prolonged survival in mouse models harboring such translocations (Abedin et al., 2016). As chromosomal aberration data analysis has not been performed within this study, this remains an interesting potential biomarker to be evaluated in prospective complementary analyses.

3.5 *NCOR2* mutations sensitize AML samples to JQ1

The most significant single-gene biomarker conferring sensitivity to JQ1 exposure in our AML samples concerned the nuclear receptor corepressor 2 gene *NCOR2*. While its precise role has been limitedly discussed in AML (perhaps due to its low mutation frequency: 1.1% in the TCGA 2013 AML study cohort (The Cancer Genome Atlas Research Network, 2013) and 3.6% in the FIMM cohort), mounting evidence has been accumulating over the last decade to support the importance of transcription factor co-repressors, and as a matter of fact, their aberrations, in cancer biology, as well as in other maladies (Battaglia et al., 2010). Specifically, *NCOR2* (also known as SMRT - *silencing*

mediator for retinoid and thyroid hormone receptors), and a similar, proto-typical co-repressor NCOR1, are known to be potent transcriptional repressors (targeted at a wide variety of transcriptional factors), functioning primarily in large complexes with histone modifiers (namely the family of histone deacetylases HDACs) at genomic enhancer and repressor sites. The activity of those proteins is delicately regulated, and selective disruptions by enhanced or hindered functions have been observed to lead to aberrant transcriptional rigidity and hence pathogenesis (and tumorigenesis). Those proteins have been observed to play key roles specifically in the pathogenesis of certain AML types, including (but most likely not limited to) APL patients (constitutive recruitment by the PML-RAR α fusion oncoprotein) and patients with the AML1/ETO fusion protein. In both cases, the abnormally recruited *NCOR1/2* genes impede normal transcriptional regulation and thus constitute driving critical oncogenic events (Wong et al., 2014; Battaglia et al., 2010). The fact that all samples with mutation to *NCOR2* in our cohort returned relatively high DSS scores with JQ1, and that the median of scores in that group was robustly and significantly higher than that of the wild-type *NCOR2* samples, provides additional support to the decisive role that transcriptional co-repressors play not only in the development of cancer, but also in its responsiveness to specific treatments, notably those affecting the transcriptional machinery of the cell, like the BETi do. This observation, detected in the FIMM cohort, was successfully validated in publicly-available cell line genomic and drug sensitivity data (Figures 12 and 18A). By considering the mode of action of JQ1 on one hand, and the role played by *NCOR2* on the other, the selection of this gene as a biomarker of sensitivity becomes fairly comprehensible, and the mechanistic basis could be speculated as follows. *NCOR2* normally recruits HDAC enzymes to deacetylate specific histone residues and hence modulate the cell's transcriptional activity. Upon *NCOR2* mutation and loss of function, an aberrant transcription of the target genes arises, resulting from persistent acetylation marks which are detected by the BET proteins (the latter being acetyl-lysine readers). However, inhibition of the BET proteins (for example by JQ1) prevents the detection of the aberrant acetylation marks in this setting, and hence spares the transcriptional disruption downstream from the *NCOR2* mutations. Special attention should also be attributed here to the insight brought upon by the incorporation of the copy number variation (CNV) data:

the patient with a homozygous deletion of *NCOR2* (similar to *NCOR2*^{-/-}) appeared to be as sensitive as the heterozygous mutants, whereas those patients with a heterozygous deletion or a gain in *NCOR2* rather clustered with the wild-type *NCOR2* samples, having returned relatively low response scores. Importantly, since mutated *NCOR2* samples return DSS scores similar to the sample having no functional *NCOR2* (high DSS), whereas wild-type *NCOR2* samples are rather similar to samples having at least one functional copy of *NCOR2* (low DSS), the possibility that *NCOR2* mutations may be of the dominant-negative type is raised, where the presence of a mutated copy possibly sequesters the other wild-type version of the protein and abolishes its activity. As such, this observation suggests the role of *NCOR2* in AML as a potential novel tumor-suppressor gene. Additional studies remain to be performed in order to further investigate the exact mechanism and cascade of events that links *NCOR2* to the BET proteins, elucidate the way the said protein modulates responsiveness to BET inhibition in AML, and verify the mutational dynamics and tumor-suppressor hypothetical role for the gene which have been raised. Taken together, our primary results offer a plausible starting point for the effective selection of sensitive AML samples to BET inhibition and hence the prediction of response based on a simple genetic biomarker.

As previously mentioned, the low literature-reported frequency of the *NCOR2* mutation detected in our 5 samples (insertion of an additional glutamine residue at proteomic position 511 following a glutamine-repeat region) combined with the borderline significance returned for the detection of that mutation by whole-exome sequencing in those samples suggested that the signal may be a sequencing artifact (perhaps due to polymerase slippage). Nevertheless, PCR amplification of the region of interest followed by capillary (Sanger) sequencing and visualization eliminated that possibility and confirmed that the mutation was well present in all of those samples. NGS used for somatic mutation calling is known to be largely dependent on the allelic fraction of the mutation, but more importantly on its coverage, which occasionally renders the technique prone to erroneous calls, notably false positives (Meyerson et al., 2010). As such, Sanger sequencing, following targeted PCR amplification, allows for a considerable increase in the abundance of the signal, and hence, more confidence in its detection, which makes it a suitable tool for the verification of NGS signals, especially when those are dubious.

3.6 A dysfunctional “CPD” system impairs ex vivo AML responses to JQ1

Isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) are frequently mutated AML genes (in up to 20% of patients) (The Cancer Genome Atlas Research Network, 2013). One of the normal functions that those enzymes perform lies in the citric acid cycle: they are responsible for the conversion of the metabolite isocitrate into α -ketoglutarate (α -KG). However, upon acquiring gain-of-function mutations, the enzymes play an oncogenic role, by abnormally further converting α -KG to 2-hydroxyglutarate (2-HG), its endogenous competitive inhibitor at several α -KG-dependent dioxygenase enzymes (Scourzic et al., 2015).

Wilms' tumor 1 (*WT1*) is also a recurrently mutated gene in AML (with a frequency of 6-8% of the patient population) (The Cancer Genome Atlas Research Network, 2013). The normal zinc finger transcription factor has been shown to be involved in the hydroxymethylation of cytosine, with loss-of-function mutations leading to aberrant patterns of the said process (Scourzic et al., 2015).

Ten eleven translocation methylcytosine dioxygenase 2 (*TET2*) is a third gene that has been reported to be expressed and commonly mutated in HM patients, particularly AML patients (in 8% according to the TCGA 2013 dataset (The Cancer Genome Atlas Research Network, 2013), and up to 27% of de novo AML cases and 32% of secondary AML patients (Scourzic et al., 2015)). *TET2* plays a crucial epigenetic role in the cell by leading to the successive oxidation of 5-methyl-cytosine (5-mC) bases into 5-hydroxymethyl-cytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxycytosine (5-caC). The detection of those higher oxidation state groups on cytosine bases in the DNA activates a DNA repair pathway: thymine DNA glycosylase (TDG) first leads to the bases' excision, creating an abasic site, which in a second step is filled up by a simple (non-methylated) cytosine residue by base-excision repair (BER).

The process described above is referred to as the passive demethylation of cytosine (as opposed to the active and reversible methylation/demethylation process controlled by DNA methyltransferase enzymes, such as DNMT3A), and is tightly regulated in a normal cell. It is specifically directed at unwanted cytosine methylation regions in the aim of restoring normal 5C to 5-mC levels. If for some reason this delicate regulation is disturbed

at any point, passive cytosine demethylation is lost, and a genome-wide hypermethylation pattern is observed, which is a typical driver for aberrant transcription (increased oncogene expression and silenced tumor-suppressor gene expression) and cellular transformation. The main reason for the loss of the cytosine passive demethylation (from now on abbreviated as CPD) is a functional loss of key enzyme TET2's activity (illustrated in (Rampal and Figueroa, 2016)). Interestingly, while being one of the main factors, *TET2* gene mutations are however not the only reason for which the enzyme's activity is lost. It has been shown that the TET2 enzyme depends on α -KG as a co-factor to perform its aforesaid activity. Hence, and based on the above, it becomes obvious that upstream oncogenic *IDH1/2* mutations in the pathway also lead to *TET2* loss-of-function, by abnormally generating an inhibitor of the enzyme (2-HG) rather than an activator (α -KG). Moreover, it has also been shown that loss-of-function mutations in the *WT1* protein lead to the same downstream effect, likely by inhibiting the binding of the activated TET2 protein to the right DNA regions for passive demethylation to occur. Taken together, it can be claimed that the described mutations in any of the four genes (*IDH1*, *IDH2*, *TET2*, or *WT1*) will lead to the same functional effect in the cell: loss of TET2 function, CPD-activity reduction, genome-wide hypermethylation patterns, and a strong epigenetic predisposition to cellular transformation and tumorigenesis. Since all of those described mutations are finally similar, and as such, redundant, it has been suggested that they display a pattern of mutual exclusivity in the cancer cells of AML. In simpler terms, a transforming AML cell needs not to mutate more than one of those genes (and hence does not), in order to reach the same downstream effect (Scourzic et al., 2015). This observation was successfully validated in the entirety of the TCGA 2013 cohort (Figure 14A), but also in our AML cohort (Figure 14B). However, three FIMM samples appeared to deviate from the general rule of mutational exclusivity for those genes: sample 4735_2 harbored somatic mutations in both the *IDH1* and *IDH2* genes, whereas samples 4599_2 and 370_2 had mutations in *IDH1* and *TET2*. Nevertheless, it is crucial to note here that the concept of mutual exclusivity is cell-, rather than sample-relevant. Thus, reported mutations in more than one of those genes for the same sample are most likely due to intra-tumor heterogeneity: the mutations are almost certainly observed in different sub-clones composing the said samples.

The above discussion provides the acceptable basis on which grouping all mutants for those genes into one functionally-similar group and comparing them to the remaining samples in the cohort was made. The disrupted CPD system did eventually turn out to be the most robust biomarker returned by the analysis, conferring exceptional resistance to JQ1 among the mutant samples with respect to the wild-type samples (where the CPD system is expected to be intact, Figure 15). Here again, the fact that this system is tightly related to epigenetics and control of transcription is in-line with the role of JQ1 and the BET proteins, even though the immediate mechanistic link between the aberration and its effect on drug response seems less readily visible (than with *NCOR2* for example) and will require further specifically-designed experiments for elucidation and sound interpretation. Nevertheless, this result does appear to have potentially robust support (especially that it has been successfully reproduced in the same direction in public cell line data), and it offers another logical factor to consider when it comes to AML patient stratification in what concerns BET inhibition attempts, both in single-agent and in drug-combination testing assays.

3.7 Summary and future prospects

This study clearly demonstrates that AML is a remarkable paradigm of the diseases of the genome. Despite the fewer somatic mutations reported for this malignancy as compared to other cancer types, the exceptionally vast heterogeneity of the underlying molecular profiles makes the AML population very diverse. This, together with the dismal results brought upon by traditional chemotherapy, has sparked significant interest in the development of therapies which are both targeted at specific molecular aberrations and, as such, personalizable among the patients, and BET inhibition is a major illustration of this. Although instrumental in the determination of diagnosis, prognosis, and disease management, clinical and experimental characteristics of patients and samples are insufficient on their own to accurately stratify patients into discrete classes reflecting different expected responses to BET inhibition, which seem to be rather dependent on the more complex genetic and molecular data. While earlier attempts at identifying response-predictive biomarkers from AML samples to BET inhibition have effectively returned certain usable signals, those findings have largely been obtained from preclinical material,

notably cell-lines and animal models, which suffer the inevitable limitations in terms of predictive validity in actual patients. This study clearly shows that the extendibility of preclinical findings to the human level needs to be done with caution: *FLT3*, but not *NPM1*, mutations conserve their predicted response patterns in the patient material (as compared to those detected in cell lines for instance). In terms of novel findings, this work contributes two major discoveries. The first one is a set of potential biomarkers or companion diagnostics to be used in the prediction of AML patients' response to JQ1, with *NCOR2* mutations being regarded as sensitizing-factors, whereas a dysfunctional CPD-system representing a resistance-conferring event. Second, the project also successfully offers a generalizable bioinformatic pipeline applicable to a wide range of diseases and drugs in the goal of discovering novel sensitivity- or resistance- conferring biomarkers from clinical, genomic and drug-response patient material.

The promising and useful outcomes of this work pave the way for functional investigations to be continued upon, both at the analytical and the experimental levels.

From a data analysis perspective, while this study has looked into genomic mutations in detail in a single gene-wise fashion, it would be of great interest to implement a more sophisticated approach that analyzes those mutations in combinations, in the purpose of detecting not only individual signals, but potentially (and very likely) specific co-occurrences, exclusivities, and interactions of them which can influence reported drug responses (synergy, additive effects, or antagonism, among other possibilities). Multiple linear or non-linear regression methods may be worth exploring in this context. In addition to somatic mutations, several other predictor classes are expected to have considerable potential to rationalize drug response, and to provide valuable additional insight, and must consequently be incorporated in future studies. Those shall include chromosomal aberrations – such as translocations, fusions, rearrangements, etc. (which would allow the evaluation of an additional previously reported biomarker concerning *MLL*-fusion patients) – and importantly, transcriptomic and pathway data from RNA sequencing studies (which would also allow the verification of biomarkers reported from that class, notably *MYC* gene expression, the WNT/ β -catenin pathway activity status, etc.). Upon a full annotation of the relevant biological processes, network pharmacology modeling

based on drug target interaction and signaling pathway information will be applied (Masoudi-Nejad et al., 2013).

From an experimental point of view, future studies are most definitely projected to entail a wet-lab component, crucial to evaluate the hypotheses raised by this study and to consolidate the presented claims. For instance, the predictive effects of the novel detected biomarkers of JQ1 response can be evaluated in unseen test specimens (from newer patient samples, genetically-manipulated animals, cell-lines material, and other suitable tools), in order to verify the reproducibility of the detected response patterns. In the case where those biomarkers turn out being even more convincingly associated with BET inhibition responses, investigations of the mechanistic basis underlying those associations shall be conducted. This may include knock-in and knock-out models of the selected genes as well as relevant downstream protein quantification methods, among others... Additionally, the implementation and utilization of prospective flow cytometry-based DSRT assays will allow for a shift from a bulk response measure to a more population-specific one. This would have the advantage of excluding the drug's effects on healthy cells in the sample from the readout and would make possible the examination of the drug activity in a single-cell or single-population fashion. Finally, suitable drug combination experiments can also be proposed and devised based on the findings, in the aim of complementing or even synergizing the effects of BET inhibition in AML. From this study, combinations of JQ1 with HDAC inhibitors (NCOR2-relevant) or with hypomethylating agents (CPD-relevant) appear to be worth exploration.

4. Materials and Methods

4.1 Sample Collection

Samples considered as candidates for inclusion in this study had been obtained from patients with newly diagnosed or relapsed acute myeloid leukemia. The patients had all provided written informed consent (*Annex II*). Each sample was either a bone marrow (BM) aspirate or a peripheral blood (PB) specimen and was matched with a corresponding skin biopsy. The samples were all extracted in the clinics according to standard protocols

approved by the local institutional review board of Helsinki University Hospital and Comprehensive Cancer Center and compliant with the Declaration of Helsinki. Samples were described according to a set of characteristics examined and reported in a data repository (*Granitics*) managed by the collaborating clinics, and those included, among others, the sample status (diagnosis versus relapsed samples), the age of the patient at the time of the sample collection, a blast content estimate (in percentage), and the risk assigned to the patient at diagnosis according to the ELN 2017 guidelines (described in Section 1.2.4). From BM and PB samples, mononuclear cells (MNCs) were isolated at FIMM by gradient centrifugation (Ficoll Paque; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

4.2 Sample Selection

A total of 170 AML samples were prospectively included in this study. The conditions for a sample's selection for inclusion were as follows: i- AML diagnosed, relapsed, refractory or transformed sample, ii- the sample was tested with the chemical probe JQ1, and returned a non-dubious drug sensitivity score DSS from the CellTiter-Glo readouts of the live cells, iii- the sample had one or more of the characteristics searched for potential biomarkers (i.e. clinical characteristics, somatic mutations, and/or CNV data). Briefly, DSS is a measure that extracts several characteristics from the multiparametric log-logistic dose-response curve of a sample treated with a drug (including the area under the curve, the drug's tested concentration-range, the minimum activity level, the curve's slope at IC₅₀, and the lower and upper asymptotes of the response) and integrates those into a single metric directly proportional with the sample's response level. A formal description of the mathematical basis for the calculation can be found in "*Quantitative scoring of differential drug sensitivity for individually optimized anticancer therapies*" (Yadav et al., 2014).

A summary of the sample characteristics is reported in Supplementary Table 2.

4.3 Drug Sensitivity Testing

MNCs from samples were cultured immediately after isolation, typically in one of two growth media: mononuclear cell medium (MCM; Promocell, Heidelberg, Germany), or

conditioned medium (CM; 25% HS-5 conditioned medium plus 75% RPMI 1640 medium mix) (Karjalainen et al., 2017). The samples were then sent for standard drug sensitivity and resistance testing (DSRT) at FIMM's High Throughput Biomedicine unit. Cells from the samples were plated on the 384-well plates constituting the drug sensitivity plate set in use at the time of the sample collection from the clinics. The plates had already been prepared with a set of selected chemically active molecules (approved therapeutic drugs in clinical use or investigational molecules/probes under development), each at five different concentrations (for JQ1, the concentration range goes from 1 to 10000 nM, following a 10-fold increase scheme). At 72 hours from plating, cell viability was measured using the CellTiter-Glo reagent (Promega, Madison, WI), according to the manufacturer's instructions, with a PHERAstar FS plate reader (BMG LABTECH, Ortenberg, Germany). Readout values allowed relevant dose-response curve fitting and calculation of a drug sensitivity score (DSS) per sample and per drug. The results were stored and made accessible for the research personnel in FIMM's main data repository (*THEDB*) and on FIMM's server (*atlas*).

4.4 Whole Exome Sequencing Pipeline

The somatic mutation and CNV data analyzed in this study was the result of a well-established whole-exome sequencing pipeline which is routinely run for all (non-APL) AML samples composing the FIMM cohort. The data is collected and stored in FIMM's main data repository (*THEDB*) and on FIMM's server (*atlas*), for use by the research personnel. The following is a brief overview of the pipeline's various stages, for more information relating to specific tools and parameters, please refer to the supplementary appendices of "*Somatic STAT3 Mutations in Large Granular Lymphocytic Leukemia*" (Koskela et al., 2012).

To start with, DNA was isolated from mononuclear cells extracted from the patients' BM or PB and skin biopsies. Using 3 µg of that DNA and various capture kits, exome capture was performed. The exomes were then sequenced on HiSeq 1500/2000/2500 instruments (Illumina, San Diego, CA, USA) using the paired-end sequencing technology. Upon completion of the runs, quality control was performed on the obtained raw Illumina reads: those were first merged then trimmed according to the quality scores from the end of the

reads. Those trimmed reads which ended up being shorter than 36 base pairs were removed. Consequently, the remaining reads were aligned against the human GRCh37 reference assembly using the Burrows-Wheeler Aligner. Reads mapping to multiple genomic positions and those which appeared to be PCR duplicates were eliminated to reduce uncertainty. Somatic mutations were finally called and annotated for each tumor sample (in reference to its matched skin sample in order to filter out germline variants). Common population variants obtained from dbSNP were also filtered out. As for the copy number aberration analysis, alignments in the BAM format and called variants were used as the inputs. Scores per gene and per sample were reported as log₂ copy number ratios of the sample divided by the reference (using the copy number data for all human genes in the Ensembl database).

4.5 Generation of Dose Response Curves

To visually examine the variation in ex vivo responses depicted by different patient samples treated with JQ1 and the amplitude of this variation, the selected 170 samples were ranked according to their JQ1 DSS. Consequently, the top and bottom 10 samples were taken forth for visualization. For each, the percentage of viability of cells at each of the five experimental doses of JQ1 was collected at 72 hours. This data was used to fit for each sample the response (percentage of viable cells) to the logarithmic value of the JQ1 concentration ($\log_{10}[JQ1]$), and to subsequently plot for that sample a standard four-parameter log-logistic curve, using the software GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The curves for all samples were then superposed on top of each other, to facilitate their comparability.

4.6 Data Collection and the Analysis Pipeline

Upon data collection, modeling in an efficient way for downstream bioinformatic and statistical analysis constituted the next step. The tool selected for this purpose was R, an efficient environment convenient for statistical computing and optimized plots' generation. A schematic representation of the general developed bioinformatic analysis pipeline is presented in Figure 19.

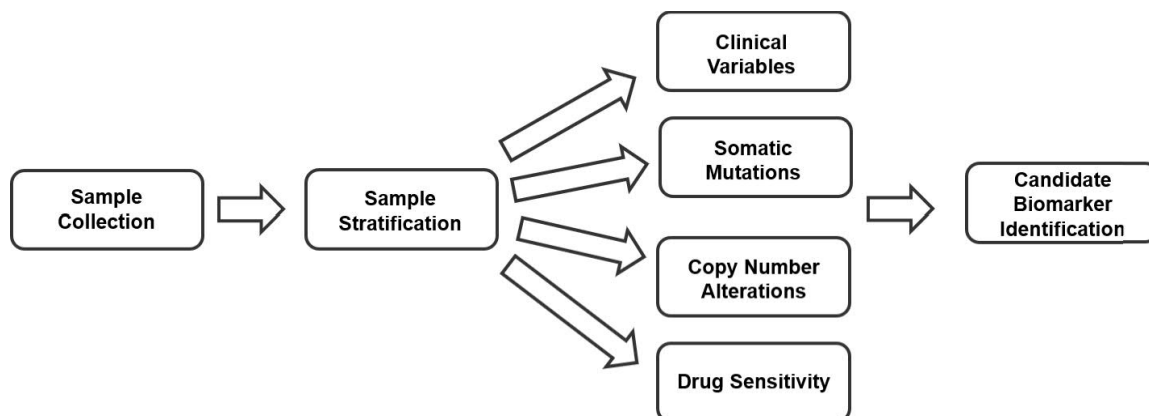


Figure 19. General bioinformatic pipeline implemented for the data analysis.

Clinical variables included the following patient and sample characteristics:

- age of the patient at the time of sample collection (modeled as a numerical discrete variable)
- ELN 2017 risk attributed to the sample’s donor at the time of diagnosis (modeled as a categorical nominal variable – three classes: favorable, intermediate, or adverse)
- Sample status (modeled as a categorical nominal variable – two classes: diagnosis or relapse). Note that the relapse group included secondary (to MDS for example), transformed, refractory, and treatment-related AML samples.
- Blast count (modeled as a numerical discrete variable – ranges from 0 to 100)
- Media used for MNCs culture (modeled as a categorical nominal variable – two classes: CM or MCM)

Assuming a distribution of DSS scores close to normal for sufficiently large groups of samples, the selection of parametric rather than non-parametric statistical tests was favored, owing to their higher degree of accuracy. Nonetheless, for small sample sizes where the normality assumption could not be confirmed, non-parametric tests were eventually utilized. Thus, the analysis of variance technique (ANOVA) was used to compare the means of the different groups of samples stratified according to the donors’ risk at diagnosis. Unpaired two-sample t-tests were used to compare the JQ1 DSS scores of the two groups of samples separated by the culture media or the sample status. Wilcoxon rank sum and Spearman correlation tests were also computed for matched

samples tested in both media. As for the age of the donor at the time of sampling and the blast count's association with JQ1, a simple linear regression as well as Pearson correlation tests were used to evaluate the existence of a potential association between the two measures.

For the somatic mutational profiles, the data was binarized per sample and per gene; i.e., each sample was assigned either a “mutant” (1) or a “wild-type” (0) status for every gene. The threshold for the inclusion of a mutation as existent for a sample was set at 0.05. A total of 4425 genes mutated in at least one of the 139 samples with genomic data were taken forward for analysis.

A mutation burden was calculated per sample, defined as the simple algebraic sum of the somatic mutations reported for the sample. Association between the mutation burden and the JQ1 response was investigated using a simple linear regression model, as well as a Pearson correlation test.

Considering the “small-n-large-p” design of the mutational data, we decided to consider every gene's mutational status' individual association with JQ1 response in a model. Since the mutational data was binarized, the model of choice was a simple logistic regression, where the JQ1 response was used as the predictor, and the mutation status as the response. Despite the inversion of the direction this model imposes, it was estimated that the existence of a robust association would still be maintained under those conditions.

For all tests performed, results were evaluated for statistical significance using associated p-values, where the threshold for significance was fixed at 0.05.

4.7 Experimental Verification – Sanger Sequencing

All five samples considered as mutated for *NCOR2* by our model had a p-value enclosed between 0.01 and 0.05 for the somatic mutation, considered as borderline in terms of significance for mutations called from NGS experiments. To verify the existence of the mutation in those samples, we decided to perform Sanger sequencing on stored DNA. Upon identification of the mutation and its genomic mutation, suitable primers were designed, such that they would both span the location of the expected mutation, but also minimize off-target replication. The design of the primers was performed using Primer-BLAST, a primer designing tool for PCR experiments. Once the primers were designed,

ordered and obtained, a PCR experiment was performed on all samples using standard conditions (the Tag polymerase used was manufactured in-house). Upon completion, PCR fragments were gel separated and the bands of interest were cut out using a standard PCR purification kit (Macherey-Nagel, Germany). The extracted PCR products per sample were then sent to the FIMM Technology Centre for capillary sequencing. The returned (.ABI) files were then examined using the plasmid editor ApE.

4.8 Biomarker Validation in Public Cell Line Repositories

To validate our novel findings from the FIMM AML cohort, the formulated hypotheses were taken forward for validation in public repositories. The purpose was to re-evaluate for the prospective genomic biomarkers detected in our dataset their predictive potential of JQ1 response. High-quality and well-characterized AML cell line genomic and drug response data was judged as the most suitable resource for this purpose. This data was obtained from two main repositories: the first one maintained by Broad Institute, with the cell line genomic data published in its Cancer Cell Line Encyclopedia (CCLE) and the drug response data in its Cancer Therapeutics Response Portal (CTRP). Similarly, the Sanger Institute also provided cell line data, with genomic profiles obtained from the Catalogue of Somatic Mutations in Cancer (COSMIC) database, and the response to drugs from the corresponding Genomics of Drug Sensitivity (GDSC) repository. To mimic the same analysis performed on the FIMM AML cohort, the following steps were followed on the data retrieved from Sanger and Broad: i- the mutation data was binarized per gene and per cell line, ii- logistic regression models accepted JQ1 response parameters as the sole predictor, and the gene of interest's mutation status as the dependent variable. Note that both response parameters to JQ1 were successively tested for their performance in the built models, and those consisted of the area under the dose-response curve measure (AUC) and the half maximal effective concentration (IC50) value. Also note that the direction of both sensitivity parameters is identical, and that it is opposite to that used in the FIMM dataset (DSS); i.e., small IC50 and AUC values reflecting high sensitivity in the sample are associated with large DSS values, and vice versa.

5. Acknowledgments

This project has been conducted at the Institute of Molecular Medicine Finland (FIMM), part of the Helsinki Institute of Life Science (HiLIFE) at the University of Helsinki.

I would like to express my most sincere gratefulness and appreciation to my supervisors, Dr. Caroline Heckman and Dr. Jing Tang. Your remarkable knowledge and expertise have allowed me to enhance my understanding of this project's central themes and other related topics every day, both at the theoretical and practical levels. Equally, your professionalism and precious advice have been crucial for the proper development of my work ethics and practice in the field of science.

Carrying out this project has taught me a whole lot, and I am thankful for each and every coworker and FIMM staff member who have contributed to its completion. I would like to express my authentic gratitude to: Juho for having continuously provided me with invaluable guidance and advice since I first joined FIMM; Samuli for having set up the whole exome sequencing bioinformatics pipeline and for having patiently explained it to me; Imre for having set up THEDB, FIMM's main data repository, and for his willingness to assist me in retrieving the data I needed from it; Alun, for his support in designing and performing the PCR and Sanger sequencing of selected samples; Alberto for sharing with me his expertise in statistical analysis; Alina for sharing her programming expertise; Wenyu for assisting in the collection of cell line data; and Dimitrios for sharing tips with me for the generation and optimization of certain figures. My special thanks also go to our lab technicians (Minna, Siv and Alun) for their tireless and professional processing of the patients' samples for all of us to make good use of, and to all of the remaining members from both the Translational Research and Personalized Medicine group (Riikka, Jarno, Ashwini, Komal, Heikki, Maija, Mamun) and the Network Pharmacology for Precision Medicine group (Yinyin, Bulat) for the weekly group meetings and discussions. It has been a real privilege to work with all of you.

I also wish to thank Orion Pharma in general (whom I continue to collaborate with), and Taija in particular, for having rendered this opportunity possible for me in the first place. Finally, my humble appreciation goes to all of the patients who have participated in this project and to their inspiring sense of altruism. I would also like to thank the Hospital

District of Helsinki and Uusimaa (HUS), and all of the collaborating clinics, for supplying the valuable patient material.

Working in FIMM for the past year has been a real delight thanks to the great working environment created by the brilliant personnel. Thank you.

Helsinki, 14/08/2018

Joseph Saad

6. List of Abbreviations

Standard abbreviations list according to the *Journal of Cell Biology* (JCB) and the following terms.

AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
AUC	Area Under the Dose-Response Curve
BET	Bromodomain and Extra-Terminal Family Proteins
BETi	Bromodomain and Extra-Terminal Family Proteins' Inhibitor
BM	Bone Marrow
BRD	Bromodomain
CM	Conditioned Medium
CNV	Copy Number Variation
CPD	Cytosine Passive Demethylation
CR	Complete Remission
CT	Clinical Trials
CTRP	Cancer Therapeutics Response Portal
DSRT	Drug Sensitivity and Resistance Testing
DSS	Drug Sensitivity Score
dDSS	differential Drug Sensitivity Score
ELN	European Leukemia Net
FIMM	Institute for Molecular Medicine Finland
GDSC	Genomics of Drug Sensitivity in Cancer
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HM	Hematologic Malignancy
IC ₅₀	Half Maximal Inhibitory Concentration
ISM	Individualized Systems Medicine
ITD	Internal Tandem Repeat
Kac	Acetyllysine /Acetylated Lysine Residue
MCM	Mononuclear Cell Medium

MDS	Myelodysplastic Syndrome
NGS	Next Generation Sequencing
NMC	NUT Midline Carcinoma
PB	Peripheral Blood
P-TEFb	Positive Transcription Elongation Factor b
PTM	Post-Translational Modification
TCGA	The Cancer Genome Atlas
TKD	Tyrosine Kinase Domain
WHO	World Health Organization

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8. Appendices

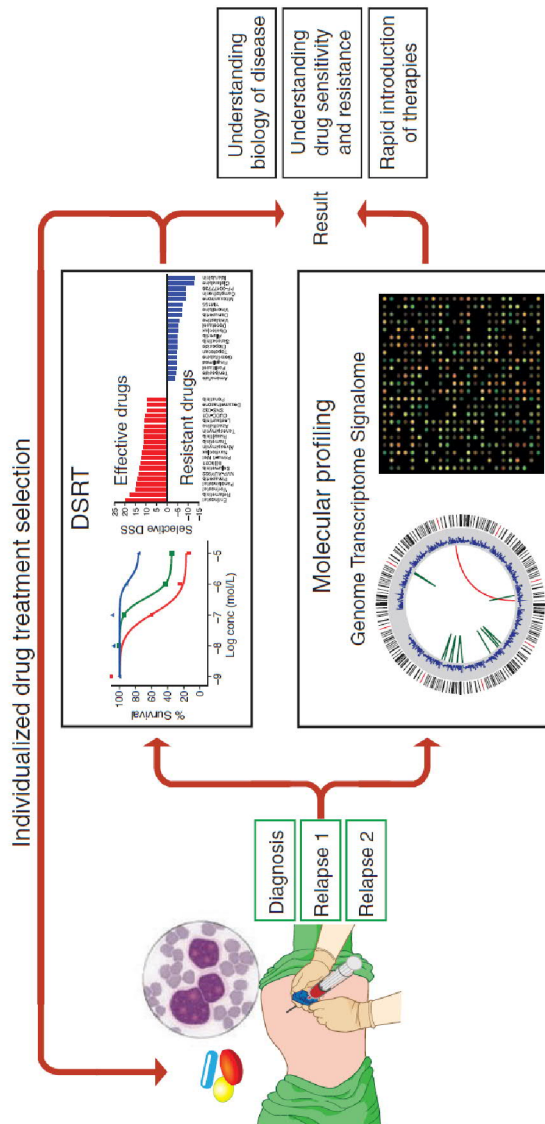


Figure 1. Functional ISM platform for improved AML therapy. The platform involves (i) comprehensive direct DSRT of 187 approved and investigational oncology compounds in *ex vivo* primary cells from serial AML samples; (ii) clinical implementation of testing results in individual patients with relapsed and refractory disease; (iii) deep molecular and genomic profiling of the patients with AML from consecutive samples before and after relapse and drug resistance for monitoring disease progression and clonal evolution; and (iv) integrating drug sensitivity, next-generation sequencing, and clinical follow-up data to understand the biology of disease, drug sensitivity, and resistance that can lead to rapid introduction of novel therapies to the clinic. DSS, Drug Sensitivity Score.

Annex I. The Functional ISM Platform Conceived for Improved Therapy

Pemovska, T., M. Kontro, B. Yadav, H. Edgren, S. Eldfors, A. Sz wajda, H. Almusa, M.M. Bespalov, P. Ellonen, E. Elonen, B.T. Gjertsen, R. Karjalainen, E. Kuleskiy, S. Lagstrom, A. Lehto, M. Lepisto, T. Lundan, M.M. Majumder, J.M.L. Marti, P. Mattila, A. Murumagi, S. Mustjoki, A. Palva, A. Parsons, T. Pirttinen, M.E. Ramet, M. Suvela, L. Turunen, I. Vastrik, M. Wolf, J. Knowles, T. Aittokallio, C.A. Heckman, K. Porkka, O. Kallioniemi, and K. Wennerberg. 2013. Individualized Systems Medicine Strategy to Tailor Treatments for Patients with Chemorefractory Acute Myeloid Leukemia. *Cancer Discovery*. 3:1416–1429. doi:10.1158/2159-8290.cd-13-0350.

Annex II. Ethical and Confidentiality Statement

The project involves the use of human samples and clinical data. The Coordinating Ethics Board of Helsinki University Hospital Comprehensive Cancer Center has approved the collection and use of the samples and data. Samples are taken from patients after informed consent and according to the Declaration of Helsinki. Prior to sampling, the patients are coded so data collected from any analyses (e.g. sequence data) cannot be used to identify the patient. These data will be kept on secure servers at FIMM. The study permits are Dnro 303/13/03/01/2011 approved on 5.11.2012 and Dnro 239/13/03/00/2010 approved on 12.10.2012. Copies of the permissions are included as appendices to the application.

Supplementary Table 1. Genes having returned a statistically significant association between their mutation status across the FIMM samples and their respective JQ1 DSS.

Gene Name	Number of Wild-Type	Number of Mutants	Gene Coefficient	Gene Standard Error	Gene p-value
RTN4	136	3	-0.362	0.145	0.013
MGA	136	3	-0.354	0.143	0.013
WRN	136	3	-0.351	0.142	0.013
SEC63	136	3	-0.347	0.141	0.014
RYR2	136	3	-0.302	0.129	0.02
FLT3	96	43	0.084	0.037	0.023
LAD1	136	3	0.454	0.203	0.025
INTS7	136	3	-0.276	0.124	0.026
AKAP6	136	3	-0.276	0.124	0.026
CACNA1E	132	7	0.204	0.092	0.026
TMTC1	133	6	0.226	0.102	0.027
NCOR2	134	5	0.257	0.116	0.027
PHF2	135	4	-0.219	0.1	0.028
PTPRG	137	2	-0.486	0.226	0.032
CRYBG3	137	2	-0.421	0.197	0.032
ABCA6	137	2	-0.421	0.197	0.032
RDX	137	2	-0.421	0.197	0.032
PRICKLE2	137	2	-0.421	0.197	0.032
AK2	137	2	-0.421	0.197	0.032
CTD-	137	2	-0.421	0.197	0.032
3193O13.9					
TRPV2	137	2	-0.415	0.194	0.032
HDAC6	137	2	-0.402	0.189	0.033
FAM81A	137	2	-0.402	0.189	0.033
FCGR3B	137	2	-0.396	0.186	0.034
FAM155A	135	4	-0.208	0.099	0.035
QSER1	137	2	-0.374	0.178	0.036
TSC2	137	2	-0.374	0.178	0.036
AC111170.2.1	136	3	-0.248	0.118	0.036
SPATA31D1	136	3	0.364	0.174	0.036
TRIO	136	3	-0.246	0.118	0.037
FLG2	137	2	-0.352	0.171	0.039
RAD50	137	2	-0.337	0.166	0.042
GPRC6A	136	3	0.341	0.168	0.042
RAPGEF4	137	2	0.73	0.363	0.044
MYOT	137	2	-0.319	0.16	0.046
MLLT4	137	2	-0.319	0.16	0.046
DMD	137	2	-0.319	0.16	0.046
IERSL	137	2	-0.319	0.16	0.046
FTH1	137	2	-0.319	0.16	0.046
MYO7B	137	2	0.892	0.45	0.048
DCAF8	137	2	-0.314	0.158	0.048
IDH2	115	24	-0.083	0.042	0.048
HDAC2	137	2	-0.311	0.158	0.049
LMBRD2	137	2	-0.311	0.158	0.049
DHX33	135	4	0.25	0.127	0.049
YEATS2	137	2	0.54	0.275	0.049

Supplementary Table 2. Summary of the FIMM cohort samples' clinical and molecular characteristics.

Characteristic	N (170)		Median	Mean	SD
Age (years)	145	-	62	56.8	15
Status	170	D: 86 (50.6%), R: 84 (49.4%)	-	-	-
Risk	110	L: 28 (25.5%), I: 47 (42.7%), H: 35 (31.8%)	-	-	-
Blast Content (%)	135	-	50	51	26
Culture Media	168	CM: 105 (62.5%), MCM: 46 (27.4%), both: 17 (10.1%)	-	-	-
Mutation Burden	139	-	42	61	66.

D: diagnosis, **R:** relapse/refractory, **L:** low, **I:** intermediate, **H:** high, **CM:** conditioned medium, **MCM:** mononuclear cell medium