ASSOCIATION OF FLT3 AND NPM1 MUTATIONS IN ACUTE MYELOID LEUKEMIA PATIENTS WITH METABOLOMIC PATTERNS DETERMINED BY MASS SPECTROMETRY

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ASSOCIATION OF FLT3 AND NPM1 MUTATIONS IN ACUTE MYELOID LEUKEMIA PATIENTS WITH METABOLOMIC PATTERNS DETERMINED BY MASS SPECTROMETRY

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ABSTRACT

ASSOCIATION OF FLT3 AND NPM1 MUTATIONS IN ACUTE MYELOID LEUKEMIA PATIENTS WITH METABOLOMIC PATTERNS DETERMINED BY MASS SPECTROMETRY

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Acute Myeloid Leukemia is a hematological cancer with high phenotypic and genotypic heterogeneity. Patients diagnosed with AML are categorized into risk groups based on cytogenetic and molecular abnormality tests, which determine the specific treatment regimes. Since risk status determination takes significant amount of time and some emergency patients require immediate treatment, a method to provide fast clinical data that will be the basis for initial treatment regime is needed in the medical community. Objective of the proposed work is to discover patterns associated with FLT3-ITD and NPM1 mutations in Acute Myeloid Leukemia patients, which will provide a fast clinical method for the proper first-response treatment. The rationale for the hypothesis is based on the previous studies which indicate a link between NPM1 and FLT3-ITD mutations with glucose and amino acid metabolism. NPM1 and FLT3 mutations were chosen based on their high frequency in AML patients and their essential role in risk group determination.

Metabolic pattern determination of the mutations was achieved by LC-MS/MS measurements of amino acid and acyl carnitine panels that are highly associated with amino acid and glucose metabolism. After preprocessing of the raw data, univariate (ANOVA) and multivariate analyses (PCA and PLS-DA) were performed to define class discrimination between sample groups. The remarkable analytes that have significant power in the discrimination were determined by VIP analysis. The developed model was validated with K-Fold cross validation method and permutation test. The most significant pathways in class discrimination were identified with pathway analysis. Visualization was accomplished via Metaboanalyst 5.0 software.

Principal Component Analysis (PCA) showed that 79% of the total variance of the sample groups was explained by the model. In order to increase class discrimination, Partial Least Squares-Discriminant Analysis (PLS-DA) was performed. R2Y and Q2 were found as 0.845 and 0.619, respectively. PLS-DA model was validated with K-fold analysis and permutation test. In all the validation experiments carried out, a low cross-validation error was observed. In VIP analysis, the most significant analytes that cause the class discrimination were found as C0 carnitine, glutamic acid, aspartic acid, tryptophan, and histidine, respectively. In the pathway enrichment analysis performed with these analytes, aminoacyl t-RNA biosynthesis, arginine biosynthesis, valine-leucine-isoleucine biosynthesis, alanine-aspartate-glutamate metabolism, histidine metabolism and arginine-proline metabolism were found as statistically significant pathways responsible for the class discrimination.

In conclusion, a preliminary model based on the targeted metabolomics approach was developed for the prediction of mutation status of NPM1 and FLT3 proteins in AML patients. Proposed model has a high fit value, validity, and strong predictive power. The reliability and validity of the model can be further increased by future multicenter studies.

Keywords: Hematological Malignancies, Acute Myeloid Leukemia, Metabolomics, Multivariate Analysis, Mass Spectrometry

ÖZ

AKUT MİYELOİD LÖSEMİ HASTALARINDA FLT3 VE NPM1 MUTASYONLARININ KÜTLE SPEKTROMETRESİ İLE BELİRLENEN METABOLOMİK DESENLERLE İLİŞKİLENDİRİLMESİ

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Akut Miyeloid Lösemi (AML), yüksek fenotipik ve genotipik heterojeniteye sahip hematolojik bir kanserdir. AML tanısı konan hastalar, spesifik tedavi rejimlerini belirleyen sitogenetik ve moleküler anormallik testlerine göre risk gruplarına ayrılır. Risk durumunun belirlenmesi önemli ölçüde zaman aldığından ve bazı acil hastaların acil tedaviye ihtiyacı olduğundan, tıp camiasında ilk tedavi rejimine temel oluşturacak hızlı klinik verileri sağlayacak bir yönteme ihtiyaç duyulmaktadır. Önerilen çalışmanın amacı, uygun ilk yanıt tedavisi için hızlı bir klinik yöntem sağlayacak olan Akut Miyeloid Lösemi hastalarında FLT3-ITD ve NPM1 mutasyonları ile ilişkili şablonları keşfetmektir. Hipotezin gerekçesi, glukoz ve amino asit metabolizması ile NPM1 ve FLT3-ITD mutasyonları arasında bir bağlantı olduğunu gösteren önceki çalışmalara dayanmaktadır. NPM1 ve FLT3 mutasyonları, AML hastalarındaki yüksek sıklıklarına ve risk grubu belirlemedeki temel rollerine göre seçilmiştir.

Mutasyonların metabolik patern belirlemesi, amino asit ve glukoz metabolizması ile yüksek oranda ilişkili olan amino asit ve açilkarnitin panellerinin LC-MS/MS ölçümleri ile sağlanmıştır. Ham verilerin ön işlenmesinden sonra, örnek grupları arasında sınıf ayrımını tanımlamak için tek değişkenli (ANOVA) ve çok değişkenli analizler (PCA ve PLS-DA) yapılmıştır. Ayırt etmede önemli güce sahip olan dikkat çekici analitler VIP analizi ile belirlenmiştir. Geliştirilen model, K-Fold çapraz doğrulama yöntemi ve permütasyon testi ile doğrulanmıştır. Yol analizi ile sınıf ayrımcılığındaki en önemli yollar belirlenmiştir. Görselleştirme Metaboanalyst 5.0 yazılımı ile gerçekleştirilmiştir.

Temel Bileşen Analizi (PCA), örneklem gruplarının toplam varyansının %79'unun model tarafından açıklandığını göstermiştir. Sınıf ayrımını artırmak için Kısmi En Küçük Kareler-Diskriminant Analizi (PLS-DA) yapılmıştır. R2Y ve Q2 sırasıyla 0.845 ve 0.619 olarak bulunmuştur. PLS-DA modeli, K-kat analizi ve permütasyon testi ile doğrulanmıştır. Gerçekleştirilen tüm doğrulama deneylerinde, düşük bir çapraz doğrulama hatası gözlenmiştir. VIP analizinde sınıf ayrımına neden olan en önemli analitler sırasıyla C0 karnitin, glutamik asit, aspartik asit, triptofan ve histidin olarak bulunmuştur. Bu analitler ile yapılan yol zenginleştirme analizinde, aminoaçil t-RNA biyosentezi, arjinin biyosentezi, valin-lösin-izolösin biyosentezi, alanin-aspartat-glutamat metabolizması, histidin metabolizması ve arjinin-prolin metabolizması sınıftan sorumlu istatistiksel olarak anlamlı yolaklar olarak bulunmuştur.

Sonuç olarak, AML hastalarında NPM1 ve FLT3 proteinlerinin mutasyon durumunun tahmini için hedeflenen metabolomik yaklaşıma dayalı bir ön model geliştirilmiştir. Önerilen model, yüksek bir uyum değerine, geçerliliğe ve güçlü bir tahmin gücüne sahiptir. Modelin güvenirliği ve geçerliliği gelecekte yapılacak çok merkezli çalışmalarla daha da arttırılabilir.

Anahtar Kelimeler: Hematolojik Maligniteler, Akut Miyeloid Lösemi, Metabolomiks, Multivaryasyon Analizi, Kütle Spektrometresi

“Well, life for none of us has been a crystal stair, but we must keep moving. We must keep going. And so, if you can't fly, run. If you can't run, walk. If you can't walk, crawl. But by all means, keep moving.”
— Martin Luther King, Jr.

To my superheroes –my mother & father-

who believe that “an investment in education gives the best returns.”

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ABBREVIATIONS	
AML	:Acute Myeloid Leukemia
CEBPA	:CCAAT Enhancer Protein Alpha
CN	:Cytogenetically Normal
ESI	:Electrospray Ionization
FDG	:Fluoro-2-deoxy-D-glucose
FLT3	:Fms-related tyrosine kinase 3
HK	:Hexokinase
HMDB	:Human Metabolome Database
IDH 1/2	:Isocitrate Dehydrogenase 1/2
ITD	:Internal Tandem Duplication
LC-MS	:Liquid Chromatography Mass Spectrometry
MALDI	:Matrix-Assisted Laser Desorption/Ionization
MRI	:Magnetic Resonance Imaging
MS	:Mass Spectrometry
NCCN	:National Comprehensive Cancer Network
NGS	:Next Generating Sequencing
NMR	:Nuclear Magnetic Resonance
NPM1	:Nucleophosmin-1
PCA	:Principal Component Analysis
PET	:Positron Emission Tomography
PLS-DA	:Partial Least Square- Discriminant Analysis
RTK	:Receptor Tyrosine Kinase
SMDB	:Serum Metabolome Database
TCA	:Tricarboxylic Acid
TKD	:Tyrosine Kinase Domain
Q	:Quadrupole
QqQ	:Triple Quadrupole
WHO	:World Health Organization
VIP	:Variance Importance Projection

LIST OF SYMBOLS

SYMBOLS

μM :Micromolar

CHAPTER 1

INTRODUCTION

1.1. Acute Myeloid Leukemia

Leukemia is the second most common hematological cancer and thirteenth among all cancer types. Acute Myeloid Leukemia (AML) accounts for 30% of all leukemia types. Symptoms such as fever, anemia, and bleeding occur in AML patients due to overproduction of immature white blood cells (myeloblasts) and a deficiency of mature white blood cells such as neutrophils and monocytes (Siegel et al., 2021; *Worldwide Cancer Data, World Cancer Research Fund*, 2022).

Despite the very important advances and developments in the field of both disease-specific treatment and supportive treatment -*infection control-related and supportive treatment-related*-, the chance of long-term complete response and/or cure with treatment cannot exceed 35-40% even in patients under the age of 60, which is a relatively better subgroup (Döhner et al., 2015). Unfortunately, in this disease, which has a median age of diagnosis of 68 and more than 50% of patients are over the age of 65, the long-term chance of complete response and/or cure in patients over 60 years of age is only between 5% and 15% (Döhner et al., 2010). In fact, it is known that the median survival of patients with AML in the elderly group who cannot receive intensive chemotherapy due to side effects is around 5 to 10 months.

There are plenty of defined cytogenetic abnormalities and mutated genes in AML. Although the genetic heterogeneity in AML has been known for almost 30 years, its enormous molecular heterogeneity has started to become evident for about 15 years. The fact that this biological heterogeneity has earned it the title of "guidance" both in the field of diagnosis and especially in the field of treatment has

been recently accepted. When the international guidelines in the last 10 years are examined, the place of molecular screening in prognostic classification and ultimately its importance in the treatment decision can be clearly seen (Saultz & Garzon, 2016).

According to accomplished studies, molecular abnormalities such as Nucleophosmin-1 (NPM1), Fms-like Tyrosine Kinase 3 Internal Tandem Duplication (FLT3-ITD), CCAAT Enhancer Protein Alpha (CEBPA), Isocitrate dehydrogenase 1/2 (IDH1/2) and KIT (c-Kit Type III Reseptor Tyrosine Kinase) are important for risk stratification not only in AMLs with a normal karyotype, but also in other AML subgroups. Some of these molecular abnormalities also affect the choice of treatment for patients with AML. As a result of the directives determined by the NCCN (National Comprehensive Cancer Network) for patients diagnosed with AML, a risk classification (Pollyea et al., 2022) is applied based on both chromosomal (cytogenetic) and protein (molecular anomaly) findings (Figure 1.1). This classification of patients has great importance in terms of prognosis and treatment.

Risk Category*	Genetic Abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Biallelic mutated *CEBPA* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}†
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}† Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}† (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*‡ Cytogenetic abnormalities not classified as favorable or adverse
Poor/Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)* -5 or del(5q); -7; -17/abn(17p) Complex karyotype,§ monosomal karyotype‖ Wild-type *NPM1* and *FLT3*-ITD^{high}† Mutated *RUNX1*¶ Mutated *ASXL1*¶ Mutated *TP53*#

Figure 1. 1. NCCN guidelines for Acute Myeloid Leukemia version 2.2022 (Pollyea et al., 2022)

1.1.1. Mutations & Risk Classification of Patients

According to cancer statistics, approximately 50% of AML cases are in the normal category when examined cytogenetically (Meyer & Levine, 2014). AML cases with normal cytogenetics are classified in the intermediate risk category in terms of recurrence and prognosis (Döhner et al., 2017). However, in terms of clinical outcome, there is a substantial heterogeneity within this group (Medinger & Passweg, 2017). When all AML classifications are taken into consideration, it can be clearly seen that FLT3-ITD is the only molecular abnormality that determines the poor risk in AML cases even with normal cytogenetics and is included in the intermediate group and classifies these cases directly to the bad risk group (Medinger & Passweg, 2017; Pollyea et al., 2022). In Figure 1.1, the only molecular abnormality in AML that determines poor prognosis and classified patients as in the poor risk group is FLT3-ITD. In other words, the presence of FLT3-ITD in a patient indicates that the patient has a poor prognosis, even if the cytogenetic classification is at good risk (Pollyea et al., 2022).

In the same figure, NPM1 which is known as a good risk predictor when seen alone, ranks first with a rate of 30-45% (Medinger & Passweg, 2017). Moreover, it is emphasized that the mutation in the NPM1 gene is seen in approximately 30% of all AML cases and in more than 50% of AML cases with normal cytogenetics (Grimwade et al., 2016). The frequencies and characteristics of these mutations and the tables are presented below (Figure 1.2).

NPM1 and FLT3 tests are primarily examined when hematopoietic stem cell transplantation is required in AML patients. In the guideline for AML hematopoietic stem cell transplantation published by the Turkish Ministry of Health in 2017 and still in force, the presence of NPM1 and FLT3 mutations among the all protein abnormalities in AML ranks first for the patients who received first-line treatment but still immediately need stem cell transplantation due to their bad prognosis (*Turkish Ministry of Health, Transplant Indications in AML*, n.d.).

In the light of this information, it can be understood that AML is a heterogeneous malignancy. However, due to their importance in risk classification and stem cell transplantation, determination of the status of FLT3 and NPM1 mutations has a primary priority in terms of prognosis and treatment.

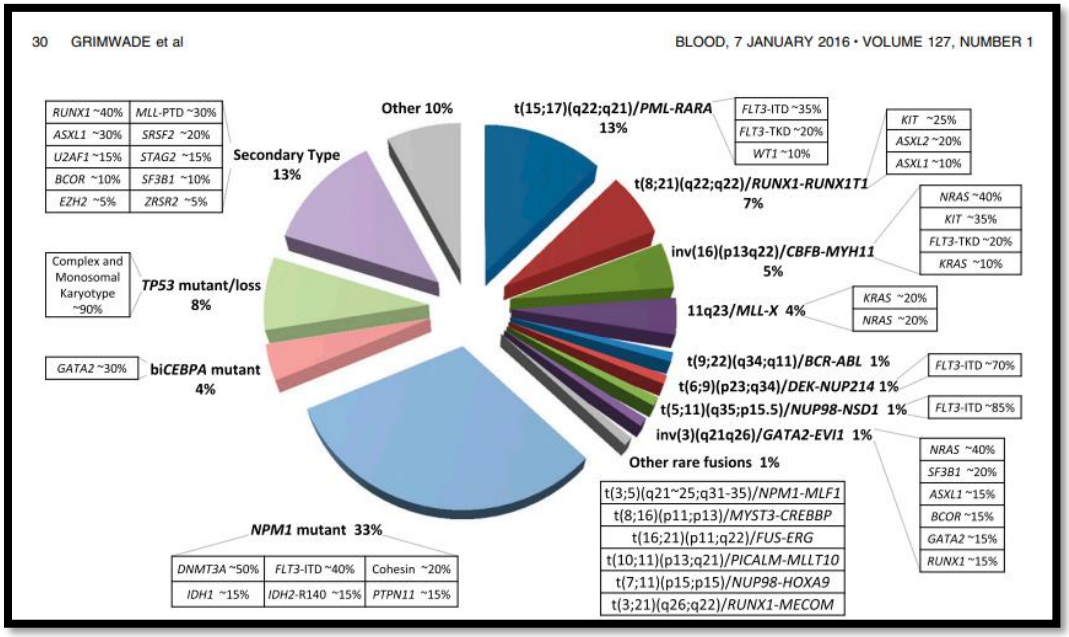


Figure 1. 2. Incidence information of mutations in AML cases (Grimwade et al., 2016)

1.1.1.1. Nucleophosmin-1 (NPM1)

NPM1 (B23, No38 or Numatrin) is shuttle phosphoprotein between nuclei and cytoplasm and exhibits two-sided function as oncogenic or tumor suppressor. Shuttling activity is crucial for normal cell function during cell cycle. It is well documented from the studies (Falini et al., 2009; Okuwaki et al., 2002) that NPM1 takes part in response to stress stimuli, ribosome biogenesis, mRNA processing, genomic stability, cell cycle progression etc. The explanation of these various roles is that NPM1 has several functional domains which provide interactions with many biomolecules such as transcription factors (IRF-1, NFkB), histones (H3, H4), proteins (p53, ARF) and enzymes (DNA Polymerase alpha). The interactions

between these molecules can explain two-sided activity of NPM1 for oncogenic and suppressor functions in the cell (Kelemen, 2022).

NPM1 can contribute to formation of cancer in three well-known mechanisms: overexpression, mutation and fusion proteins with NPM1. Chromosomal translocation and mutation of the NPM1 frequently occur in hematological malignancy. For instance, abnormal formation of the fusion protein NPM-ALK is observed in anaplastic large cell lymphoma (Andraos et al., 2021).

On the other hand, a mutation in exon 12 of NPM1 (Type A mutation) is unique to AML, resulting in abnormal localization of the protein in the cytoplasm. There is a frameshift in the region encoding C-terminus of the protein. This abnormal issue results in formation of a nuclear export signal. Within this signal, expression of the mutated NPM1c+ protein is increased. NPM1 mutations are a founder genetic abnormality in formation and also in disease maintenance. There are approximately 50 different kinds of NPM1 mutations in AML. All these mutations create cytoplasm-dislocated NMP1c+ protein. This protein has importance in AML in terms of oncogenicity (Y. Chen & Hu, 2020).

NPM1 mutations are the most frequent genetic abnormalities in AML patients. These mutations have a prognostic significance. According to international guidelines for AML classifications, in the absence of ITD of FLT3 gene in cytogenetically normal AML (CN-AML) cases, these mutations have been associated with favorable prognosis (Y. Chen & Hu, 2020). As distinct from other myeloid mutations, NPM1 is exceptional for its specificity to a subtype of AML which is identified as a specific diagnostic feature by WHO classification of myeloid cancers (Verhaak et al., 2005) .

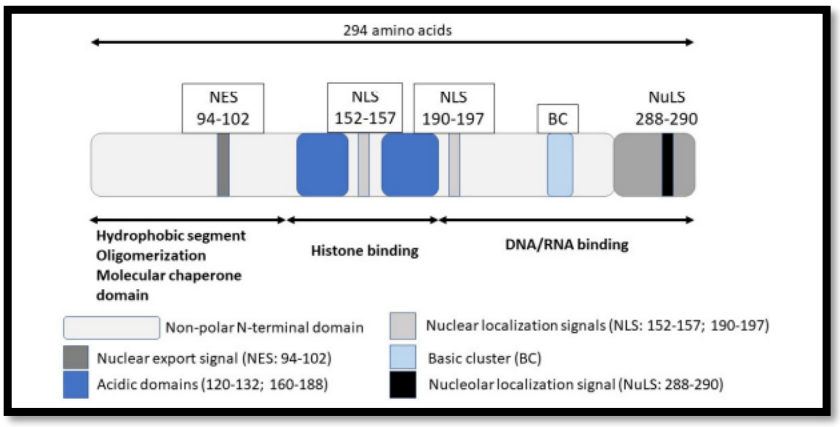


Figure 1. 3. Functional domains of NPM1 (Kelemen, 2022)

The link between increased stem-cell associated genes (HOXA, HOXB, MEIS1, etc.) and NPM1c+ leukemia cells have been studied in order to define genetic characterization of the mutated cells. Within relocalization of NPM1c+ from cytoplasm to the nucleus caused in a downregulation of the HOX/MEIS1. These findings show the importance of therapeutic potential of inducing NPM1 (Y. Chen & Hu, 2020).

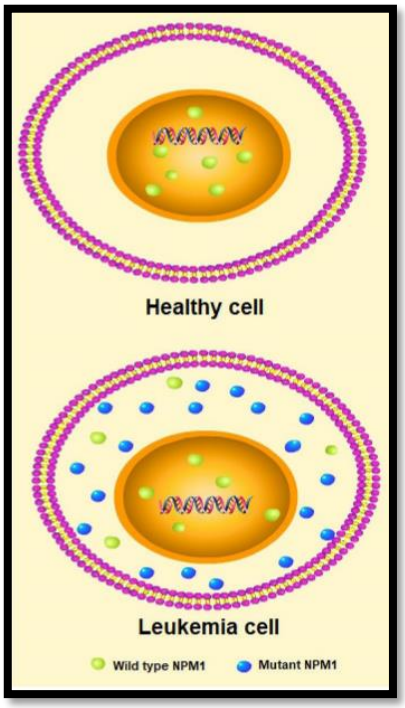


Figure 1. 4. Cellular location of NPM1 protein for healthy and leukemia cell (Y. Chen & Hu, 2020)

Mutated NPM1 is also important to monitor disease status in AML. The mutation disappears with complete remission. However, it reappears at relapse. Based on this information, a large cohort of treated AML patients further showed that NPM1 mutation can be also used for prediction of residual disease(Ivey et al., 2016). If the patient's NPM1 mutation status still cannot disappear after the second cycle of chemotherapy, it has been associated with a high risk of relapse after 3 years of follow-up. NPM1 mutations were found in 95 % of AML relapsed patients. In the light of this information about NPM1 mutations, it can be obviously said that NPM1 measurement is not only important in determination of risk classification of AML patients at the beginning of prognosis, but also important in monitoring disease status.

1.1.1.2. FMS-like Tyrosine Kinase 3 (FLT3)

FLT3 is a member of the subfamily Platelet-derived Growth Factor Receptor (PDGF-R) of receptor tyrosine kinases (RTK) also known as Stem Cell Kinase-1. This transmembrane ligand activated RTK member was first recognized by its expression in hematopoietic stem cells (HSCs). With the following studies, its importance in normal HSC function is well known now (Levis & Small, 2003).

In order to preserve normal function of HSCs, it is important to regulate microenvironment with network of hematopoietic growth factors (IL-3, IL-6, SCF, LIF, FLT-3 ligand), signaling pathways (Wnt, Notch), cell cycle regulators (p21,p53) and transcription factors (RUNX1, HOX) (Roboz & Guzman, 2009). When the network is considered, it is not surprising that FLT3 has an important role in AML.

Upon binding of FLT3 ligand (FL) to FLT3, homodimerization and conformational changes occur, within formation of autophosphorylation signals promotes cell survival, proliferation and differentiation through downstream molecular pathways such as PI3K, RAS, STAT5 (Knight et al., 2022). After dimerization and activation of the receptor, it is immediately internalized and degraded.

FLT3 mutations are found in approximately 30 % of *de novo* AML patients. All FLT3 mutations cause constitutive activation of the receptor, resulting in survival and proliferation of AML (Daver et al., 2019).

One of the common mutations in FLT3 is ITD (Internal Tandem Duplications) mutations for AML cases. In FLT3-ITD, additional amino acids are inserted into the juxtamembrane domain. Hence, the auto-regulatory function of this domain is disrupted. Receptor can be dimerized, autophosphorylated and constitutively activated in absence of the ligand (Grafone et al., 2012). When constitutively activated receptor signal and wild type (WT) activated FLT3 receptor signal are compared, downstream pathway activations differ. FLT3-ITD causes repression of two transcription factors, important for myeloid maturation (PU.1 and C/EBP) (Gruszka et al., 2019; Knight et al., 2022). Another difference is that higher mRNA levels of Fizzled-4 receptor in Wnt pathway were found in FLT3-ITD cells compared with FLT3-WT (Gruszka et al., 2019). In addition, high β-Catenin levels are found in FLT3-ITD mutations. In the light of this information, there is a synergy between FLT3-ITD mutation and Wnt pathway in AML (Gruszka et al., 2019).

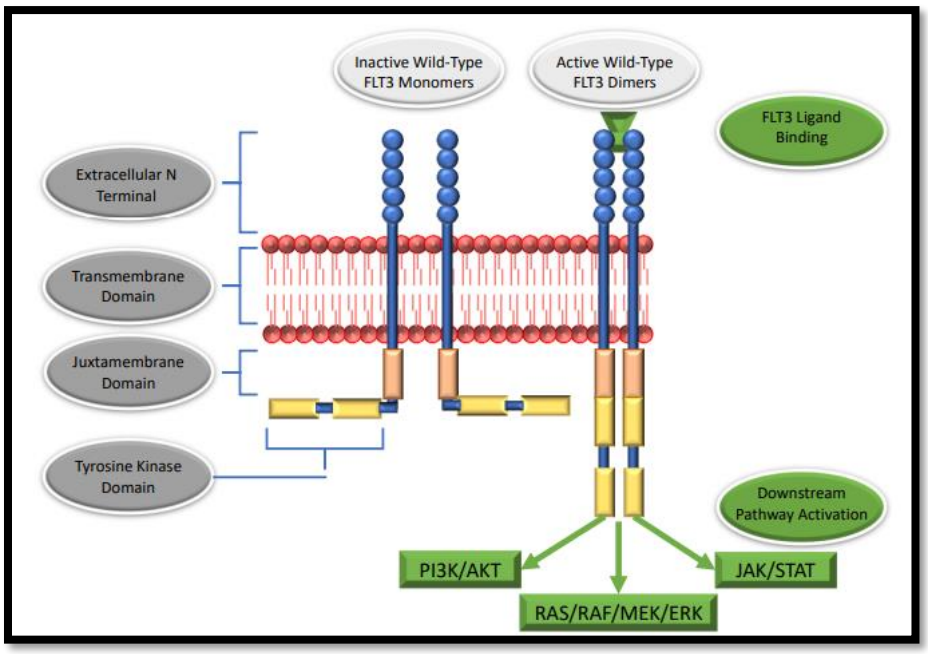


Figure 1. 5. Inactive and active form of wild type FLT-3 (Knight et al., 2022)

The relation between constitutively activated Wnt signaling and cancer cell metabolism were studied. According to these studies, Wnt network is a regulator of cancer cell metabolism (Lee et al., 2012; Pate et al., 2014; Sherwood et al., 2014). The results show that there is a crosstalk between Wnt and c-MYC -downstream protein of Wnt- pathways in cancer cells. It means that β-catenin-mediated c-Myc expression causes high expression of rate-limiting glycolytic genes via Lactate Dehydrogenase (LDH), glucose transporter 1 (GLUT-1), and the M2 isoform of pyruvate kinase in order to boost aerobic glycolysis in cancer (Sherwood, 2015). Consequently, FLT3-ITD mutation affects downstream signaling pathways resulting in significant changes in cancer cell metabolism.

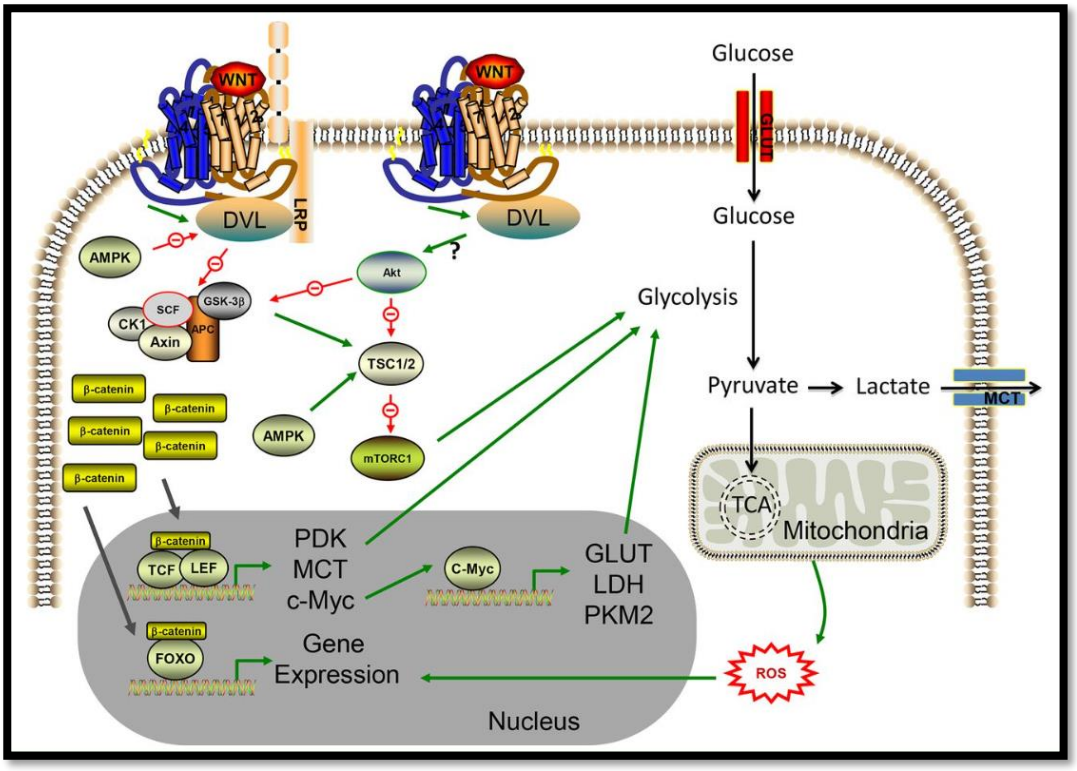


Figure 1. 6. Wnt signaling affects cancer cell metabolism via downstream pathways (Sherwood, 2015)

Based on these significant effects on cancer cell metabolism, proliferation and survival, FLT3-ITD mutation is associated with poor prognosis in AML cases and has a negative impact on the management of patients (Daver et al., 2019).

1.1.2. Clinician's Perspective on AML Patients

AML is a hematological malignancy that develops and progresses rapidly in a few weeks. Management of AML includes standard therapy (according to international guidelines e.g., NCCN), palliative care and clinical trials. According to established prognostic algorithms of NCCN, all data belonging to new drugs are evaluated and compared with the results of the previous year. Then, new treatment options are offered to clinicians (Estey, 2020). As seen in Figure 1.7, treatment strategies and options are clearly given according to risk evaluations.

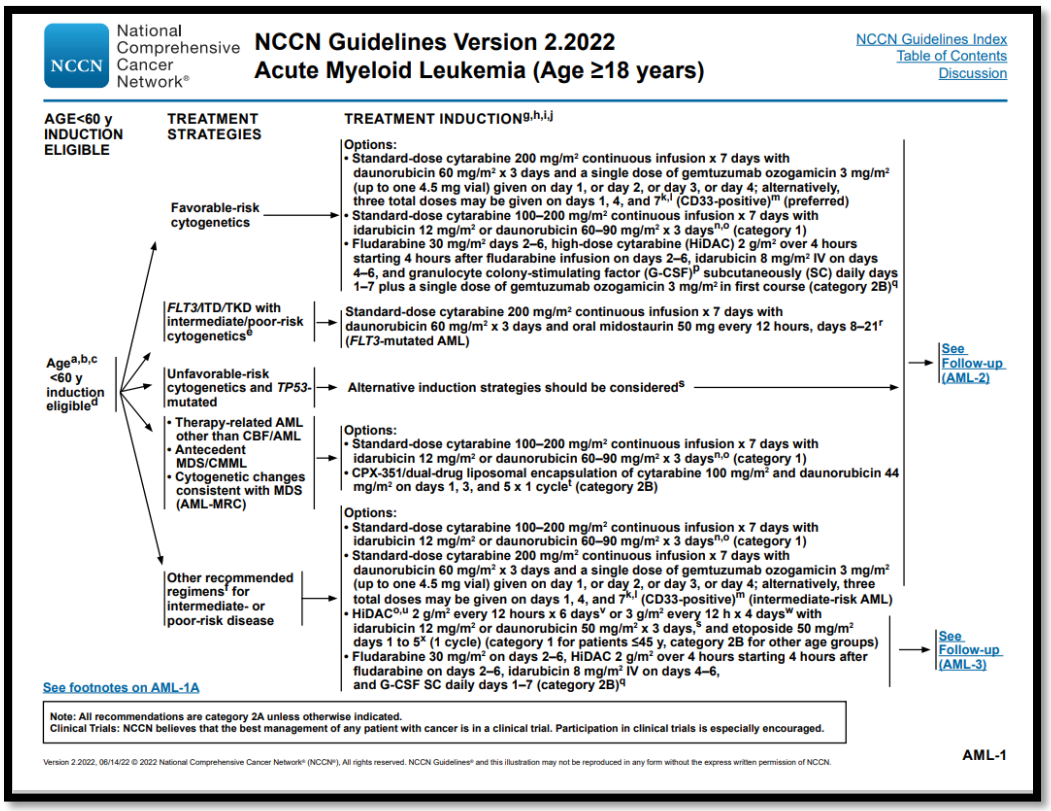


Figure 1. 7. NCCN Guideline to define risk stratification of AML cases (*NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®), Acute Myeloid Leukemia Version 2.2022,* 2022)

In the first sight of a newly diagnosed AML patient, clinicians must take into consideration whether to suggest standard therapy, clinical trial and allogeneic transplantation after induction therapy. The path to be chosen by the clinician should be determined as a result of the risk/benefit analysis of all treatment options.

It should not be forgotten that current therapies were clinical trials even 1-2 years ago.

AML is a rapidly progressing cancer type that requires urgent treatment. Obtaining testing results of the cytogenetic & mutations panel takes approximately 14-21 days. In case of suspicious results, the sample is measured again. In some cases, it is impossible to wait for genetic testing results. According to Röllig et al., the risk in awaiting testing results is less than the risk in beginning therapy before results are available (Röllig et al., 2019).

As mentioned above, according to NCCN classification, NPM1, FLT3-ITD, CEBPA, IDH1/2, DNMT3A, KIT and other mutations are important for prediction of all AML subsets (*NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®), Acute Myeloid Leukemia Version 2.2022*, 2022). However, in Turkey, all these mutations are not checked as a routine test in the AML panel of hematology centers. Besides that, measurement of the mutation panel is only performed in major hospitals. Even though a number of university hospitals in major cities are capable to define molecular abnormality tests via Next Generation Sequencing (NGS), obtaining results can prolong up to 4-8 weeks (*Erciyes University, Laboratory Test Guideline*, 2017; *Medipol AML-NPM1 Sequence Analysis*, n.d.).

Although there are plenty of mutations that affect the risk stratification in AML, the parameters which are preferentially measured at Ankara University Hematology Department -due to their incidence and strategic importance- are given below in Figure 1.8 (Grimwade et al., 2016).

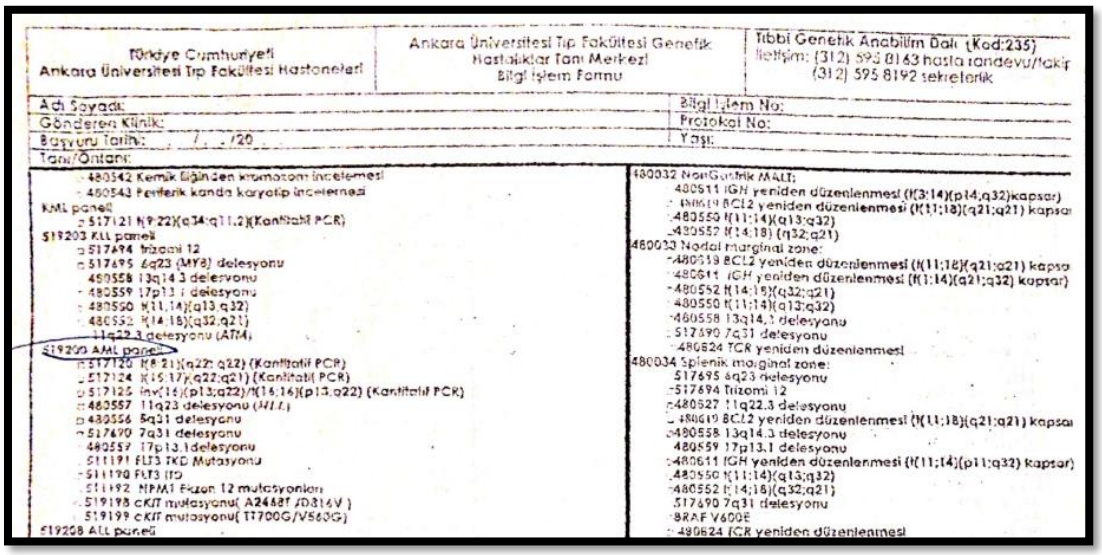


Figure 1. 8. The parameters of AML panel which are preferentially measured in Ankara University Hematology Department

In the light of these information, until obtaining whole mutation panel results of *de novo* AML patient, rapid diagnosis techniques are needed, primarily for FLT3 and NPM1 and later for other critical mutations in order to guide the clinician quickly (Röllig et al., 2019).

1.2. Metabolomics

Omic technologies refer to universal detection of characteristics of cellular molecules in a specific biological sample. For instance, genomics is the first step of omics cascade. Genomics discovers structure, function, mapping of genomes and characterizes features and their relation with production of related proteins. On the other hand, metabolomics is the last step of omics cascade and identifies/quantifies cellular metabolites (<1500 Da) using high throughput analytical techniques such as Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography Mass Spectrometry (GC-MS), Nuclear Magnetic Resonance (NMR) with sophisticated statistical methods (multivariate analysis) in order to obtain significant information from huge data stack. Since it is the last step of the cascade, information which is obtained from metabolomics studies is closer to predict phenotype than other omic areas.

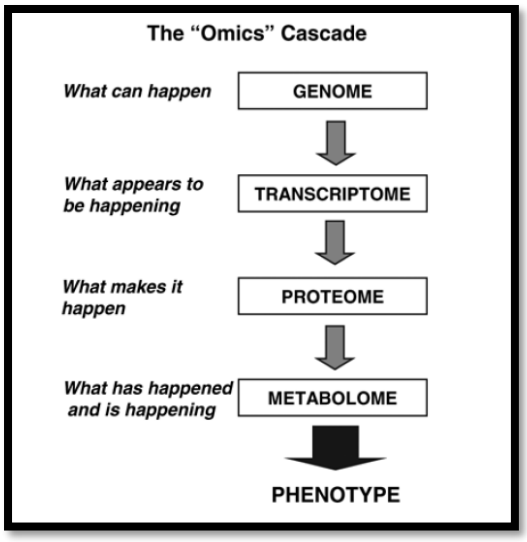


Figure 1. 9. Omics cascade (Anton Pervukhin, 2009)

Pathological disorders, organ dysfunctions or tumors can affect chemical and protein content of biological samples such as blood, plasma and serum. Since most of today's clinical tests (e.g., lactate, phenylalanine, Free T3) are based on the analysis of biological samples, metabolomics studies have crucial clinical importance for new diagnostic tests in future (Psychogios et al., 2011).

Serum Metabolome Database (SMDB) and Human Metabolome Database (HMDB) are examples for free sources in order to obtain information about metabolite names, level of verification, concentration ranges and related diseases (Psychogios et al., 2011).

1.2.1. Cancer Metabolomics

In 1927, Warburg et al., published a milestone study about a distinct feature of cancer cell metabolism. In this study, which will be referred to as the Warburg effect in the following years, it was shown that cancer cells consume 200X more glucose than healthy cells (Warburg et al., 1927). On average, glycolysis is 10-100 times faster than oxidative phosphorylation (Liberti & Locasale, 2016) in cancer

cells. Since cancer cells need a rapid energy source for biosynthetic pathways in order to proliferate and survive, glycolysis is a reasonable choice.
However, interest in this distinct metabolic phenotype significantly decreased in the 1960s. The fact of "cancer is a metabolic disease" has been faced again in the last 15 years. Due to the increased technology of instrumental techniques changed the way of thinking about cancer studies. With these innovations, cancer biology was re-focused on the importance of signaling pathways and transcription factors in order to understand control of tumor growth and proliferation rate. However, in the last few years, the question that "how disrupted metabolism can contribute to cancer pathogenesis" has been investigated. This question was arisen because of the understanding of disrupted pathways and changes at gene level affect metabolic pathways (Dang & Semenza, 1999; Hsu & Sabatini, 2008). With increasing metabolomics studies, the term "oncometabolite" has been used in the area to define small molecules of metabolism that are closely interacted with tumor microenvironment.

Oncometabolites are endogenous molecules that specifically related with tumor growth and metastasis. 2-hydroxyglutarate (2HG) was the first oncometabolite found in high concentration in glioma and AML (Ward et al., 2010). After the discovery of 2HG, many other oncometabolites were discovered such as fumarate (renal cell carcinoma)(Panarsky et al., 2020), sarcosine (prostate cancer) (Sreekumar et al., 2009) and succinate (paraganglioma) (Imperiale et al., 2013) .

During the production of oncometabolites, equilibrium of metabolism and redox is taken into consideration. For instance, succinate dehydrogenase/fumarate hydratase depleted cancers cause the loss of key enzymes for Krebs cycle and related metabolites resulting in increased Warburg-like metabolism (Yong et al., 2020).

The common point of all these discovered oncometabolites is that they are required for cancer-related metabolic pathways or are produced as a result of these pathways. In addition to being the final products of complex oncogenic pathways,

oncometabolites can influence pathways through feedback signals (Yang et al., 2013).

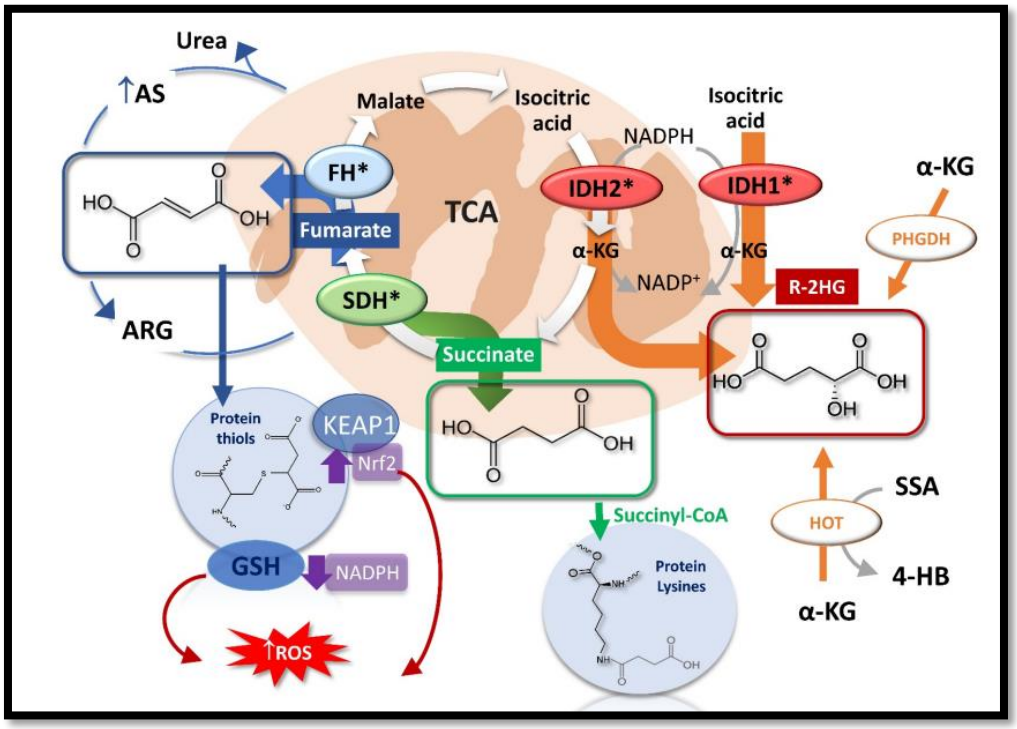


Figure 1. 10. Production of oncometabolites and related reactions (di Gregorio et al., 2021)

Metabolomics also enables pattern recognition with rapid, accurate and precise analytical techniques such as LC-MS, GC-MS and NMR. These techniques are capable of detecting hundreds of biomolecules in a short time at the same run. Through this capability, metabolomics is a useful approach in order to discover patterns even for cancer subtypes (Díaz et al., 2021; Erlic et al., 2021; Inoue et al., 2015; Michálková et al., 2018; Weckwerth & Morgenthal, 2005; P.-C. Zhou et al., 2020).

Besides the discovery of oncometabolites as a biomarker and cancer related patterns, metabolomics can be beneficial for imaging metabolic biomarkers. As we know, X-ray, positron-emission tomography (PET), magnetic resonance imaging (MRI), magnetic resonance spectroscopy imaging (MRSI), and ultrasound

are useful imaging techniques to diagnose cancer (Brindle, 2008). PET uses a radiolabeled glucose - 2-[18F] fluoro-2-deoxy-D-glucose (FDG)- based on the Warburg effect (Friess et al., 1995). Another example of metabolomics studies in imaging techniques is MRSI detection of choline in breast tumors. With this imaging technique, unnecessary breast biopsies are reduced (Bartella & Huang, 2007).

The last but perhaps the most important application of metabolomics in cancer is targeted drug therapy. The first drugs for the treatment of cancer are based on use of similar structures of metabolites that can interfere with their related pathway. For instance, Cytarabine is an anti-metabolite in AML treatment that targets late-stage DNA synthesis. 2-deoxyglucose interferes with glucose and targets the glycolysis enzyme, hexokinase (HK) (Beger, 2013). Different examples are given in Table 1.1.

Table 1. 1. Examples for antimetabolite drugs.

Metabolic Pathway	Target	Drug	Stage of Development
Nucleic acid synthesis	Dihydrofolate reductase (DHFR)	Methotrexate	Approved
Nucleic acid synthesis	5-Phosphoribosyl-1-pyro-phosphate (PRPP)	Mercaptourine, thioguanine	Approved
Glycolysis	GLUT1	WZB117 (Liu et al., 2012), BAY-876 (Siebeneicher et al., 2016)	Preclinical
Glycolysis	Hexokinase	2-Deoxyglucose(Zhong et al., 2008)	Phase 1/2
Amino acid transport and biosynthesis	Circulating asparagine	L-asparaginase (*FDA Approves Asparaginase Erwinia Chrysanthemi (Recombinant) for Leukemia and Lymphoma*, 2021)	Approved

1.2.2. Strategies for Metabolomics

Within the increased technology level of analytical techniques, there are 2 main strategies in metabolomics called as untargeted and targeted metabolomics (Figure 1.11).

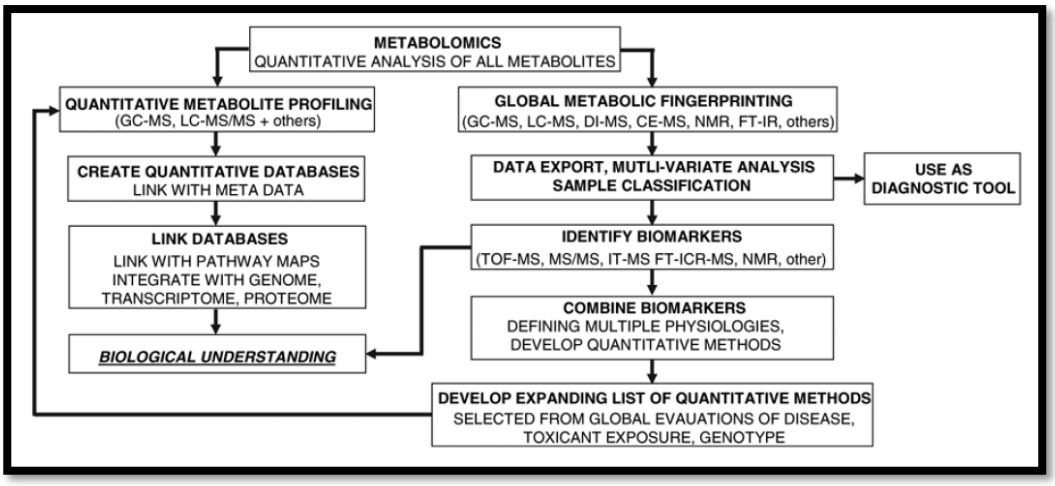


Figure 1. 11. Flow chart of metabolomic approaches

Untargeted metabolomics is based on screening of biological samples in order to obtain fingerprints of the patient. Plenty of metabolites can be measured in the same run. Defined/non-defined metabolites can be monitored from the same run. Hence, it can be anticipated that regarding the disease status some metabolites will change and reflect characteristics of the patient group. Besides that, in untargeted metabolomics, there is no need to suggest a hypothesis. Hypothesis can be shaped according to preliminary results. Therefore, new potential biomarkers can be identified with this strategy (Benito et al., 2018; Gika et al., 2019; Ward et al., 2010). If high throughput analytical techniques are also added to the same sample, full information about sample metabolome can be obtained (Barber et al., 2019).

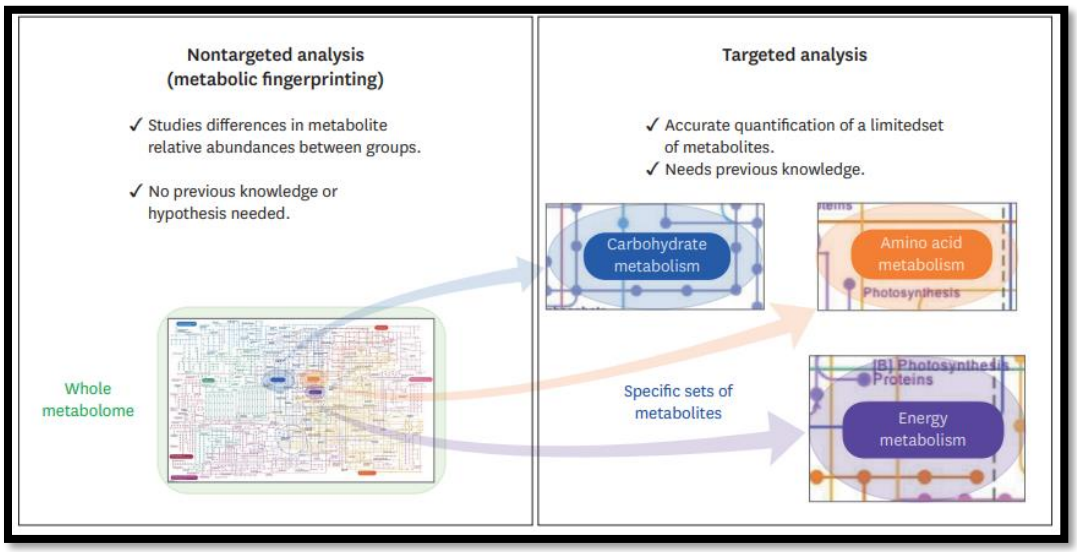


Figure 1. 12. Targeted vs. Untargeted Metabolomics (Barber et al., 2019)

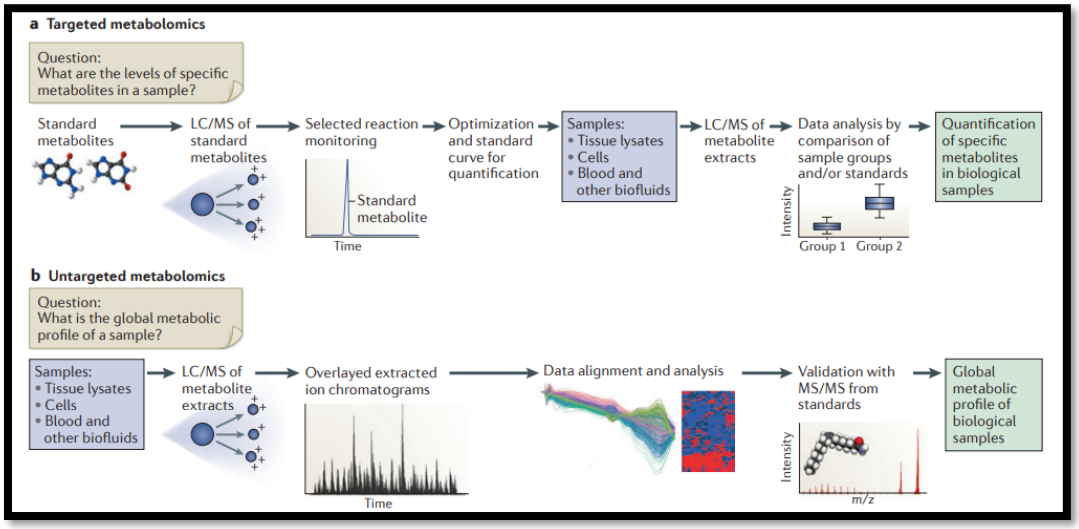


Figure 1. 13. Workflows of targeted/untargeted metabolomics (Patti et al., 2012)

Metabolome profiling is quite a complex process. First of all, some metabolites may be spatial or have circadian fluctuations. Secondly, diet dependent variability should be taken into consideration. Last of all, current analytical techniques may be not sensitive enough for trace level components. Thus, the technology level of the current instruments is the restrictive step for metabolomics (Dettmer et al., 2007).

On the other hand, targeted metabolomics is based on quantification of preselected sets of metabolites (Jasbi et al., 2019; Plewa et al., 2019; Wang et al., 2021).

Preselected metabolites can be chosen according to knowledge obtained from literature. The novelty -compared with classical biochemistry- is based on measurement of many metabolites at the same run with high sensitivity (Barber et al., 2019).

As seen in Table 1.2, both methods have unique disadvantages/advantages.

Table 1. 2. Comparison between targeted and untargeted metabolomics

Features	Untargeted	Targeted
Metabolite coverage	Excellent	Poor
Hypothesis Need	No	Yes
Linear Range	Poor	Good
Repeatability	Poor	Good
Sensitivity	Poor	Good
Need for reference standard	No	Yes
Data analysis	Hard	Easy
High resolution instrument need	Yes	Depends on metabolite concentration in sample
New biomarker discovery	Yes	No

1.2.3. Platforms for Metabolomics Studies

Metabolome is defined as the complete metabolite profile (<1500 Da) of specified biological samples. Biological matrices are generally complex matrices. This complexity is caused by a great number of metabolites with a broad range of physicochemical properties. The following techniques are mostly used in metabolomics: NMR, MS (LC-MS, UHPLC-MS, Capillary Electrophoresis (CE-MS), GC-MS) and Fourier Transform Infrared (FTIR) spectroscopy. Each analytical technique has its own advantages and disadvantages (Figure 1.11). For instance, volatile metabolites are important for our hypothesis, LC-MS is not suitable for our purpose. Due to these reasons, it is difficult to develop a sample

preparation and analysis method that covers all metabolites (Segers et al., 2019). In order to provide metabolite coverage, combined techniques (e.g., LC-MS and GC-MS) can be used for the same sample.

Property	Technique					
	NMR	HPLC–MS	UHPLC–MS	SFC-MS	CE–MS	GC–MS
Sensitivity	-	++	+++	+++	+	+++
Reproducibility	High	Lower than NMR				
Resolution	Low	Higher than NMR				
Quantification	Yes	Yes; internal standard needed				
Amount of sample preparation	Low	Medium	Medium	Medium	Medium	Extensive
Sample volume for injection	100–200 µl	±10 µl	±2-10 µl	±1–10 µl	1–20 nl	±1–2 µl
Range of metabolites	Polar and nonpolar	RPLC: nonpolar HILIC: polar		Polar and nonpolar	Polar	Volatile polar and nonpolar
Identification of metabolites	Easy	Difficult (databases need to be improved)				Easy (spectral libraries)

HILIC: Hydrophilic-interaction liquid chromatography; RPLC: Reversed-phase liquid chromatography; SFC-MS: Supercritical fluid chromatography mass spectrometry; UHPLC–MS: Ultrahigh-performance liquid chromatography mass spectrometry.

Figure 1. 14. Advantages/Disadvantages of analytical techniques in metabolomics (Segers et al., 2019)

1.2.3.1. LC-MS Based Metabolomics

Since its advantages of speed, sensitivity, dynamic range over other analytical techniques in medical research, the most common technique used in metabolomics is MS. Depending on types of basic units (e.g., ionization source, mass analyzer and ion detector), there are plenty of combinations for MS systems (Figure 1.15 and Table 1.3).

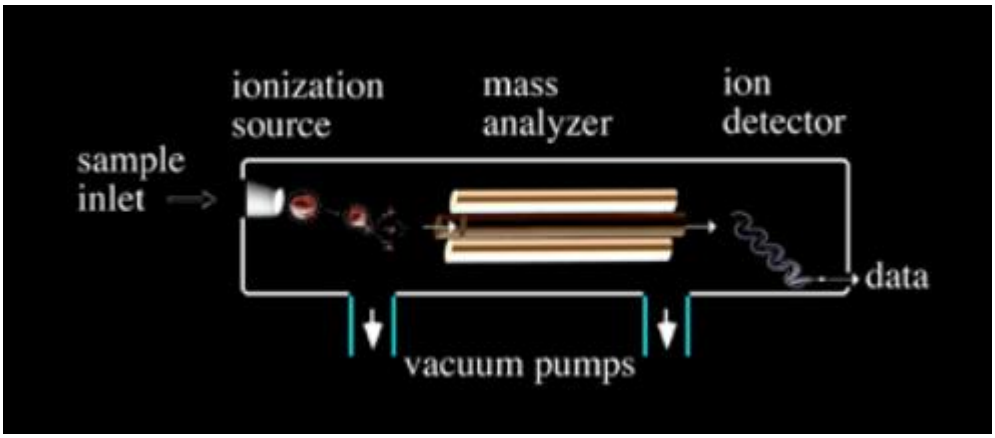


Figure 1. 15. Basic units of Mass Spectrometry

MS-based metabolomics provides quantitative results with high sensitivity, high reproducibility and saves time. Additionally, in case of combined with a separation technique, complexity of mass bulk is reduced. Through this reduction, additional information can be obtained from results. For instance, according to retention times, metabolites can be compared with each other regarding their physicochemical properties.

Table 1. 3. Subunits of mass spectrometry

Sample introduction	Ionization Source	Analyzer Type	Detector
Direct Infusion Direct Ionization	Electrospray Ionization	Quadrupole	Electron Multiplier
Gas chromatography	Chemical Ionization	Ion trap	Faraday Cup
Liquid chromatography	Soft Laser Desorption (MALDI, SELDI, DIOS)	Time of Flight (TOF)	Array Detector
Capillary Electrophoresis	Inductively Coupled Plasma (ICP)	Tandem/Hybrid: Triple Quadrupole (QqQ), Q-TOF), etc.	Charge Detector

Figure 1.13 summarizes advantages/disadvantages of MS types in metabolomics studies. GC-MS is a combination of Gas Chromatography and Mass Spectrometry. Firstly, sample is vaporized into the gas phase. Then, components of the sample are separated using a column. Separated components are carried by an inert gas such as helium, nitrogen or hydrogen. GC-MS is excellent for volatile & non-polar metabolites (e.g., sterols) in a single analysis. Also, it provides high resolution. The most important disadvantage of GC-MS is that inability to detect thermolabile metabolites. In addition, non-volatile compounds must be derivatized before introduction to the instrument. On the other hand, LC-MS usually does not need

derivatization of metabolites and provides detection of thermolabile compounds (Ren et al., 2018).

Liquid chromatography (LC) is based on the interactions of the sample with the mobile and stationary phases. Compared to other separation methods, LC offers a robust, fast and reproducible analysis opportunity. It is also relatively easy to combine with an MS or other measuring instruments.

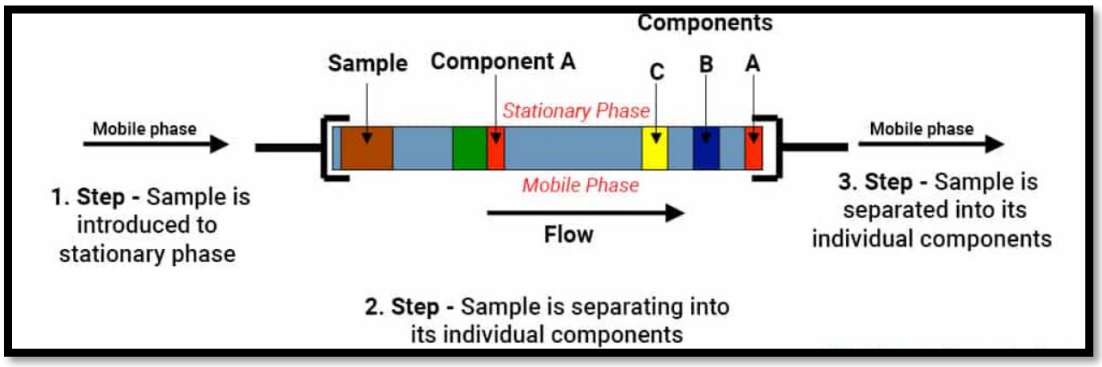


Figure 1. 16. Basic principle of Liquid Chromatography (High Performance Liquid Chromatography (HPLC): Principle, Types, Instrumentation and Applications, 2019)

LC-MS is an analytical technique which is a combination of Liquid Chromatography and Mass Spectrometry. LC provides a physical separation based on hydrophobic interactions, ion exchange or other phsico-chemical features of the target compound in a liquid sample/solution using a column. Separation of coumpouns is explained with “Retention Time”. It is the time taken for a solute to pass through the column. After separation of the target compound, it is ionized via ionization source (Figure 1.15 and Table 1.3). Through ionization, charged compounds are formed. These charged particles then migrate under high vacuum through a series of mass analyzers (quadrupole) by applying electromagnetic fields. The charged particles are detected via mass detector according to their mass/charge ratio. The resulting MS spectrum shows the mass to charge ratio plotted against the peak intensity.

	Advantages	Disadvantages
GC-MS	Suitable for the detection of volatile metabolites Highly repeated retention times Universal database facilitates the identification of the structure	Unsuitable for non-volatile and thermally unstable metabolites Sample pre-processing process is tedious and often requires derivatization
LC-MS	Simple sample preparation Can be matched with multiple MS detectors Wide coverage of detectable metabolites	Ion suppression Metabolites can not be detected without form adduct ions
CE-MS	Low sample volume required for the detection, especially suitable for precious samples	System stability is less stable than LC-MS, GC-MS Affected by salt in the sample
IM-MS	Isomers can be distinguished Fast detection speed The three-dimensional structure of metabolites can be determined in dynamic motion	Determination structural information indirectly from CCS values
DI-MS	High-throughput detection of samples The data processing process is relatively simple	Ion suppression Isomers cannot be distinguished
MALDI-MS	Short sample analysis time Low sample consumption High salt tolerance	Reproducibility is greatly affected by the matrix and sample processing Quantitative analysis is difficult to achieve
MSI	Enables *in situ* detection and presentated *in situ* metabolites information	Imaging effect is affected by resolution It takes longer time to complete the detection under the high resolution
DART-MS	Samples can be analyzed directly without the extraction process Low sample consumption Sample analysis cycle was sharply shortened	Polar compounds are difficult to ionize Ion suppression

Figure 1. 17. Advantages/disadvantages metabolomics studies depends on Mass Spectrometry techniques (Ren et al., 2018)

After obtaining raw data from LC-MS, one of the most important steps is data processing before starting statistical analysis. This step involves outlier screening, filtering, baseline correction, peak alignment, ion annotation and normalization.

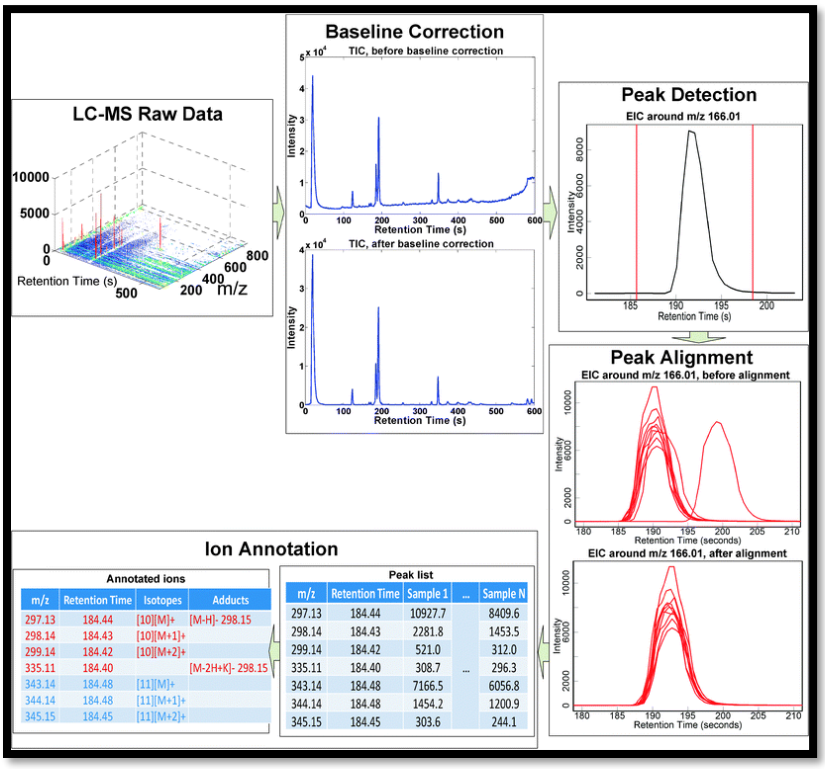


Figure 1. 18. Before statistical analysis, steps for raw data processing in LC-MS (B. Zhou et al., 2012)

Outlier Screening: The aim is to eliminate abnormal peaks compared to their biological or analytical replicates. Principal Component Analysis (PCA) is mostly used for outlier screening in metabolomics. The detailed information about PCA will be given in the next chapter.

Filtering: It is used for elimination of background noise or contaminants in untargeted metabolomics. There are plenty of filtering approaches such as median filtering, Gaussian, Savitzky-Golayor or moving average filtering. Since this thesis is based on targeted metabolomics, the detailed information is not given about filtering.

Baseline correction: The correction is based on estimation of low-frequency baseline from the raw signal. Baseline shift can be often observed as a gradual increase in baseline intensity. It is mainly caused by changes in temperature, long term chemical contamination of the detector, mobile phase contamination or deficiency in column equilibration (B. Zhou et al., 2012). Examples of baseline shifts are given in Figure 1.19.

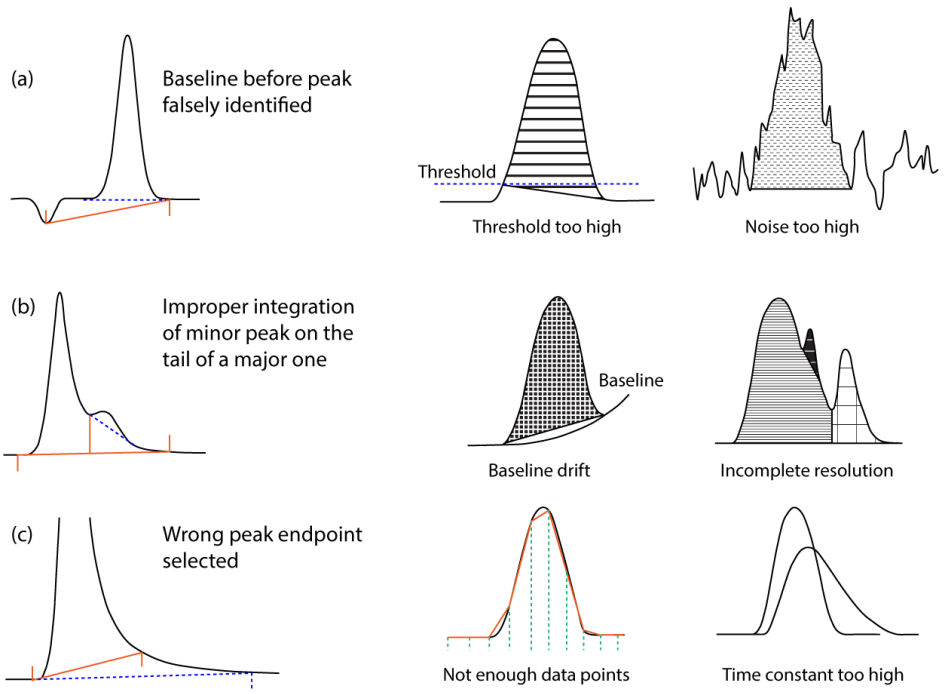


Figure 1. 19. Examples for baseline shift (B. Zhou et al., 2012)

Peak detection: This step is the transformation of raw data that comes from an MS detector. It is defined as peak point of each ion. Peak detection is carried out in two steps. First, the centroids of peaks over m/z range are calculated. Then, according to retention time ranges, peaks are searched. Regarding this step, most of leading device manufacturers have their own peak detection algorithms according to their device properties (B. Zhou et al., 2012).

Peak alignment: Retention time of a metabolite can shift across different samples even in analytical replicates. The shifts can not be controlled, and its direction can occur in unexpected ways. In order to make a correct comparison between samples, a peak alignment step should be performed. The corrections for the retention time shifts are carried out in order to ensure that the same ion is compared between samples. Addition of reference compounds- in similar structure with target ion – into the sample is generally used as a landmark to align peaks (B. Zhou et al., 2012).

Ion annotation: In raw chromatogram, there are many groups of ions with similar retention times as in our target ion but with different molecular weights. Presence of isotopes, adducts, and neutral-loss fragments can cause differences in molecular weights of the same ion. In order to eliminate these unwanted peaks of the same metabolite, ion annotation steps should be performed in untargeted analysis (B. Zhou et al., 2012). Since this thesis is based on targeted metabolomics, the detailed information is not given about this step.

Normalization: It aims to eliminate sample to sample variability. The changes in instrument variability, biological handling, metabolite extraction, and ionization power may cause variation between runs. In order to reduce systematic variation between runs, all results must be normalized with each other (B. Zhou et al., 2012). The most common example for normalization in clinical chemistry is metabolite detections in urine samples. For instance, catecholamine concentration in urine depends on patient's water consumption or urine osmolality, kidney function or sample time. If a patient's urine has low osmolality, it is assumed that creatinine

concentration is also low in the same sample. Namely, creatine is a reference analyte in urine samples. The concentration of any metabolite in the urine sample of individuals is normalized by dividing the creatine concentration in the same sample. In other words, the metabolite concentration is normalized to the creatinine concentration. After this step, it is checked whether the normalized metabolite concentration is within the normal range. (Li et al., 2022).

When metabolomics is compared with proteomics or genomics, normalization is more difficult due to the high variety of metabolite structures. To date, there is no standard method for directly measuring total metabolites, such as total protein measurement, which is commonly used for proteomics. When developing or implementing a sample normalization method for metabolomics, the overall performance of the method is often evaluated by minimizing variations within the same sample group. The easiest and most common way to evaluate variations is the PCA score chart.

There are 2 main approaches for normalization in metabolomics: Sample based, and Data based (Figure 1.20) (Misra, 2020). In this thesis, a sample-based approach (addition of internal standards) was applied in order to normalize results. In this method, a chemical with precisely known concentration is added to each sample as a reference material. This material can be a stable molecule that will not interfere with our analytes in chromatogram, or it can be our isotope-labeled target analyte. Disadvantage of this normalization method is that it has high cost.

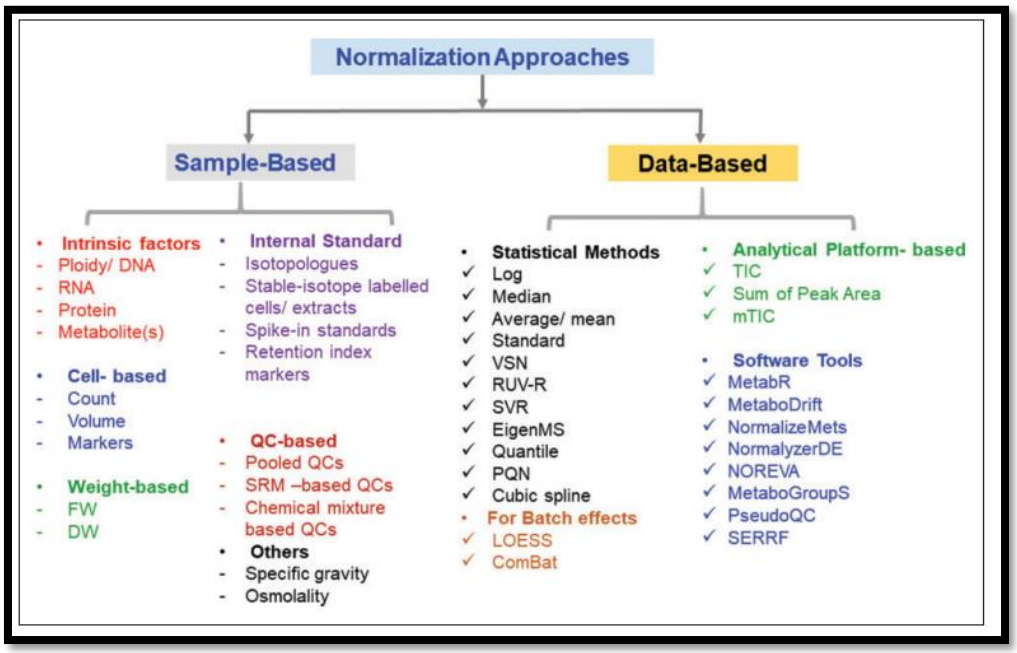


Figure 1. 20. Normalization approaches in metabolomics (Misra, 2020)

1.2.4. Statistical Analysis

After processing of raw data, results are summarized as a peak list. In this step, the aim is the detection of peaks whose levels are significantly different between groups. There are 2 main groups for statistical methods: Univariate and multivariate analysis. As well known, univariate analysis is based on evaluation of statistical importance of each peak, separately (B. Zhou et al., 2012). The most common methods for univariate analysis are t-test, fold change analysis, analysis of variance (ANOVA), etc.

If the aim of study is about metabolomic profiling of a specific group, multivariate analysis must be performed since plenty of metabolites will be evaluated and compared simultaneously (B. Zhou et al., 2012).

1.2.4.1. Multivariate Analysis

The most important step after the identification of metabolites is the evaluation of hundreds of metabolite signals between groups, including pattern recognition and

clustering steps. In this context, multivariate analysis methods are used to analyze and visualize very large metabolomic datasets in an understandable way, taking into account all metabolic features with each other and at the same time. Although multivariate analyses are divided into unsupervised (e.g., PCA) or supervised (e.g., PLS-DA, OPLS-DA), both methods are defined as pattern recognition methods used to detect patterns associated with the variables examined (Wang et al., 2012).

1.2.4.1.1. Principal Component Analysis (PCA)

PCA is an unsupervised method used in metabolomics. Unsupervised methods are described as a method to find and explore the groups or trends in our results. "Unsupervised" means that there is no information about class membership in data. Thus, we can obtain only a few prior assumptions and a little prior knowledge of the data. Unsupervised methods are commonly used as an overlook method during the first step of metabolomics analysis and these methods can help visualize the data or verify any unintended issues.

Since more than 3 variables and groups are compared at the same time in metabolomics, dimensions are not enough to explain results. Thus, PCA is the most common technique in metabolomics to achieve a linear transformation from the original data as much as possible by preserving the variance of the original data, even by reducing the size in the direction of maximum variance (Wang et al., 2012). PCA is used to explain the variance in a dataset with fewer principal components (PCs). Since PCA does not use class label information, this leads to the conclusion that the PCA model is not always successful in emphasizing the differences between the sample groups that are interested in.

The method focuses on the differences between samples. In other words, if the differences between samples within a group are larger, the model may not focus on the differences between groups. This is particularly the case when the normal metabolic variation between samples is much greater than the metabolic disruption caused by a disease (Wang et al., 2012).

The questions below can be explained by PCA:

- *Why do some samples cluster together?*
- *Why do different groups (clusters) separate from each other?*
- *How is the trend between groups?*
- *Is there any information for future studies in my data at all?"* (Brad Swarbrick, 2012)

As seen in Figure 1.21, PCA was used in order to define outliers and eliminate them from the raw data. To sum up, PCA is used to observe trends, clusters and outliers. In this thesis, it was used to get an overview of the data, e.g. to assess clustering of samples in the same group and outliers.

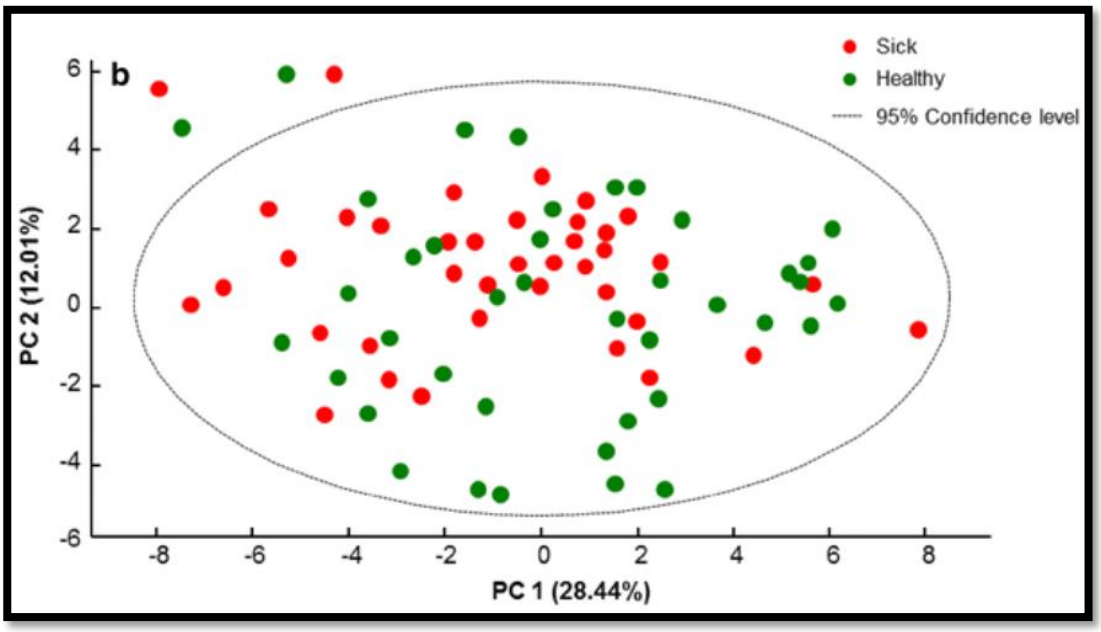


Figure 1. 21. Before performing of the main statistical analysis, 4 outlier samples were identified using PCA and eliminated from the pool in order to proceed statistical analysis correctly with remaining results (Pasamontes et al., 2016).

1.2.4.1.2. Partial Least Square-Discriminant Analysis (PLS-DA)

Due to the unsupervised nature of the PCA algorithm, it has the ability to make a dimensional reduction only when the within-group variation is significantly lower

than the between-group variation. Therefore, partial least squares discriminant analysis (PLS-DA, Partial Least Squares- Discriminant Analysis) has come to be important in metabolomic studies, especially in fingerprint studies.

The PLS-DA model reveals the difference between groups clearer than PCA. The most important reason is that the distinction between groups in PLS-DA is overly optimistic. Therefore, post-PLS-DA validation steps need to be followed to ensure that the differences identified between groups are significant. Due to the success of PLS-DA in group differentiation, validation steps must be performed especially in case of low within-group variability. However, as the number of PLS-DA groups increases, the error rate also increases as the distinction becomes more complex. As in all chemometric methods, the discriminative power of the model parameters (Q2, R2, CV-ANOVA) should be evaluated in order to find a statistically significant difference between the two classes in PLS-DA (Szymańska et al., 2012; Trygg et al., 2007).

The calculated factors “R2Y” and “Q2Y” are used to evaluate the PLS-DA model and the reliability of the test. R2Y describes the fraction of variation. On the other hand, Q2Y is a measure of the predictability of the model. R2Y and Q2Y scores range from 0 to 1, and an R2Y value of 1 indicates that 100% of the variance can be explained by the model. If the Q2Y value is close to 1, it shows that the model is highly reliable in the validation tests. R2 score is always greater than Q2 score. Studies have requested that R2 and Q2 scores should be as close to 1 as possible and the difference between them should be less than 0.3 (Szymańska et al., 2012). For classification purposes, Y is a dummy matrix, i.e., 0s and 1s are often used to represent the group assignment of samples. It is important to note that variable importance in projection (VIP) values estimate the importance of each variable in the projection used in a PLS model and are often used for variable selection.

When measured metabolite numbers are higher than sample numbers at least twice, PLS-DA can perfectly separate groups by chance. This separation is explained with

the “curse of dimensionality”, that predicts the sparsity of the data to grow increasingly faster with the number of dimensions.
For example, about the “curse of dimensionality” term from Vishwesh, K., *“It’s easy to catch a caterpillar moving in a tube (1 dimension). It’s harder to catch a dog if it were running around on the plane (two dimensions). It’s much harder to hunt birds, which now have an extra dimension they can move in. If we pretend that ghosts are higher-dimensional beings, those are even more difficult to catch”.*

This term can be seen only in a range of ratios from 2:1 to 1:200. In plenty of omics studies especially in genomics, gene:sample ratios can even exceed 1:1000 (i.e., data sets with 25 samples and 25,000 genes are common). For this reason, sample size determination is a crucial step during designing an experiment plan.

1.2.4.1.3. Validation of Modeling Method

As mentioned in the previous chapter, PLS/OPLS models try to find a linear relationship between a X predictor matrix (e.g., spectrometric data of biological samples) and a Y response matrix (e.g., clinical results, treatment...). Generally, in metabolomics studies, the X predictor matrix has more columns (predictor variables) than rows (individuals) (Triba et al., 2015). Thus, results may have false-positive predictive power (overfitted & overestimated), as preliminary studies are conducted with a small sample size and plenty of metabolites are tested at the same time. Therefore, post-assessment validation of appropriate modeling is essential to understand the actual performance of a model and potential biomarkers.

In order to prove the ability of estimation power of the model, the only way to predict Y values of new individuals is to predict individuals from an independent dataset (i.e., that were not used to build this model). It means that the data set should be divided into 2 groups as training and test set. The training set is used for building the model and the test set is used for the estimation of predictability.

The purpose of validation is to measure the predictive ability of the model. Validation can be done in two ways, internally and externally. In external verification, new data is collected and analyzed in the same way as the samples after processing. In internal validation, the existing data set is divided into two separate groups for modeling and validation. If there is no test set (Triba et al., 2015), the cross-validation analysis is the alternative strategy to evaluate the quality of a model. Different cross validation procedures exist.

Cross validation method is a technique that can be applied to different types of models, including classification models. The most used cross-validation techniques are leave-one-out cross-validation-LOOCV and k-fold cross-validation. In a study where the sample size is N in LOOCV, 1 sample is left out each time, and a total of N - 1 sample is used as a training set, and the remaining sample is used for testing. As a result, this process is repeated N times.

1.2.5. Literature Survey of Metabolomics Studies in AML

After the discovery of 2-hydroxyglutarate as a mutation biomarker in IDH mutated AML patients via metabolomics, it has not only been used as a mutation marker in AML, but has also enabled the discovery of inhibitor drugs for isocitrate dehydrogenase mutation and the follow-up of treatment through this oncometabolite (Abou Dalle & DiNardo, 2018; Madala et al., 2018). In line with this example, the discovery of new oncometabolites enables the discovery of new cancer biomarkers as well as the identification of protein targets in relevant metabolic pathways. Since these proteins provide prognostic information such as drug resistance and metastasis risk in the disease mechanism, they may help in the development of new drugs. For these reasons, cancer metabolomics studies have gained importance in recent years and large-scale studies are carried out today (Avuthu & Guda, 2022; Ketavarapu et al., 2022; Roth & Powers, 2022).

The reason for performing studies on metabolite screening on AML patient samples is because of the effect of protein anomalies observed in patients on the

increase/decrease of biomolecules in metabolism, especially carbohydrate, amino acid and lipid metabolism (W.-L. Chen et al., 2014; Fultang et al., 2021; Muscaritoli et al., 1999a, 1999b; Tabe et al., 2019).

To give an example for each mutation, in the study conducted by Ju et al. (Ju et al., 2017) in leukemia cells, it was shown by cell culture studies that the FLT3-ITD mutation increases the expression of the mitochondrial hexokinase-2 enzyme, increasing the dependence on glycolysis and making them highly sensitive to glycolytic inhibition.

The most comprehensive metabolomic study conducted until 2022, in terms of the number of patients and covering all AML subgroups, is published by Chen et al. in Blood Journal (W.-L. Chen et al., 2014). This AML study is intended to find a common metabolomic signature in all AML patients. For this purpose, 229 AML patients were used. In the cytogenetically normal AML group, 6 metabolites were significantly different (Lactate, 2-oxoglutarate, pyruvate, 2-HG, glycerol-3-phosphate, citrate) compared with healthy controls. After cytogenetically risk grouping, metabolomic studies were performed according to molecular abnormalities in the cytogenetically normal patient group, which is the most common group, since they did not see a significant metabolomic difference between the groups.

After this comprehensive validation study, Wang et al. published in 2019 "Identification of novel serum biomarker for the detection of acute myeloid leukemia based on LC-MS". In this study, the aim was to find a common metabolite biomarker. For this purpose, samples of 55 AML patients were scanned by LC-MS and untargeted metabolomic analysis was performed. According to their results, metabolic differences associated with AML are mainly involved in amino acid metabolism such as alanine, aspartate, phenylalanine, glutamate, taurine and tryptophan metabolism; d-Glutamine and d-glutamate metabolism and also fatty acid metabolism.

In another study, plasma and mononuclear cell samples of 8 individuals with FLT3-ITD mutation were compared in individuals with AML but wild type for FLT3 by LC-MS/MS-based untargeted metabolomic profiling. It was found that the amount of 21 and 33 different metabolites varied between the two groups in plasma and cell samples, respectively (Stockard et al., 2018). When metabolite-related pathway analysis was performed, it was emphasized that these metabolites, were associated with pathways such as purine and tryptophan metabolism, and fatty acid oxidation. It was stated that the study was the first exploratory study in terms of metabolomic profiling of the FLT3-ITD mutation, and it was stated that a comprehensive cohort study should be conducted to carry out validations.

According to the study belonging Gallipoli et al., it has been shown by LC/MS metabolomic profiling and cell culture studies that when mutant AML cells are inhibited using specific tyrosine kinase inhibitors, a decrease in TCA pathway activity is observed and the cell begins to take glutamine uninterruptedly into the cell for use in main carbon metabolism and use it as fuel (Gallipoli et al., 2018).

1.2.5.1. Amino acids in Metabolism

After the removal of amino groups, carbon skeleton of amino acids can be degraded by dehydrogenation, decarboxylation, and other reactions. With these ways, their carbon backbones are utilized to citric acid cycle. Then, they can be degraded to acetyl-CoA. At the end, they are completely oxidized to carbon dioxide and water via oxidative phosphorylation (Nelson, 2008).

Some amino acids can be degraded to ketones, some to glucose or some to both. In specific conditions, some amino acids may be a significant source of metabolic energy. Consequently, amino acid metabolism is fused into intermediary metabolism (Nelson, 2008).

The carbon backbones of amino acids are included in citric acid cycle via five intermediates: acetyl CoA, alpha-ketoglutarate, succinyl CoA, fumarate and

oxaloacetate. Alanine, cysteine, glycine, serine, threonine, and tryptophan can be directly degraded to pyruvate, then they can be converted to acetyl-CoA or oxaloacetate. On the other hand, isoleucine, leucine, lysine, threonine, and tryptophan can be directly degraded to acetyl-CoA (Nelson, 2008).

Arginine, glutamate, glutamine, histidine and proline can form alpha-ketoglutarate. Isoleucine, methionine, threonine and valine can form succinyl-CoA. Phenylalanine and tyrosine can produce fumarate. Asparagine and aspartate can produce oxaloacetate (Nelson, 2008).

As a detailed example, the carbon structure of asparagine and aspartate are utilized in citric acid cycle as oxaloacetate mentioned above. In the first step, asparaginase catalyzes the conversion of asparagine to aspartate. Then, aspartate aminotransferase catalyzes the conversion of aspartate to oxaloacetate. In the meantime, alpha-ketoglutarate is converted to glutamate using pyridoxal phosphate. At the end of the catabolic pathway for asparagine and aspartate, the formation of glutamate and oxaloacetate is achieved. Therefore, glutamate and aspartate metabolism are tightly connected (Nelson, 2008).

Tryptophan can be cleaved in 4 steps to form alanine. Then, alanine can be converted to pyruvate. As a second path for tryptophan, it can be degraded to alpha-ketoadipate to produce acetoacetyl-CoA, resulting formation of acetyl-CoA. Acetyl-CoA and carnitine merge to form acyl-CoA in cytoplasm. With this reaction, it can pass through mitochondria membrane via acylcarnitine transferase.

Since arginine and histidine contain five adjacent carbons, the degradations of these amino acids are quite complex. Arginine is converted into ornithine by urea cycle. In next steps, ornithine can be converted to glutamate. On the other hand, histidine is degraded to glutamate in four steps.

Glutamate is one of the most abundant amino acids in human serum. It is the major nitrogen donor, especially through aspartame and glycine. Nitrogen availability is

crucial for biosynthesis of secondary metabolites. In addition, glutamate is also used in biosynthesis of glutathione. Since cancer cells need more biofuel for increased energy requirement and antioxidant, these important contributions mentioned above are vital for cancer cells as well as healthy cells. Many studies showed that most of cancer cells are glutamate dependent (Quek et al., 2022; Roma et al., 2022; Tabatabaee & Menard, 2022). In some cancer types, glutamate concentration is correlated with progression of the cancer (Koochekpour et al., 2012). As mentioned above, glutamate and aspartate metabolism are tightly connected. Gorgoglione et al. reviewed how glutamine-derived aspartate biosynthesis is crucial for cancer cells (Gorgoglione et al., 2022).

With the light of these information, amino acids are irreplaceable precursors for both cancer and healthy cells.

1.3. Aim of Study

Problem definition: As written in chapter 1.1.2, AML is rapidly progressing cancer type that requires urgent treatment. In order to define mutation status of newly diagnosed patients, cytogenetic tests must be performed. Since the obtaining of testing results take 14-21 days, rapid diagnosis tests are extremely desired by clinicians in Turkey. Also, it should be noted that, according to Röllig et al., the risk in awaiting testing results is less than the risk in beginning therapy before results are available (Röllig et al., 2019). Due to the high incidence and strategic importance in risk stratification of patients, investigation of NPM1 and FLT3 mutations in AML patients compared to healthy individuals was chosen for this thesis.

The rationale for the hypothesis is based on the previous studies (given in the section 1.2.5), which indicate a link between NPM1 and FLT3-ITD mutations with glucose and amino acid metabolism.

Cancer metabolism focuses on central carbon metabolism, especially on glycolysis and citric acid cycle. Since the relation of amino acids with the components of citric acid cycle is well-known, new studies have shed light on the importance of amino acids for most types of cancer. Several studies have indicated that changes in amino acid levels for AML patients are remarkable (Bjelosevic et al., 2021; Gregory et al., 2019; Muscaritoli et al., 1999b; Mussai et al., 2015).

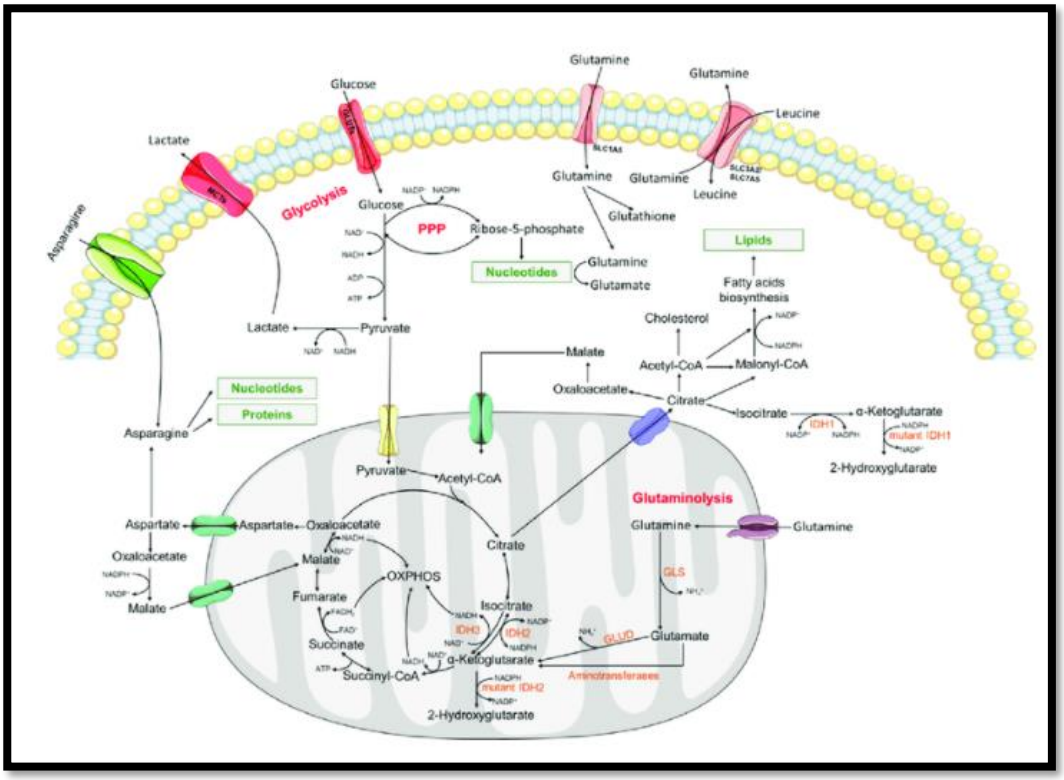


Figure 1. 22. Amino acids as precursors for the human metabolism (Fultang et al., 2021)

Fatty acid oxidation is the main source of energy in AML cells for their survival. Carnitine palmitoyl transferase 1A (CPT1A) and carnitine transporter CT2 (SLC22A16) proteins are rate limiting actors of fatty acid oxidation. These proteins are overexpressed in AML. Therefore, targeting acylcarnitine metabolism is one of the new therapeutic approaches for AML treatment.

Aim of this thesis is to develop a prediction method associated with FLT3 and NPM1 mutations in Acute Myeloid Leukemia patients using LC-MS/MS targeted

metabolomics approach, which might provide fast clinical data for the proper first-response treatment until obtaining mutation results for *de novo* AML patient.

In accordance with this aim, the measurements of amino acids and acylcarnitines were performed to define a pattern for discrimination of the mutations in AML serum and whole blood samples.

CHAPTER 2

MATERIAL AND METHOD

2.1. Material

2.2. Chemicals and Consumables

Chemical	Supplier
Acetonitrile	Merck
Methanol	Merck
Ammonium Formate	Sigma
Hydrochloric Acid	Kimetsan
1-Propanol	Merck
Pyridine	Merck
Whatman paper-CF10	Gelifesciences
Silica Gel	Sigma
Wax Paper	VWR
Nitrogen Tube	Linde Gaz
EDTA & Serum Tubes	BD Vacutainer

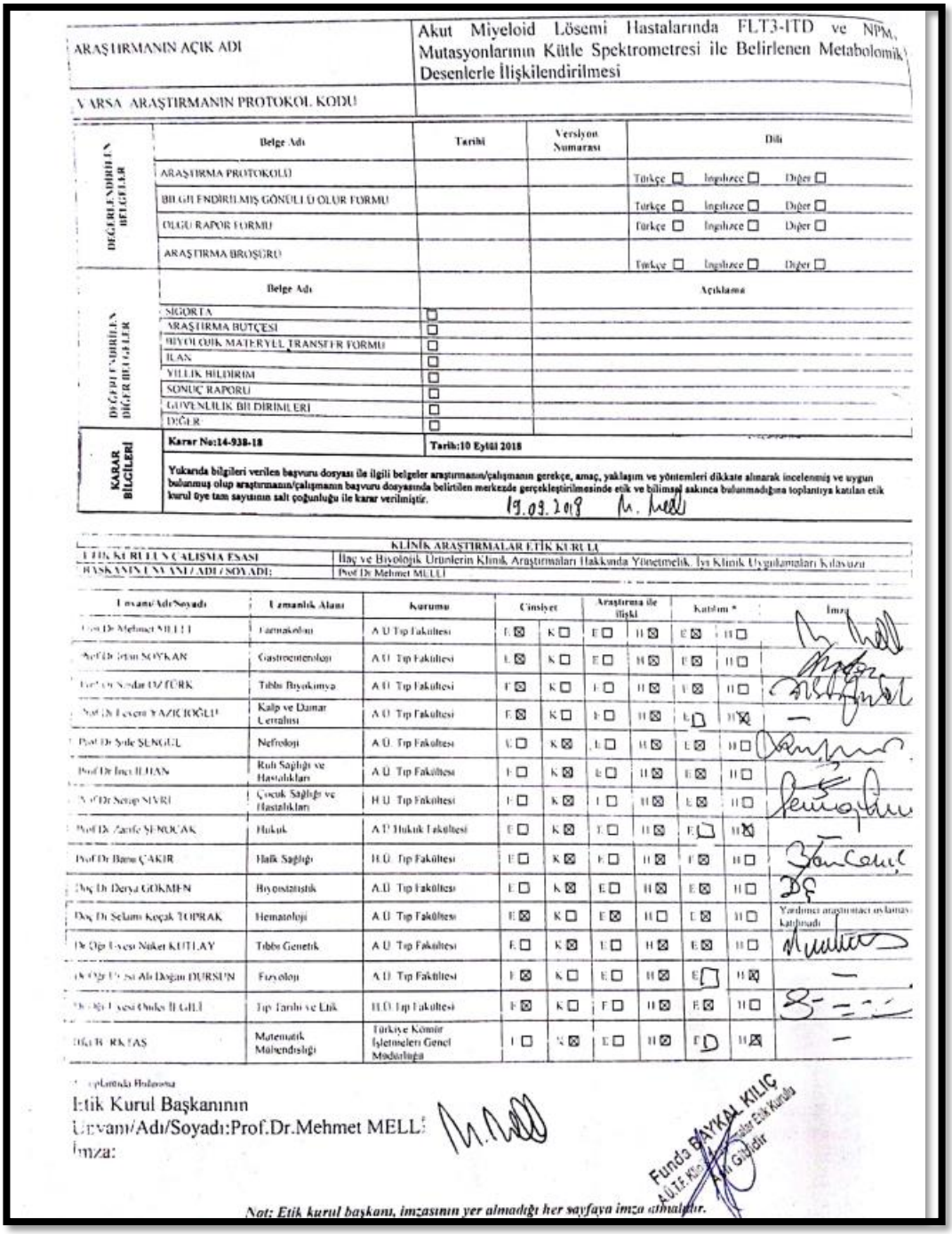
In order to quantify the amino acid panel, Bome Trivitron (Turkey) in house QAA kit was used. For the measurements of acylcarnitine panel, Bome Trivitron (Turkey) in house Newborn Screening kit was used.

2.2.1. Equipment

Equipment	**Brand**
Centrifuge	Thermo Scientific (USA), Megafuge 40
Cutter	HZMM (China)
Deionized Water System	Sartorius (Germany)
ESI Triple Quadrupole LC-MS	Shimadzu (Japan), 8040
Freezer	GFL (Germany), 6445
Microcentrifuge	Sigma (Germany), 1-14
Micropipette Set	Brand (Germany), Transferpette S series
Nitrogen blowdown evaporator	VLM (Germany), EVA-EC2S
pH meter	Hanna (USA), HI110
Precision Scale	Kern (Germany), ABS120
Refrigerator	Vestel (Turkey)
Thermal mixer	Biosan (Latvia), TS100C
Waterbath	Nüve (Turkey)
Vortex	Daihan Scientific (Korea), VM-10

2.2.2. Patient Samples

All AML patient (n=42) and control serum& blood samples (n=16) were obtained from Ankara University Medical Faculty Hematology Department according to the ethics committee approval as seen in Figure 2.1.



ARAŞTIRMANIN AÇIK ADI	Akut Miyeloid Lösemi Hastalarında FLT3-ITD ve NPM1 Mutasyonlarının Kütle Spektrometresi ile Belirlenen Metabolomik Desenlerle İlişkilendirilmesi
VARSA ARAŞTIRMANIN PROTOKOL KODU	

DEĞERLENDİRİLEN BELGELER	Belge Adı	Tarihi	Versiyon Numarası	Dili
	ARAŞTIRMA PROTOKOLÜ			Türkçe ☐ İngilizce ☐ Diğer ☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU			Türkçe ☐ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU			Türkçe ☐ İngilizce ☐ Diğer ☐
	ARAŞTIRMA BROŞÜRÜ			Türkçe ☐ İngilizce ☐ Diğer ☐

DİĞER İNCELENEN BELGELER	Belge Adı		Açıklama
	SİGORTA	☐	
	ARAŞTIRMA BÜTÇESİ	☐	
	BİYOLOJİK MATERYEL TRANSFER FORMU	☐	
	İLAN	☐	
	YILLIK BİLDİRİM	☐	
	SONUÇ RAPORU	☐	
	GÜVENLİLİK BİLDİRİMLERİ	☐	
	DİĞER	☐	

KARAR BİLGİLERİ	Karar No:14-938-18	Tarih:10 Eylül 2018

Yukarıda bilgileri verilen başvuru dosyası ile ilgili belgeler araştırmanın/çalışmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş ve uygun bulunmuş olup araştırmanın/çalışmanın başvuru dosyasında belirtilen merkezde gerçekleştirilmesinde etik ve bilimsel sakınca bulunmadığına toplantıya katılan etik kurul üye tam sayısının salt çoğunluğu ile karar verilmiştir. 19.09.2018

KLİNİK ARAŞTIRMALAR ETİK KURULU

ETİK KURULUN ÇALIŞMA ESASI	Beşeri Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu
BAŞKANIN UNVANI/ADI/SOYADI:	Prof.Dr.Mehmet MELLİ

Unvanı/Adı Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile İlişki		Katılım *		İmza
Prof.Dr.Mehmet MELLİ	Farmakoloji	A.Ü. Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof.Dr.Şeref SOYKAN	Gastroenteroloji	A.Ü. Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof.Dr.Nesrin DİZDAROĞLU	Tıbbi Biyokimya	A.Ü. Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof.Dr.Ercan YAZICIOĞLU	Kalp ve Damar Cerrahisi	A.Ü. Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	—
Prof.Dr.Şule ŞENGÜL	Nefroloji	A.Ü. Tıp Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Prof.Dr.İnci İLHAN	Ruh Sağlığı ve Hastalıkları	A.Ü. Tıp Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Prof.Dr.Serap SİVRİ	Çocuk Sağlığı ve Hastalıkları	H.Ü. Tıp Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Prof.Dr.Zerrin ŞENOCAK	Hukuk	A.Ü. Hukuk Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☐	H ☒	
Prof.Dr.Banu ÇAKIR	Halk Sağlığı	H.Ü. Tıp Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç.Dr.Derya GÖKMEN	Biyoistatistik	A.Ü. Tıp Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç.Dr.Selami Koçak TOPRAK	Hematoloji	A.Ü. Tıp Fakültesi	E ☒	K ☐	E ☒	H ☐	E ☒	H ☐	Yardımcı araştırmacı uygulama katılmadı
Dr.Dişhekimi Nüket KUTLAY	Tıbbi Genetik	A.Ü. Tıp Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Dr.Öğr.Üyesi Ali Doğan DURSUN	Fizyoloji	A.Ü. Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	—
Dr.Öğr.Üyesi Onder İLGİLİ	Tıp Tarihi ve Etik	H.Ü. Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Dilek IŞIKTAŞ	Matematik Mühendisliği	Türkiye Kömür İşletmeleri Genel Müdürlüğü	E ☐	K ☒	E ☐	H ☒	E ☐	H ☒	—

Etik Kurul Başkanının
Unvanı/Adı/Soyadı:Prof.Dr.Mehmet MELLİ
İmza:

Funda BAYKAL KILIÇ
A.Ü.T.F. Klinik Araştırmalar Etik Kurulu
Asıl Üyedir

Not: Etik kurul başkanı, imzasının yer almadığı her sayfaya imza atmalıdır.

Figure 2. 1. Ethics committee approval

The samples of *de novo* AML patients who did not receive any treatment were collected. Other inclusion criteria are as follows:

- Patients >18 years old
- Having no metabolism disorders (e.g., type 1/2 diabetes, glycogen storage disorders, fructose metabolism disorders, lipid metabolism disorders, any type of liver/kidney/pancreas/ malfunction)
- Patients whose have sample collection time less than 24 hours

Two types of venous blood samples were collected from all patients, one into the EDTA tube as a whole blood sample and one into the gel tube as a serum sample. In order to store serum samples; gel sample tube was immediately separated at 2500 rpm in 15 minutes via centrifugation. Supernatant was aliquoted and freezed at -80°C until analysis.

In order to store whole blood samples in a stabilized form, EDTA tube samples were prepared according to "Dried Blood Spot" method (Figure 2.2.) that is peculiar in newborn screening prepared with heel lance blood. In this method, 50 µL blood was aspirated with micropipette. Then, the blood was dropped onto Whatman Paper without touching the filter paper directly with the tip. During this step, obtaining a fully saturated blood circle is important. This procedure was repeated until the all sample was finished. After dropping, Whatman paper was dried for 12-24 hours in a humidity-controlled environment in order to prevent moisture-induced degradation. After the drying step, whatman paper was rolled with wax paper and placed into an Aluminium foil bag with a silica gel. Then, in order to prevent air contact, the foil bag was sealed. DBS samples were also stored at -80°C until analysis time.

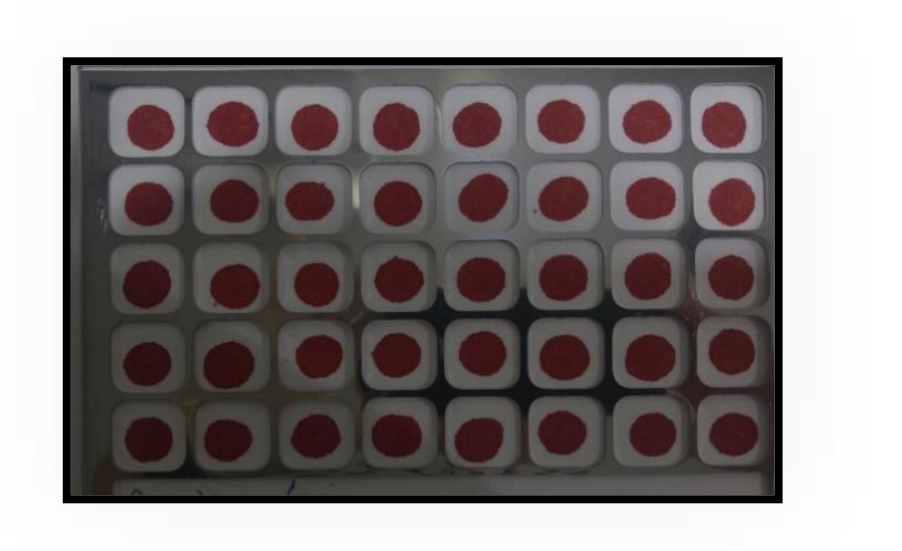


Figure 2. 2. After dropping of EDTA sample onto Whatman paper

2.3. Method

2.3.1. Risk Stratification of AML Patients

All *de novo* AML patients were classified according to the risk classification algorithm of NCCN AML Guideline. As also mentioned in Chapter 1.1.1, there are 3 risk classes: Favorable, Intermediate, Unfavorable.

Patient's risk status and the detailed clinical diagnosis and mutation reports were obtained from the hospital. The clinical features of the participants are given in Chapter 3.

FLT3 and NPM1 mutation tests are routinely performed in Ankara University Hematology Department. For NPM1 mutation tests, the target region was amplified by PCR reaction using specific primers. The obtained product was visualized and evaluated after agarose gel electrophoresis. PCR was performed for sequence analysis using a pair of primers. Sequence analysis of the obtained PCR products was performed by BigDye Terminator (Thermo, USA) method using ABI 3130 (Thermo,USA) analyzer. For FLT3 mutation tests. The same approach is used to determine FLT3 mutations.

In this study, there are 5 patient groups:

1. Healthy individuals as a control group
2. NPM1 (-), FLT3 (-) AML patients
3. NPM1 (+), FLT3 (+) AML patients
4. NPM1 (+), FLT3 (-) AML patients
5. NPM1 (-), FLT3 (+) AML patients

2.3.2. Targeted Metabolomics Studies

To define metabolomic patterns for NPM1 and FLT3 mutations, the panel of amino acid and acylcarnitine were measured from serum samples. Three technical replicates were prepared for each panel measurement and measured in different runs.

2.3.2.1. Amino Acid Panel

Bome Trivitron (Turkey) in house Quantitative Amino Acid LC-MS/MS kit was used for targeted amino acid measurements. The method is based on derivatization of amino acids and measured with Multiple Reaction Monitoring (MRM) -also known as Selective Reaction Monitoring- mode of LC-MS/MS system.

Triple quadrupole MS is used in the MRM method. Firstly, the ion which is corresponded with our compound of interest is targeted for fragmentation to obtain daughter ions (one or more). These daughter ions which are specific to our target molecule can be selected for quantification (Figure 2.3). By ignoring all other ions, working with the MRM mode increases the specificity and sensitivity of the measurement of the target molecule.

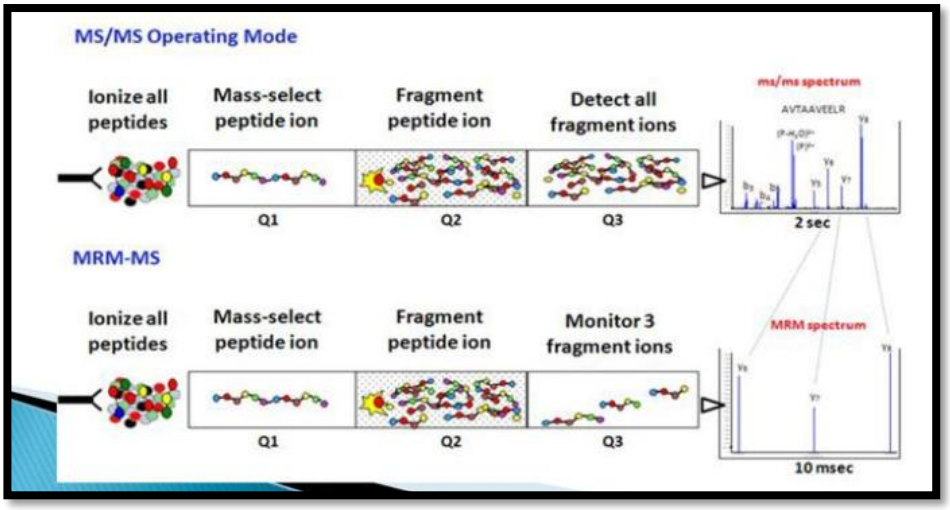


Figure 2. 3. MRM mode in MS/MS

During all measurements, as an internal quality control, bilevel Bome Trivitron (Turkey) amino acid control materials with assigned values were prepared in the way as same as the patient samples. QC results must be within ±1SD range to be acceptable. For each analyte of the panel, isotope labeled analytical standards were used for normalization and quantification process.

Amino acid panel is given in Table 2.1. The flow of sample preparation is given in Figure 2.4. The details about analytical conditions are given in Table 2.2.

Table 2. 1. Amino acid panel

Alanine	Hydroxylysine
Alpha amino adipic acid	Hydroxyproline
Alpha amino butyric acid	Isoleucine
Alpha amino pimelic acid	Leucine
Arginine	Lysine
Argininosuccinic acid	Methionine
Asparagine	1-methyl Histidine
Aspartic Acid	Ornithine
Beta alanine	Phenylalanine

Table 2.1. (continued)

Beta-aminoisobutyric acid	Proline
Citrulline	Sarcosine
Cystathionine	Serine
GABA	Taurine
Glutamine	Threonine
Glutamic acid	Tryptophan
Glycine	Tyrosine
Histidine	Valine

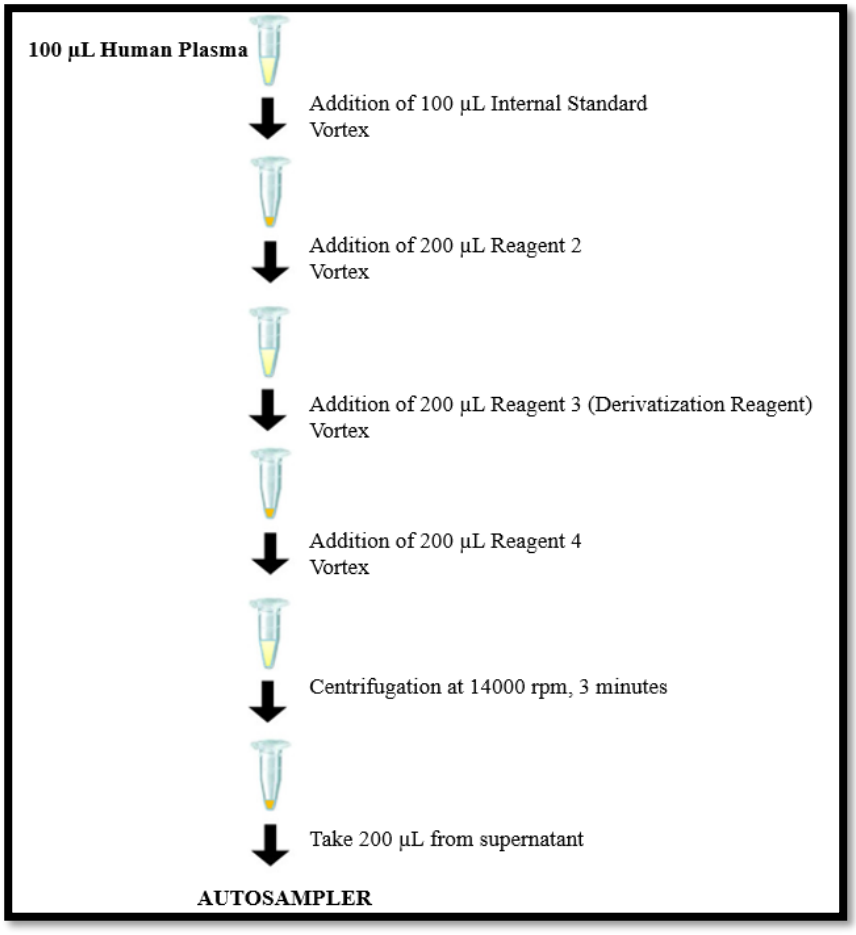


Figure 2. 4. Sample preparation for the amino acid measurements

Table 2. 2. Analytical conditions for amino acid measurements

<table>
<tr><td>Device</td><td>Shimadzu 8040 LC-MS/MS</td></tr>
<tr><td>Ion Source</td><td>ESI</td></tr>
<tr><td>Interface Temperature</td><td>300°C</td></tr>
<tr><td>DL Temperature</td><td>250°C</td></tr>
<tr><td>Heater Temperature</td><td>400°C</td></tr>
<tr><td>Drying Gas</td><td>Nitrogen 10 L/min.</td></tr>
<tr><td>Nebulizer Gas</td><td>Nitrogen 3 L/min.</td></tr>
<tr><td>Heating Gas</td><td>Air 10 L/min.</td></tr>
<tr><td>Column</td><td>Trimaris QAA Kit Column
(Commercial Secret)</td></tr>
<tr><td>Column Temperature</td><td>40°C</td></tr>
<tr><td>Flow Rate</td><td>0.25 mL/min.</td></tr>
<tr><td>Sample amount</td><td>200 µL</td></tr>
<tr><td>Gradient</td><td>
<table>
<tr><td>Duration</td><td>A %</td><td>B %</td></tr>
<tr><td>0:00</td><td>65</td><td>35</td></tr>
<tr><td>12:00</td><td>35</td><td>65</td></tr>
<tr><td>12:01</td><td>5</td><td>95</td></tr>
<tr><td>16:00</td><td>5</td><td>95</td></tr>
<tr><td>16:01</td><td>65</td><td>35</td></tr>
<tr><td>20:00</td><td>65</td><td>35</td></tr>
</table>
</td></tr>
</table>

Calculation of analyte concentration is given as a formula:

$$Sample\ Concentration = \frac{(Analyte\ area\ in\ sample) * (Internal\ std\ area\ in\ calibrator) * (Calibrator\ concentration)}{(Internal\ std\ area\ in\ sample) * (Analyte\ area\ in\ calibrator)}$$

2.3.2.2. Acylcarnitine Panel

Since determination of fatty acid oxidation defects via tandem MS is important for newborn screening programs globally, Bome Trivitron in house Expanded

Newborn Screening LC-MS/MS kit was chosen to measure targeted acylcarnitine panels. It is important to note that the tandem LC-MS/MS method for the expanded newborn screening panel is a standard, open-source method in all newborn screening laboratories.

The total body carnitine pool is composed of L-Carnitine, C2, short-chain (C3–C5), medium chain (C6–C12), and long-chain (C14–C20) acylcarnitine [7]. The conventional abbreviation for acylcarnitine is shown as C followed by the chain length number, the number of saturated bonds after the colon, DC indicates a dicarboxylic acid, and an OH represents a hydroxyl group (e.g., C16:1-OH, 16 carbons with 1 double bond and a hydroxyl attached to the acyl group) (McCann et al., 2021).

For this analysis, a DBS sample should be used. The flow for sample preparation of DBS was given in Chapter 2.1.3. Dried blood samples that are irregular in shape and not homogeneously distributed should not be used.

The sample preparation for acylcarnitine panel is as follows:

- A sample of 3.2 mm diameter is punched via puncher (Figure 2.5.) from the dried blood sample and placed into a 96-well plate.
- Add 200 µl of Reagent 1 (Metabolite Extraction Solvent) to each well containing the DBS. Then, the plate is covered and mixed at 300 rpm for 30 min at room temperature.
- After mixing, the liquid extracts are transferred to a clean well and evaporated under nitrogen until there is no liquid left.
- Add 60.0 µl of Reagent 2 (Internal Standards for each metabolite of panel) to the dried wells. The well plate is covered well again. It is mixed at 300 rpm for 20 min at 65 °C.
- After mixing, it is evaporated until there is no liquid in the wells.

- 200 µl of Reagent 3 is added to the dried wells and the samples are dissolved. 10.0 µl is injected into the chromatography system. Prepared samples are stable for 24 hours.

During all measurements, as an internal quality control, bilevel commercial quality control materials with assigned values were prepared under the same conditions with patient samples. QC results must be within ±1SD range to be valid.

The isotope dilution method is used to calculate the results. Analyte concentrations are determined by dividing the known concentrations by the area of their related internal standards. It is recommended to use Newborn Screening software suitable for the model of the MS device used for easy calculation. In this thesis, Shimadzu Neonatal Solution was used for automatic calculation.

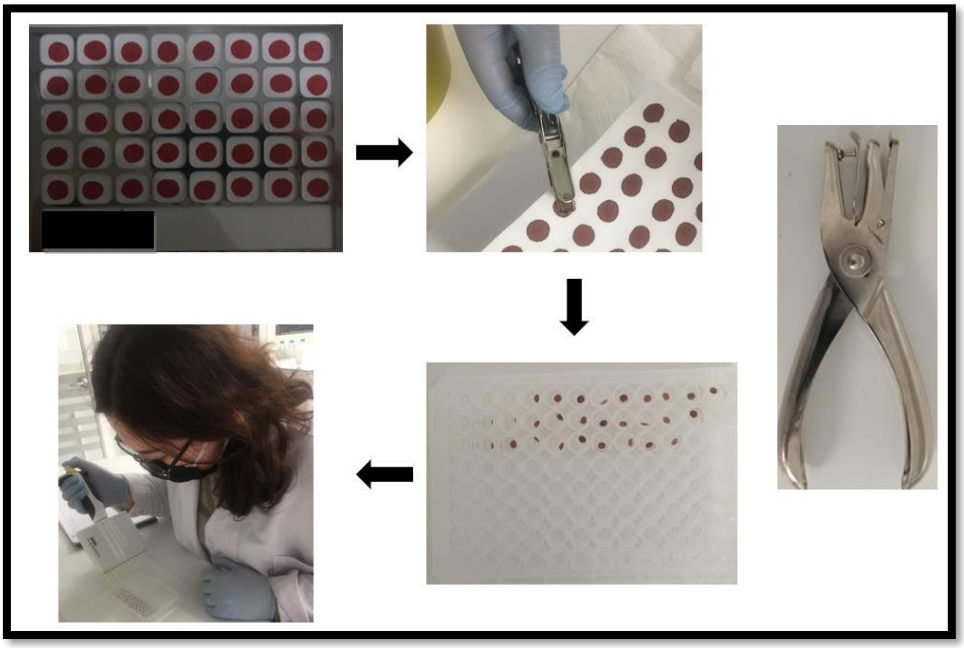


Figure 2. 5. Punching process from DBS samples

Acylcarnitine panel is given in Table 2.3. The details about analytical conditions are given in Table 2.4.

Table 2. 3. Acylcarnitine panel

C0 Carnitine (free carnitine)	C8 Carnitine
C2 Carnitine	C3DC Carnitine
C3 Carnitine	C10:1 Carnitine
C4 Carnitine	C10 Carnitine
C5 Carnitine	C4DC Carnitine
C4OH Carnitine	C5DC Carnitine
C6 Carnitine	C12 Carnitine
C5OH Carnitine	C6DC Carnitine
C8:1 Carnitine	C14:2 Carnitine

Table 2. 4. Analytical conditions for acylcarnitine measurements

Device	SHIMADZU 8040		
Ionization source	ESI		
Interface Temperature	300 °C		
DL Temperature	250 °C		
Heater Temperature	400 °C		
Drying Gas	10 L/min.		
Nebulizer Gas	3 L/min.		
Heating Gas	10 L/min.		
Column	No analytical column Guard column is used for protecting device from contaminants.		
Flow rate	0,125 mL/min.		
Sample volume	10 µL		
Isocratic program	Duration	% A	% B
	0:00	0	100
	2:00	0	100

2.3.3. Data Analysis

Spectral data processing (baseline correction, peak identification, peak alignment, normalization) and analyte quantification was performed with the “LabSolutions” software developed by Shimadzu.

Before starting with statistical analysis, preprocessing steps (centering, scaling, normalization) were applied via “Simca 17 (Sartorius, Germany)” software.

Then, basic statistical calculations (Mean, SD, CV%, etc.) were performed with “Excel”. Univariate statistical analysis (ANOVA) was performed and visualized as bar graphs with the “GraphPad Prism 8 (USA)” software. It is assumed that the variation of each sample group is not equal.

In order to find patterns and correlations between several variables (analytes) simultaneously, multivariate statistical analyses (PCA, PLS-DA), VIP, cross validation and pathway analysis were performed with “Simca-P+” and “Metaboanalyst 5.0” software. Firstly, score graphs created via PCA and PLS-DA methods have provided comprehensible visual graphs by dimension reduction of complex spectral data. Secondly, VIP analysis was performed. The analytes were ranked in order of their contributions according to the model obtained from the PLS-DA method.

In addition to being compatible with the dataset used in the model, a good PLS-DA model should also be in harmony with new data. Our training model may have overfitting or underfitting of unknown samples. Therefore, cross validation was performed to test the reliability of our model. There are different types of cross validation techniques. However, the basis of these techniques is same:

- split data into subsets,
- then hold out a subset and test the model with remaining sets.

In this study, Leave One Out Cross Validation (LOOCV) technique was applied to our model. This technique leaves 1 data point out of training data. For instance, if a data set have n samples, then, n-1 samples are used to train the model and p points are used as the validation set. This is repeated for all combinations in which the original sample can be separated this way, and then the error is averaged for all trials, to give overall effectiveness.

The analytes with a value of >1 in the VIP analysis were tested in databases (KEGG) and the most prominent pathways were identified and visualized via Metaboanalyst 5.0.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Clinical Characteristics

Serum and whole blood EDTA samples were collected from 41 AML patients at diagnosis prior to initiation of any chemotherapy. As a negative control, serum and whole blood samples were also collected from 16 healthy donors. The clinical features of participants are given in Table 3.1. The biochemistry and Complete Blood Counting (CBC) results of all healthy donors were in the reference range. On the other hand, hyperglycemia in the nondiabetic individuals was observed in some AML cases. Recent studies have indicated that in patients with severe infections, hyperglycemia can be caused by a catabolic state with disruption of glucose uptake.

According to patient criteria given in Chapter 2, sample numbers of each group are as below:

1. Healthy individuals as a control group: 16
2. NPM1 (-), FLT3 (-) AML patients: 28
3. NPM1 (+), FLT3 (+) AML patients: 3
4. NPM1 (+), FLT3 (-) AML patients: 3
5. NPM1 (-), FLT3 (+) AML patients: 7

Table 3. 1. Clinical characteristics of AML patients and healthy donors

	Healthy Controls n=16	**AML n=42**	**Reference Ranges**
Gender			
- *Female*	11	14	N/A
- *Male*	5	27	
Age			
- *Median*	36	50	N/A
- *Range*	22-47	20-80	
White Blood Cells			
- *Median*	6.15	22.6	4.50-11.0
- *Range*	4.21-8.39	0.65-141	(10^9cells/L)
Red Blood Cells			
- *Median*	4.29	2.78	3.80-5.30
- *Range*	3.54-5.27	1.37-4.75	(10^12cells/L)
Hemoglobin			
- *Median*	13.5	8.69	11.7-16.0
- *Range*	11.6-15.7	5.10-13.8	g/dL
Platelet			
- *Median*	251	73.2	150-400
- *Range*	164-361	11.0-452	(10^9cells/L)
ALT			
- *Median*	16.3	27.0	0.00-35.0
- *Range*	7.00-29.0	7.00-148	(U/L)
AST			
- *Median*	14.7	27.0	0.00-35.0
- *Range*	9.00-26.0	8.00-105	(U/L)
ALP			
- *Median*	68.3	91.0	35.0-105
- *Range*	47.0-101	23.0-461	(U/L)
GGT			
- *Median*	17.9	49.8	6.00-42.0
- *Range*	8.00-29.0	9.00-400	(U/L)
LDH			
- *Median*	161	470	135-214
- *Range*	137-184	134-2500	(U/L)
Urea			
- *Median*	19.9	33.4	12.8-42.8
- *Range*	14.0-31.0	13.0-71.0	(mg/dL)
Uric Acid			
- *Median*	3.50	5.25	2.40-5.70
- *Range*	2.50-5.10	2.00-10.00	(mg/dL)
Serum Creatinine			
- *Median*	0.66	0.81	0.50-0.90
- *Range*	0.51-0.81	0.34-1.42	(mg/dL)

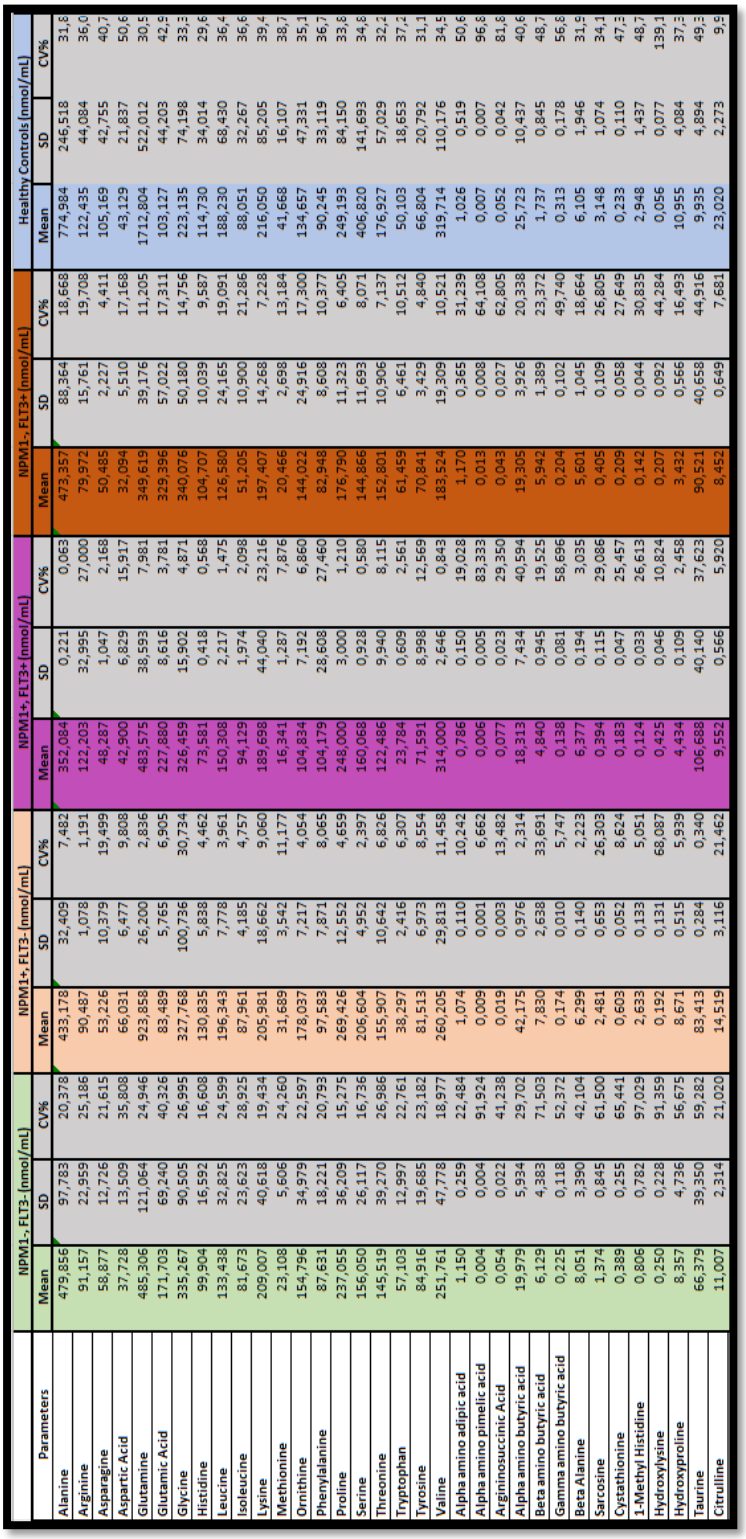
Table 3.1. (continued)

Glucose			
- *Median*	87.9	112	84.0-172
- *Range*	78.0-96.0	84.0-162	(mg/dL)
Triglycerides			
- *Median*	90.1	140	0.00-150
- *Range*	48.0-141	55.0-242	(mg/dL)
Cholesterol			
- *Median*	131	147	0.00-180
- *Range*	101-155	118-214	(mg/dL)
CRP			
- *Median*	0.69	59.6	0.00-5.00
- *Range*	0.10-1.60	0.3-311	(mg/L)

3.2. Amino Acid Profile

36 parameters in the amino acid panel were measured. All samples were studied in the same run with three technical replicates. The summary of amino acid results (Mean, Standard Deviation and CV%) for each group is given in Table 3.2. According to these results, Brown-Forsythe and Welch ANOVA were performed with Dunnett's method in order to make comparison between sample groups. ANOVA results of the analytes that show significant differences compared with mutation groups are given in Figure 3.1.

Table 3. 2. The summary of amino acid results



Parameters	NPM1-, FLT3- (nmol/mL)			NPM1+, FLT3- (nmol/mL)			NPM1+, FLT3+ (nmol/mL)			NPM1-, FLT3+ (nmol/mL)			Healthy Controls (nmol/mL)		
	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
Alanine	479,856	97,783	20,378	433,178	32,409	7,482	352,084	0,221	0,063	473,357	88,364	18,668	774,984	246,518	31,8
Arginine	91,157	22,959	25,186	90,487	1,078	1,191	122,203	32,995	27,000	79,972	15,761	19,708	122,435	44,084	36,0
Asparagine	58,877	12,726	21,615	53,226	10,379	19,499	48,287	1,047	2,168	50,485	2,227	4,411	105,169	42,755	40,7
Aspartic Acid	37,728	13,509	35,808	66,031	6,477	9,808	42,900	6,829	15,917	32,094	5,510	17,168	43,129	21,837	50,6
Glutamine	485,306	121,064	24,946	923,858	26,200	2,836	483,575	38,593	7,981	349,619	39,176	11,205	1712,804	522,012	30,5
Glutamic Acid	171,703	69,240	40,326	83,489	5,765	6,905	227,880	8,616	3,781	329,396	57,022	17,311	103,127	44,203	42,9
Glycine	335,267	90,505	26,995	327,768	100,736	30,734	326,459	15,902	4,871	340,076	50,180	14,756	223,135	74,198	33,3
Histidine	99,904	16,592	16,608	130,835	5,838	4,462	73,581	0,418	0,568	104,707	10,039	9,587	114,730	34,014	29,6
Leucine	133,438	32,825	24,599	196,343	7,778	3,961	150,308	2,217	1,475	126,580	24,165	19,091	188,230	68,430	36,4
Isoleucine	81,673	23,623	28,925	87,961	4,185	4,757	94,129	1,974	2,098	51,205	10,900	21,286	88,051	32,267	36,6
Lysine	209,007	40,618	19,434	205,981	18,662	9,060	189,698	44,040	23,216	197,407	14,268	7,228	216,050	85,205	39,4
Methionine	23,108	5,606	24,260	31,689	3,542	11,177	16,341	1,287	7,876	20,466	2,698	13,184	41,668	16,107	38,7
Ornithine	154,796	34,979	22,597	178,037	7,217	4,054	104,834	7,192	6,860	144,022	24,916	17,300	134,657	47,331	35,1
Phenylalanine	87,631	18,221	20,793	97,583	7,871	8,065	104,179	28,608	27,460	82,948	8,608	10,377	90,245	33,119	36,7
Proline	237,055	36,209	15,275	269,426	12,552	4,659	248,000	3,000	1,210	176,790	11,323	6,405	249,193	84,150	33,8
Serine	156,050	26,117	16,736	206,604	4,952	2,397	160,068	0,928	0,580	144,866	11,693	8,071	406,820	141,693	34,8
Threonine	145,519	39,270	26,986	155,907	10,642	6,826	122,486	9,940	8,115	152,801	10,906	7,137	176,927	57,029	32,2
Tryptophan	57,103	12,997	22,761	38,297	2,416	6,307	23,784	0,609	2,561	61,459	6,461	10,512	50,103	18,653	37,2
Tyrosine	84,916	19,685	23,182	81,513	6,973	8,554	71,591	8,998	12,569	70,841	3,429	4,840	66,804	20,792	31,1
Valine	251,761	47,778	18,977	260,205	29,813	11,458	314,000	2,646	0,843	183,524	19,309	10,521	319,714	110,176	34,5
Alpha amino adipic acid	1,150	0,259	22,484	1,074	0,110	10,242	0,786	0,150	19,028	1,170	0,365	31,239	1,026	0,519	50,6
Alpha amino pimelic acid	0,004	0,004	91,924	0,009	0,001	6,662	0,006	0,005	83,333	0,013	0,008	64,108	0,007	0,007	96,8
Argininosuccinic Acid	0,054	0,022	41,238	0,019	0,003	13,482	0,077	0,023	29,350	0,043	0,027	62,805	0,052	0,042	81,8
Alpha amino butyric acid	19,979	5,934	29,702	42,175	0,976	2,314	18,313	7,434	40,594	19,305	3,926	20,338	25,723	10,437	40,6
Beta amino butyric acid	6,129	4,383	71,503	7,830	2,638	33,691	4,840	0,945	19,525	5,942	1,389	23,372	1,737	0,845	48,7
Gamma amino butyric acid	0,225	0,118	52,372	0,174	0,010	5,747	0,138	0,081	58,696	0,204	0,102	49,740	0,313	0,178	56,8
Beta Alanine	8,051	3,390	42,104	6,299	0,140	2,223	6,377	0,194	3,035	5,601	1,045	18,664	6,105	1,946	31,9
Sarcosine	1,374	0,845	61,500	2,481	0,653	26,303	0,394	0,115	29,086	0,405	0,109	26,805	3,148	1,074	34,1
Cystathionine	0,389	0,255	65,441	0,603	0,052	8,624	0,183	0,047	25,457	0,209	0,058	27,649	0,233	0,110	47,3
1-Methyl Histidine	0,806	0,782	97,029	2,633	0,133	5,051	0,124	0,033	26,613	0,142	0,044	30,835	2,948	1,437	48,7
Hydroxylysine	0,250	0,228	91,359	0,192	0,131	68,087	0,425	0,046	10,824	0,207	0,092	44,284	0,056	0,077	139,1
Hydroxyproline	8,357	4,736	56,675	8,671	0,515	5,939	4,434	0,109	2,458	3,432	0,566	16,493	10,955	4,084	37,3
Taurine	66,379	39,350	59,282	83,413	0,284	0,340	106,688	40,140	37,623	90,521	40,658	44,916	9,935	4,894	49,3
Citrulline	11,007	2,314	21,020	14,519	3,116	21,462	9,552	0,566	5,920	8,452	0,649	7,681	23,020	2,273	9,9

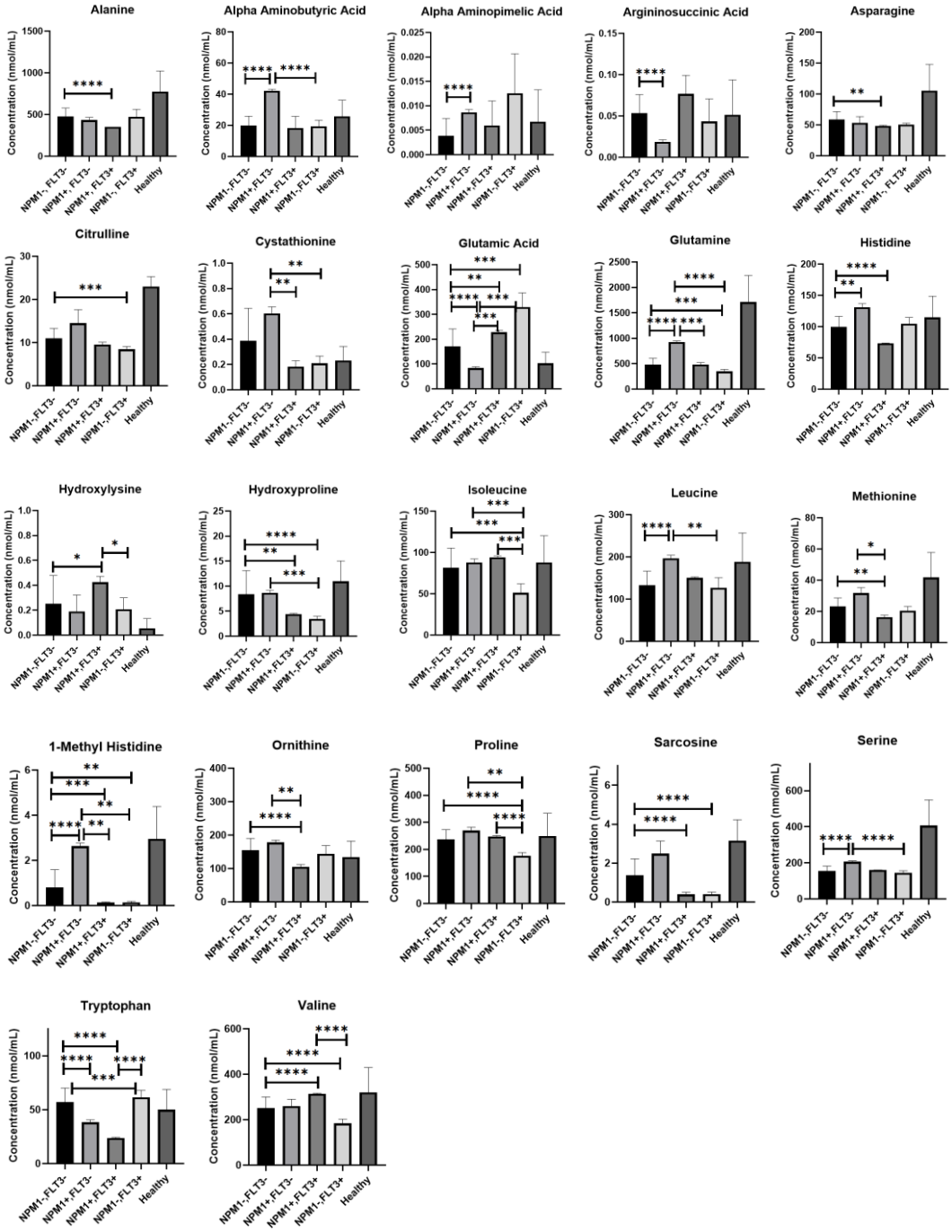


Figure 3. 1. ANOVA results in amino acid panel. Asterisks * denote statistical significance between sample groups at $p<0.05$.

When compared more than two sample groups with a high number of analytes, it is hard to interpret obtained results with univariate analysis. Nevertheless, univariate analysis is a useful method to take a glance at the data before multivariate analysis.

Regarding the evaluation of amino acid results, no study was found comparing the amino acid levels between mutation groups.

According to Figure 3.1, certain analytes may have a higher contribution to our model to be developed that provide the classification of patient samples. For instance, as seen in Figure 3.1, since some analytes have only significance for one mutation -alanine, alpha-aminobutyric acid, asparagine, leucine, tryptophan, serine- these parameters may be more important in terms of class discrimination.

Methionine levels in mutation groups were found significantly lower than healthy group. This finding is also similar with previous studies in literature (Muscaritoli et al., 1999c; X. Zhou et al., 2020). Hypermethylation of histone and DNA is a characteristic feature of myeloid malignancies (Goldman et al., 2019; Holz-Schietinger et al., 2012). The decreased level of methionine in mutation groups is explained by increased transmethylation levels in myeloid cells and their dependency on methionine.

Glutamine is the most abundant amino acid in human serum (Cruzat et al., 2018). Due to increased energy demand and anabolic reactions, cancer cells use glutamine as a precursor for *de novo* glucose and other amino acids synthesis. Glutamine is also used as a precursor for alpha-ketoglutarate (α-KG) conversion. Through α-KG formation, glutamine contributes in TCA cycle with its carbon skeleton. Glutamate can be produced from glutamine via glutaminases (GLS1 and GLS2) in human cells. GLSs are overexpressed in AML. The inhibition of GLSs is used as a therapeutic approach using bis-2-(5-phenylacetamido-1,2,4thiadiazol-2-yl)ethyl sulfide (BPTES) derivative manufacturing by Calithera Biosciences (Cai et al., 2016; Matre et al., 2016). As seen in Figure 3.1, glutamine levels in mutation groups were found significantly lower than healthy group. On the other hand, glutamate levels in mutation groups were found significantly higher than healthy group. Based on the knowledge of overexpression of glutaminases in AML cells, the findings are consistent with the literature (Kreitz et al., 2019; Matre et al., 2016).

In the most type of cancer cells, Tryptophan is degraded as the precursor for kynurenine pathway. According to Figure 3.1, tryptophan levels were found significantly lower in NPM1(+), FLT3 (-) and NPM1(+), FLT3 (-) mutation groups compared with the healthy group. On the other hand, the remained mutation groups have higher levels of tryptophan. Regarding this finding, no study has been found in literature.

Kikushige et al. (2022) showed that acute leukemia cells are dependent to Branched Chain Amino Acid (BCAA) metabolism to maintain stemness (Kikushige et al., 2022). According to the study, in acute leukemia cell, BCAA transaminase-1 (BCAT1) level is higher than control group. BCAT1 actively catabolizes BCAA into Branched Chain Keto Acid (BCKA). As seen in Figure 3.1, valine levels were found significantly lower in mutation groups compared with healthy group, except NPM1 (+), FLT3 (+). For Isoleucine, when healthy group was compared with mutation groups, the only significant difference was observed for NPM1 (-), FLT3 (+) group. Contrary to the literature, decreases in BCAA levels were not observed for all mutation groups.

After accomplishing multivariate studies, this approach will be re-evaluated according to be obtained VIP score of each analyte.

3.3. Acylcarnitine Profile

18 parameters in the acylcarnitine panel were measured from DBS samples prepared from whole blood donations. All samples were studied in the same run with three technical replicates. The summary of acylcarnitine results (Mean, Standard Deviation and CV%) for each group is given in Table 3.3. According to these results, Brown-Forsythe and Welch ANOVA were performed with Dunnett's method in order to make comparison between sample groups. ANOVA result of the analytes that shows significant differences compared with mutation groups are given in Figure 3.2.

Table 3. 3. The summary of acylcarnitine results

Parameters	NPM1-, FLT3- (nmol/mL)			NPM1+, FLT3- (nmol/mL)			NPM1+, FLT3+ (nmol/mL)			NPM1-, FLT3+ (nmol/mL)			Healthy Controls (nmol/mL)		
	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
C0	28,414	5,326	18,744	42,348	3,853	9,098	28,769	0,905	3,146	14,761	0,667	4,517	14,692	1,632	11,1
C2	10,837	3,414	31,506	16,760	11,940	71,239	15,423	1,249	8,098	8,747	0,634	7,246	16,185	1,536	9,5
C3	0,849	0,379	44,635	0,535	0,169	31,589	0,606	0,014	2,310	1,310	0,048	3,629	0,042	0,007	16,4
C4	0,195	0,055	28,223	0,383	0,153	39,783	0,401	0,090	22,338	0,090	0,011	12,196	0,075	0,009	11,9
C5	0,100	0,044	44,311	0,121	0,010	8,264	0,123	0,004	2,863	0,042	0,006	14,019	0,043	0,007	15,4
C4OH	0,042	0,024	57,878	0,107	0,077	71,274	0,182	0,007	3,846	0,013	0,002	15,181	0,010	0,002	18,6
C6	0,044	0,013	29,866	0,074	0,010	13,514	0,091	0,010	10,989	0,023	0,002	7,922	0,031	0,006	19,6
C5OH	0,159	0,056	35,543	0,197	0,080	40,287	0,078	0,066	83,618	0,096	0,015	15,912	0,023	0,002	7,9
C8:1	0,082	0,033	40,384	0,062	0,018	29,032	0,081	0,002	1,894	0,054	0,005	8,866	0,025	0,006	25,4
C8	0,050	0,011	21,894	0,067	0,008	11,940	0,050	0,006	12,000	0,031	0,002	7,079	0,052	0,011	21,5
C3DC	0,099	0,021	21,614	0,102	0,025	24,100	0,084	0,068	80,952	0,062	0,006	9,219	0,086	0,009	10,9
C10:1	0,096	0,021	22,049	0,141	0,015	10,638	0,068	0,007	9,615	0,063	0,004	6,620	0,059	0,010	17,3
C10	0,072	0,020	27,614	0,112	0,006	5,357	0,050	0,002	3,076	0,035	0,002	5,599	0,057	0,010	17,5
C4DC	0,350	0,094	26,772	0,350	0,076	21,714	0,328	0,033	10,061	0,194	0,006	2,839	0,025	0,001	5,4
C5DC	0,082	0,024	28,900	0,128	0,043	33,594	0,137	0,002	1,460	0,048	0,003	7,245	0,080	0,015	18,4
C12	0,037	0,010	27,705	0,052	0,004	7,692	0,069	0,002	2,899	0,022	0,001	6,611	0,021	0,004	19,5
C6DC	0,037	0,016	41,548	0,032	0,009	26,304	0,036	0,002	5,556	0,008	0,001	8,474	0,092	0,012	12,8
C14:2	0,043	0,012	28,728	0,075	0,002	2,667	0,064	0,003	3,953	0,020	0,002	9,262	0,013	0,003	23,0

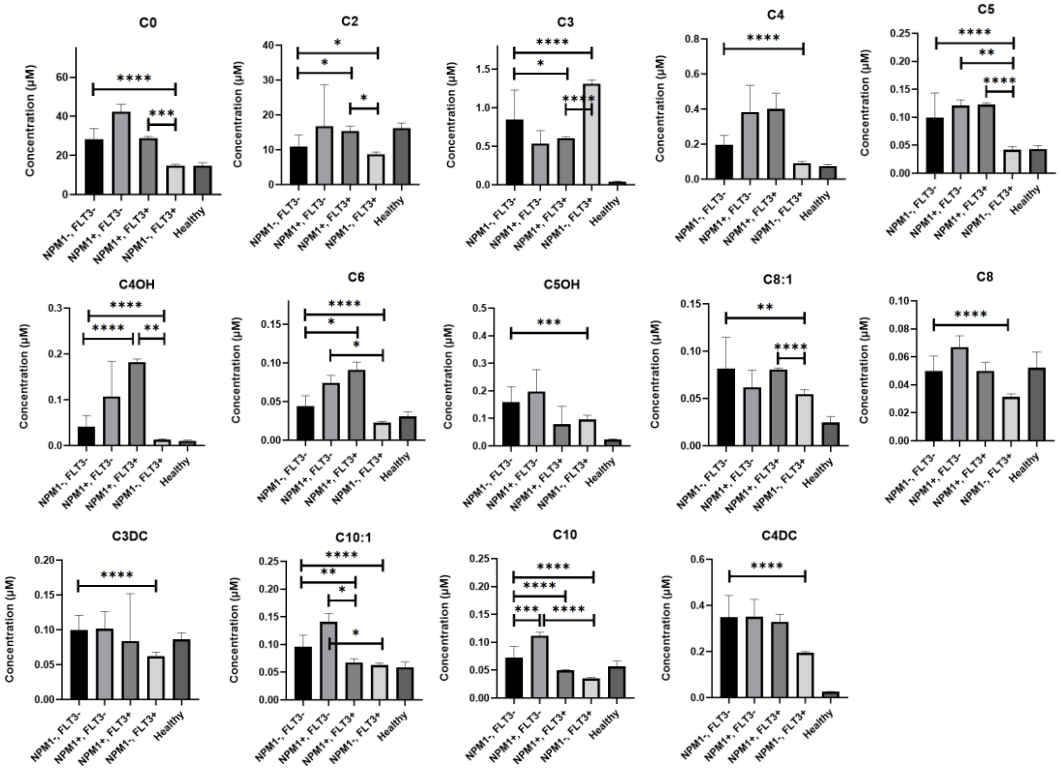


Figure 3. 2. ANOVA results in acylcarnitine panel. Asterisks * denote statistical significance between sample groups at $p<0.05$.

As mentioned in amino acid results, certain analytes may have a higher contribution to our model. Considering 52 analytes and 58 samples, interpretation of the obtained results is quite hard. Therefore, an advance statistical analysis, Multivariate analysis were performed. It is used to find patterns and correlations between several variables at the same time. After accomplishing multivariate studies, univariate results will be re-evaluated according to be obtained VIP score of each analyte.

3.4. Multivariate Analysis

To find a pattern for the classification of 4 patient groups, all panel metabolites must be evaluated and compared simultaneously by multivariate analysis. Firstly, a PCA model was applied to provide an overview of the results, where no outlier was found (Figure 3.3).

The first principal component is the direction in space along which projections have the largest variance. The second principal component is the direction which maximizes variance among all directions orthogonal to the first.

As a result of the PCA model, the most important 2 principal components with the highest variance explained 79.0% of the total variance. Namely, 79.0 % of the variance of the dependent variable being studied was explained by the variance of the independent variable. R2 and Q2 values were calculated as 0.793 and 0.732, respectively.

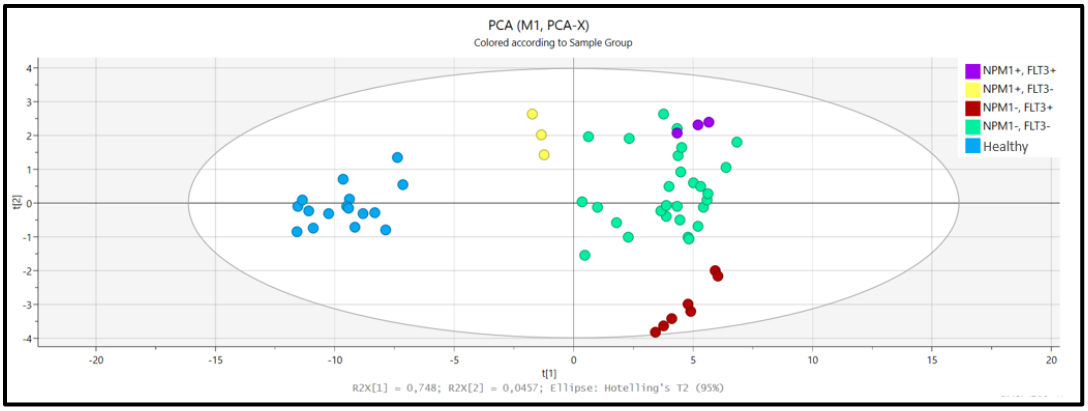


Figure 3. 3. PCA 2D Score Plot. Each dot represents a patient sample. R2X[1] and R2X[2] symbolizes for Principal Component 1 and Principal Component 2, respectively. Each sample group was indicated with a different color. The first component explains 75 % of the variation, and the second component 5 %.

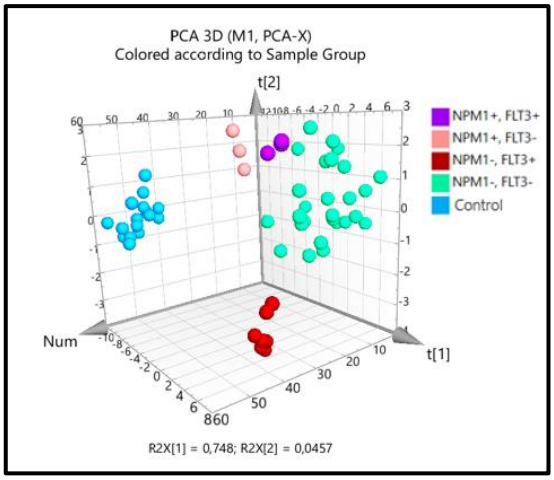


Figure 3. 4. PCA 3D score plot

The R2 values which are greater than 0.75 (> 0.75) are considered as “strong”, the values from 0.50-0.75 is considered as moderate” and the values range from 0.30-0.49 is considered as “weak” factor loadings. According to this information, the obtained PCA model is considered strong.

The last chemometric method used to measure the discriminating power of metabolites in sample groups and to visualize possible metabolic changes is PLS-DA. Obtained result is given in Figure 3.5. The model was explained with 5 components. R2X, R2Y and Q2 values were calculated as 0.869, 0.845 and 0.619, respectively. A close value of Q2 to R2 (difference <0.3) shows that the model has high prediction power.

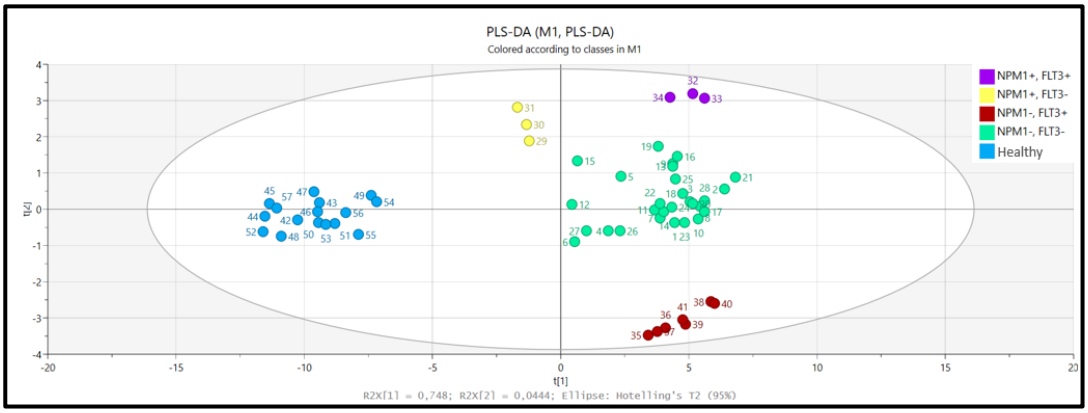


Figure 3. 5. PLS-DA Score plot. Each dot represents a patient sample. R2X[1] and R2X[2] symbolizes for Principal Component 1 and Principal Component 2, respectively. Each sample group was indicated with a different color.

As mentioned in chapter 1.2.4.1.2, if the Q2Y value is close to 1, it shows that the model is highly reliable in the validation tests. R2 score is always greater than Q2 score. Studies have requested that R2 and Q2 scores should be as close to 1 as possible and the difference between them should be less than 0.3 (Szymańska et al., 2012).

To define the important analytes for class discrimination, loading plot and VIP score results are given in Figure 3.6 and 3.7, respectively. Loading plot of the PLS-DA model. The loading plot is complementary to the score plot and explains how

the X-variables relate to each other as well as to group belonging (Y-variable symbolized by a group dot). X-variables located near a group dot are positively associated with that group. For instance, the control group is characterized by high values for Glutamine. On the other hand, NPM1+, FLT3+ group is characterized by low values of Glutamine since it is located on the opposite side of the origin of the graph.

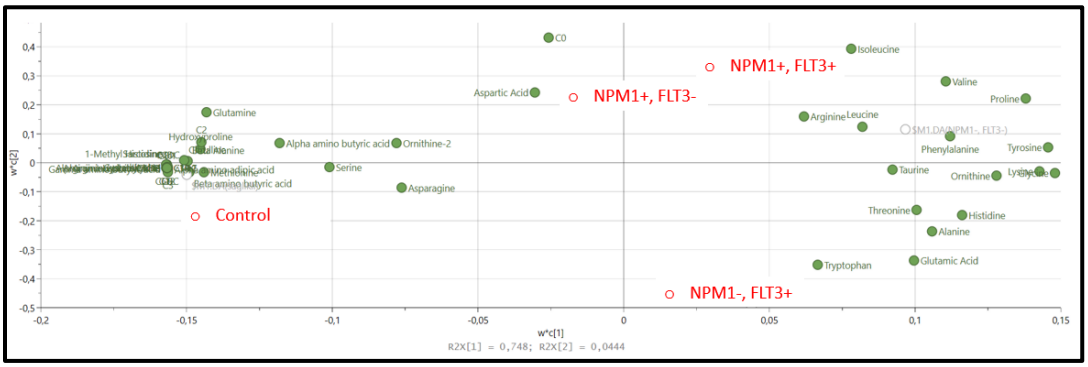


Figure 3. 6. Loading plot of PLS-DA model. X variables (analytes) represents with green dot, Y variables (classes) represents with red dot.

VIP analysis is a weighted sum of squares of the PLS-DA loadings considering the amount of explained Y-variable in each dimension. The value of VIP score which is greater than 1 is the criteria for identification of significant variables in order to define patterns.

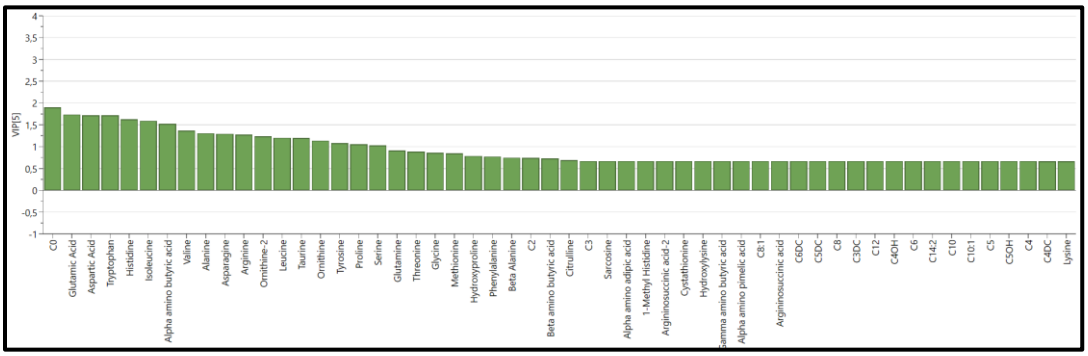


Figure 3. 7. The results of VIP analysis

According to Figure 3.7, the parameters that have a VIP score greater than >1 are given in Table 3.4.

Table 3. 4. Significant analytes in the PLS-DA model

Analyte	**VIP Score**
C0 carnitine	1.90
Glutamic acid	1.74
Aspartic acid	1.72
Tryptophan	1.72
Histidine	1.63
Isoleucine	1.59
Alpha-aminobutyric acid	1.53
Valine	1.36
Alanine	1.31
Asparagine	1.30
Arginine	1.28
Ornithine	1.24
Leucine	1.21
Taurine	1.20
Tyrosine	1.08
Proline	1.06
Serine	1.03

Fatty acid oxidation is the main source of energy for most cancer cells in order to survive. Thus, they express high levels of fatty acid oxidation enzymes in the related reactions. In these reactions, the cofactor carnitine is essential since it serves as a “shuttle-molecule” to allow fatty acid acyl moieties entering the mitochondrial matrix where these molecules are oxidized via the β-oxidation pathway. Therefore, the role of carnitine in cancer cell metabolism is important. Fatty acid oxidation is also main source of energy in AML cells for their survival. Carnitine palmitoyl transferase 1A (CPT1A) and carnitine transporter CT2 (SLC22A16) proteins are

rate limiting actors of fatty acid oxidation. These proteins are overexpressed in AML. According to this information, in the VIP result, it is not surprising to see free carnitine in the first rank (Carracedo et al., 2013; Shi et al., 2016; Wu et al., 2015).

Amino acids are not only essential for proteins but also intermediate metabolites fueling multiple biosynthetic pathways. Muscaritoli et al. showed that glycine, glutamic acid, ornithine and tryptopan levels are significantly higher in AML patients compared with healthy subjects (Muscaritoli et al., 1999a). According to the obtained results seen in Table 3.4, the changes in the mentioned amino acids were found remarkable for class discrimination.

In another study published by Tabe et al., the aim of article was focused on the deprivation of 4 key amino acids (arginine, asparagine, glutamine and cysteine) in the important pathways of hematological malignancies as a new therapeutic approach (Tabe et al., 2019). The results showed that the deprivation of these amino acids increase the vulnerability of cancer cells in AML. Since these amino acids are important for the biosynthesis of proteins that are necessary for the survival and proliferation of AML cells, the finding that they are significantly important in class difference is consistent with the literature.

In order to promote proliferation in AML cells, protein modifications and biosynthesis reactions including nitrogen anabolic processes are necessary. These steps can also be rate limiting for cell proliferation. The nitrogen required biosynthetic pathways consume high amounts of amino acids as a nitrogen source. In glutaminolysis, glutamate is formed by degradation of glutamine. Glutamate can be directly participated in biosynthesis or indirectly provide nitrogen source for biosynthetic reactions. Due to its contributions of glutamate, glutaminolysis provides the ultimate mechanism to maintain the cytosolic homeostasis of glutamate. Regarding the importance of glutaminolysis in AML, Gallipoli et al. (Gallipoli et al., 2018) showed that glutaminolysis is a metabolic dependency in FL3 mutations in the *in vivo* model of AML. In Table 3.4, the VIP result showed

that glutamic acid/glutamate is the second important analyte for class discrimination. As seen in Figure 3.6, glutamic acid have importance for the discrimination of FLT3+ mutation groups.

When the univariate and multivariate analysis are compared, the parameters (alanine, alpha-aminobutyric acid, asparagine, leucine, tryptophan, serine) that were significantly different in a one mutation type, have also remarkably importance for the class discrimination in the VIP analysis.
Even though the obtained results seem to be consistent with the previous studies, the developed PLS-DA model for the discrimination of mutation groups in AML samples should be validated.

3.4.1. Validation

In case of absence of validation data set or small sample population, permutation tests are effective for verifying the developed model. As mentioned in chapter 2.2.3, in a permutation test, the class labels and samples are permuted, they are randomly assigned to different individuals. With the 'wrong' class labels, again a classification model is calculated. The basis of the permutation test is that with the wrong class labels, the newly calculated classification model should not be able to predict the classes very well. As the groups are formed in a random way, the assumption is that no difference exists between them.

In Figure 3.8, Permutation tests with 1000 cycles were performed to validate the PLS-DA model. The histogram shows the distribution results of permutated samples. The arrow indicates our original sample. The right direction of the x-axis shows that the separation power between groups becomes stronger. Figure 3.8 indicates that our data set have more discriminating power than the permutated set.

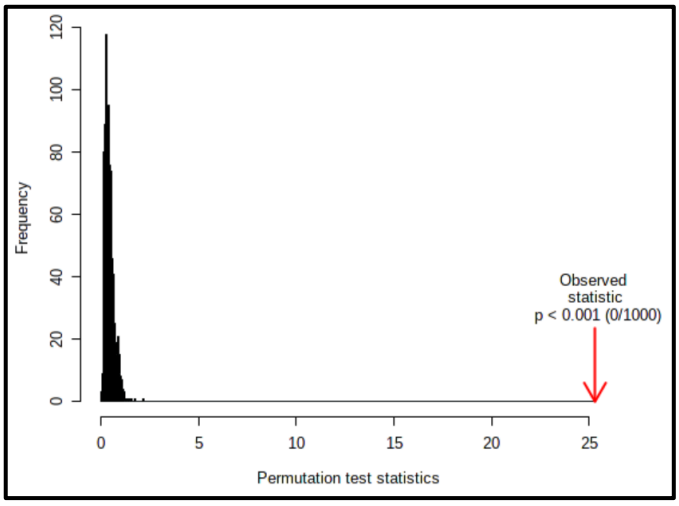


Figure 3. 8. The result of permutation test

In addition to the permutation test, K-fold cross validation method was also performed in order to validate the PLS-DA model. In Figure 3.9, accuracy, R2 and Q2 values were calculated for each component in the model. According to the calculated parameters, it is seen that the compatibility and predictive ability of the model are strong.

PLS-DA cross validation details:

Measure	1 comps	2 comps	3 comps	4 comps	5 comps
Accuracy	0.77193	0.85965	0.85965	0.92982	0.98246
R2	0.80922	0.9265	0.95796	0.96755	0.97388
Q2	0.79551	0.87878	0.89585	0.89772	0.89351

Figure 3. 9. K-fold cross validation results

3.4.2. Pathway Analysis

Pathway analysis was performed to detailly examine in detail to evaluate pathways including the parameters responsible for class discrimination. In accordance with this purpose, Pathway enrichment analysis was performed using Metaboanalyst 5.0. The enrichment ratio is calculated as the number of hits within a particular

metabolic pathway divided by the expected number of hits. Figure 3.10 shows the MetaboAnalyst 5.0 pathway enrichment analysis used to evaluate the metabolic pathways most implicated in the results. Statistically significant pathways are given in Figure 3.11.

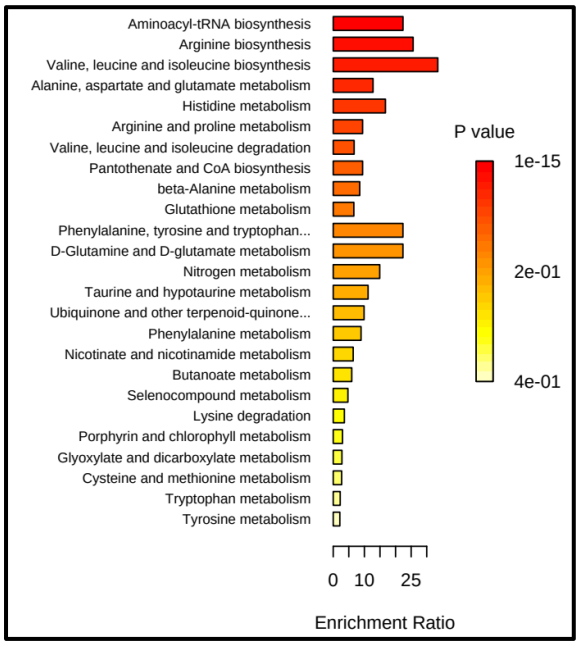


Figure 3. 10. The result of pathway enrichment analysis

	total	expected	hits	Raw p	Holm p	FDR
Aminoacyl-tRNA biosynthesis	48	0.54	12	1.25E-15	1.05E-13	1.05E-13
Arginine biosynthesis	14	0.16	4	9.99E-06	8.29E-04	4.19E-04
Valine, leucine and isoleucine biosynthesis	8	0.09	3	6.27E-05	5.14E-03	1.76E-03
Alanine, aspartate and glutamate metabolism	28	0.31	4	1.85E-04	1.50E-02	3.89E-03
Histidine metabolism	16	0.18	3	5.93E-04	4.75E-02	8.73E-03
Arginine and proline metabolism	38	0.42	4	6.24E-04	4.93E-02	8.73E-03

Figure 3. 11. Significant pathways obtained from enrichment analysis

Since biosynthetic pathways are important for survival of cancer cells, it is not surprising to see Aminoacyl-tRNA biosynthesis pathways that play an important role in protein synthesis. Aminoacyl-tRNA synthetases are involved in a variety of physiological and pathological processes, especially tumorigenesis. Stockard et al. showed that aminoacyl-tRNA biosynthesis was also found significant in FLT mutated AML patient samples (Stockard et al., 2018). The result of pathway analysis for aminoacyl-tRNA biosynthesis is consistent with the literature.

According to the study accomplished by Mussai et al., arginine concentrations in AML patients were significantly low compared with healthy controls both at diagnosis and during treatment (Mussai et al., 2019). The decrease in arginine level was also observed in this thesis (Figure 3.1). Also, the results belonging to Mussai et al. show that the low arginine microenvironment acted as a metabolic brake on T cell function and expansion. When all this information is taken into consideration, arginine metabolism seems to have a potency for class discrimination according to Figure 3.11. As seen in Figure 3.6, arginine has a potency for the discrimination of FLT3 mutation. The obtained result for arginine biosynthesis is consistent with the literature.

Valine is a branched-chain amino acid (BCAA) and essential for self-renewal of leukemic stem cells. It plays an important role in protein synthesis and cell proliferation via interacting with mTOR signaling pathways. Through catabolism of BCAAs, their nitrogen groups can be transferred to alpha-ketoglutarate resulting in formation of glutamate or they can be completely converted to form TCA cycle metabolites, succinyl CoA or acetyl CoA. Due to these important roles, in the VIP score analysis (Figure 3.11), valine metabolism was found significantly important for the class discrimination. In addition, in the univariate analysis of the amino acid panel, valine concentration was found in low levels compared with healthy subjects. Decrease in the concentration can be explained by degradation of valine to provide its nitrogen source or supply energetic precursors.

Stockard et al. showed that proline metabolism is affected by FLT3 mutations in pediatric AML samples (Stockard et al., 2018). Glutamate can be also converted from BCAAs and proline. Since glutamate concentration is tightly controlled and the biosynthesis of several non-essential amino acids is interlinked with each other by glutamate, it was expected to see proline biosynthesis as statistically significant pathway in the rank list of pathway analysis.

Limited information is available about the role of alanine metabolism in myeloid leukemias.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTION

AML is a rapidly progressing cancer type that requires urgent treatment. In order to define the risk class of newly diagnosed patients, mutation tests must be performed. Since the obtaining of testing results take 14-21 days, rapid diagnosis tests are extremely required by clinicians in Turkey. Also it should be noted that, according to Röllig et al., the risk in awaiting testing results is less than the risk in beginning therapy before results are available (Röllig et al., 2019). Until obtaining all mutation results in the AML test panel, fast and reliable prediction methods to determine mutations status of *de novo* patient are required by clinicians in order to apply proper first-response treatment. Due to the high incidence and strategic importance in risk stratification of patients, NPM1 and FLT3 mutations were chosen for this thesis.

In this thesis, the patterns associated with FLT3 and NPM1 mutations in *de novo* AML samples were investigated using the LC-MS/MS system. All samples were collected from Ankara University Hematology Department with ethics committee approval (Document Number & Approval Date: 14-938-18/ 19.09.2018). For this purpose, amino acid and carnitine panels that are closely related with energy metabolism were measured in AML serum and DBS samples. Sample groups were categorized as in 5 different populations:

- Healthy subjects
- FLT3+, NPM1+ de novo AML patients
- FLT3-, NPM1- de novo AML patients
- FLT3+, NPM1- de novo AML patients
- FLT3-, NPM1+ de novo AML patients

Thesis studies were accomplished in three stages: Data collection, data processing and data analysis. In the data collection step, defined analytes (34 amino acids and 18 acylcarnitines) were measured via LC-MS/MS system. The raw data was processed in terms of spectral corrections (baseline correction, alignment, normalization, quantification, etc.) with Labsolution software developed by Shimadzu. Subsequently, univariate (ANOVA) and multivariate analysis (PCA and PLS-DA) were performed with the corrected data set in order to define patterns between sample groups. Using VIP method, remarkable analytes that are important in class discrimination were defined. The developed model was validated with K-Fold cross validation method and permutation test.

In univariate analysis of the amino acid panel, no significant difference between mutation groups was observed for alpha-aminoadipic acid, beta-alanine, beta-aminoisobutyric acid, GABA, lysine, phenylalanine, taurine, threonine, and tyrosine ($p>0.05$). In addition, C5DC, C12, C6DC and C12 were not also found significantly different between mutation groups ($p>0.05$).

In multivariate analysis, the PCA model explains 79.0 % of the total variance. R2 and Q2 values of the PCA model were calculated as 0.793 and 0.732, respectively. Furthermore, R2Y and Q2 values of the PLS-DA model were calculated as 0.845 and 0.619, respectively. According to these results, it has been determined that the model has high coherence, validity, and predictive power. In order to determine significantly important analytes in the developed PLS-DA model, VIP analysis was performed. According to the VIP results, C0 carnitine, glutamic acid, aspartic acid, tryptophan and histidine metabolites had the most effect on the classification between the 5 groups.

The developed PLS-DA model was validated with K-fold cross validation and permutation test method. In the 1000 cycle of permutation test performed for the validation of the PLS-DA model, the permutated data sets did not perform better

than the original data. K-fold result shows that R2 and Q2 values of the PLS-DA model are 0.93 and 0.90 respectively. These results indicated that the fitting and validity of the model are high, and the predictive power is strong.

Pathway enrichment analysis was performed with the analytes that are significantly important in VIP analysis (score >1.00). The result indicates that the pathways of aminoacyl tRNA, arginine, valine-leucine-isoleucine biosynthesis were found significantly important ($p<0.05$) in terms of the discrimination of mutation groups. As a result, with the measurement of fifty-two analytes closely related with energy metabolism, five sample groups were successfully separated by LC-MS/MS using metabolomics methods. The developed model is a basis as a preliminary study in terms of defining mutation status of NPM1 and FLT3 proteins in AML patients. In order to increase reliability and validity of the model, the study must be expanded as a multi-centered study. In this way, biological variance in sample groups would be observed more clinically relevant.

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FOREIGN LANGUAGES

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PUBLICATIONS

1. Durusu İZ, Hüsnügil HH, Ataş H, Biber A, Gerekçi S, Güleç EA, Özen C. "Anti-cancer effect of Clofazimine as a single agent and in combination with cisplatin on U266 Multiple Myeloma Cell Line", Leukemia Research, 55:33-40 (2017). doi: 10.1016/j.leukres.2017.01.019

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