MOLECULAR INVESTIGATION OF THE EFFECTS OF INTERLEUKIN-6 (IL-6) AND URIDINE 5'-DIPHOSPHO-GLUCURONYLTRANSFERASE 1 (UGT1A1) MUTATIONS ON THE FORMATION OF HEART FAILURE AND DIURETIC RESISTANCE

by
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ABSTRACT

MOLECULAR INVESTIGATION OF THE EFFECTS OF INTERLEUKIN-6 (IL-6) AND URIDINE 5'-DIPHOSPHO-GLUCURONYLTRANSFERASE 1 (UGT1A1) MUTATIONS ON THE FORMATION OF HEART FAILURE AND DIURETIC RESISTANCE

Furosemide is a diuretic and is used for treatment of patients with heart failure. It has been found that in some patients, the drug does not work in sufficient quantities. This condition is named as furosemide resistance. In this study, it is aimed to investigate the relationship between *UGT1A1* and *IL-6* mutations with furosemide resistance in patients with heart failure. Sixty heart failure patients using furosemide (patient group) and 30 healthy individuals (control group) were enrolled in this study. Patients were divided into 2 subgroups as furosemide resistant (non-responders) group (n=30) and the non-resistant (responders) group (n=30). Mutations in the first exon of *UGT1A1*; rs1800795 and rs1800796 variations in *IL-6* were analyzed by direct sequencing and real-time polymerase chain reaction (RT-PCR), respectively. At the end of the study, eleven mutations were detected in *UGT1A1*, of which nine of them are novel. Also, rs1800795 and rs1800796 variations were detected in all of the groups. When patient and control groups were compared with each other, rs1800796 variation in *IL-6* was found statistically high in the patient group (p=0,027). When the three groups were compared with each other, similarly rs1800796 variation in *IL-6* was found statistically high in the non-responders group (p=0,043). When allele distributions were compared between patient and control groups, the C allele of rs1800796 variation in *IL-6* was found statistically high in the patient group (p=0,032). When allele distributions were compared between the three groups, 55 T-insertion in *UGT1A1* was found statistically high in the non-responders group (p=0,017). Three mutations were found deleterious and six mutations were detected as probably damaging to protein functions. It is thought that our study will contribute to the elucidation of pharmacogenetic features (drug response-gene relationship) and the development of individual-specific treatment strategies in heart failure patients using furosemide.

ÖZET

INTERLÖKIN-6 (IL-6) VE URIDIN 5' - DIFOSFO-GLUKURONOILTRANSFERAZ 1 (UGT1A1) MUTASYONLARININ KALP YETMEZLIĞI VE DIÜRETIK DIRENCI OLUŞUMUNA ETKISININ MOLEKÜLER DÜZEYDE ARAŞTIRILMASI

Furosemid bir diüretiktir ve kalp yetmezliği olan hastaların tedavisinde kullanılır. Hastaların bir kısmında ilacın yeterli miktarda (optimal dozda) işe yaramadığı belirlenmiştir. Bu durum furosemid direnci olarak bilinmektedir. Bu çalışmada kalp yetmezliği olan hastalarda *UGT1A1* ve *IL-6* genindeki mutasyonlar ile furosemid direnci arasındaki ilişkinin araştırılması hedeflenmiştir. Çalışmaya furosemid kullanan 60 kalp yetmezliği hastası (hasta grubu) ve 30 sağlıklı birey (kontrol grubu) dahil edilmiştir. Hastalar furosemid direnci geliştiren (ilaca cevap vermeyen) (n=30) ve geliştirmeyen grup (ilaca cevap veren) (n=30) olarak 2 alt gruba ayrılmıştır. *UGT1A1*'in ilk ekzonundaki mutasyonlar ve IL-6'daki rs1800795 ve rs1800796 varyasyonları sırasıyla dizi analizi ve Gerçek-Zamanlı Polimeraz Zincir Reaksiyonu (RT-PCR) ile analiz edilmiştir. Çalışmanın sonunda *UGT1A1*'de dokuz tanesi yeni olan toplam onbir mutasyon tespit edilmiştir. rs1800795 ve rs1800796 varyasyonları da tüm gruplarda tespit edilmiştir. Hasta ve kontrol grupları birbirleri ile karşılaştırıldıklarında, *IL-6*'da rs1800796 varyasyonunun istatistiksel olarak anlamlı düzeyde yüksek olduğu tespit edilmiştir (p=0,027). Üç grup birbiri ile karşılaştırıldığında, benzer olarak *IL-6*'daki rs1800796 varyasyonu ilaca yanıt vermeyen grupta istatistiksel olarak yüksek bulunmuştur (p=0,043). Hasta ve kontrol gruplarının allel dağılımları karşılaştırıldığında, *IL-6*'daki rs1800796 varyasyonunun C alleli hasta grubunda istatistiksel olarak yüksek bulunmuştur (p=0,032). Allel dağılımları üç grup arasında karşılaştırıldığında, *UGT1A1*'deki 55-T insersiyonu ilaca cevap vermeyen (dirençli) grupta istatistiksel olarak anlamlı düzeyde yüksek bulunmuştur (p=0,017). Üç mutasyonun zararlı, altı mutasyonun ise protein fonksiyonuna hasar verdiği tespit edilmiştir. Çalışmamızın furosemid kullanan kalp yetmezliği hastalarında, farmakogenetik özelliklerin (ilaç yanıtı-gen ilişkisinin) aydınlatılmasına ve bireye özgü tedavi stratejilerinin geliştirilmesine katkı sağlayacağı düşünülmektedir

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LIST OF SYMBOLS/ABBREVIATIONS

A	Adenine
ACE	Angiotensin-converting enzyme
Ala	Alanine
Asp	Aspartic acide
Asn	Asparagine
Arg	Arginine
BNP	Brain Natriuretic Peptide
BUN	Blood urea nitrogen
C	Cytosine
Cys	Cysteine
DNA	Deoxy ribonucleic acid
ECG	Electrocardiogram
ECO	Echocardiogram
ERK	Extracellular signal regulator kinase
FAM	6-carboxyfluorescein
G	Guanine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HGB	Hemoglobine
HDL	High density lipoprotein
HF	Heart Failure
His	Histidine
HTC	Hematocryte
ICD	Implantable cardioverter defibrillators
IL-6	Interleukin-6
Ile	Isoleucine
JAK	Janus kinase
K	Potassium
LDL	Low density lipoprotein
Leu	Leucine

Lys	Lysine
μL	Microliter
mg/dL	Miligram/Deciliter
Met	Methionine
MRI	Magnetic resonance imaging
ng/mL	Nanogram/Mililiter
Na	Sodium
nmol/L	Nanomol/Liter
NT-proBNP	N-terminal proB-type Natriuretic Peptide
NYHA	New York Heart Association
OD	Optical density
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol -3-kinase
Phe	Phenylalanine
Pro	Proline
RTPCR	Real time polymerase chain reaction
S	Serine
SAFE	Survivor Activation Factor Enhancement
SD	Standard deviation
SNP	Single nucleotide polymorphism
SPSS	Statistical package for the social science
T	Thymine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UGT1A1	Uridine 5'-diphospho-glucuronyltransferase
USA	United States of America
UVB	Ultraviolet B
Val	Valine

1. INTRODUCTION

1.1. AIM OF STUDY

Diuretics are used drugs to prevent the accumulation of waste material and prevent edema due to heart failure and related circulatory problems. Furosemide is used as the most common diuretic for the treatment of patients with heart failure. It has been found that in some patients with heart failure using furosemide, the drug does not work in sufficient quantities and patients develop resistance to this drug. This condition is named furosemide resistance. Furosemide resistance may be related to various gene mutations. Therefore, it was aimed to investigate the genetic factors which affect furosemide resistance in heart failure patients who use furosemide for treatment. It is thought that the mutations in the *UGT1A1* gene which encodes UDP-5'- diphospho glucuronyltransferase (UGT) enzyme and *IL-6* gene which produces *IL-6* are effective for furosemide metabolism. For this reason, it was aimed to investigate *UGT1A1* and *IL-6* gene mutations. In this study, rs1800795 and rs1800796 in *IL-6* gene variations were detected by Real-Time Polymerase Chain Reaction (RT-PCR). Because of *UGT1A1* gene is directly related to furosemide metabolism, 1st exon of the gene was investigated by the direct sequencing method. Heart failure is a polygenic and multifactorial disease. It is thought that many factors such as nutrition, cigarette, and alcohol consumption are effective in this process. Therefore it was aimed the collect information about risk factors (Hemoglobin (HBG), sodium, potassium, ventricular natriuretic peptide (NT-proBNP), hematocrit, blood ürea nitrogen (BUN), creatinine), and evaluation of whole parameters with genotype data at the end of the study.

1.2. HEART FAILURE (HF)

Heart failure (HF) is the heart's inability to pump the amount of blood needed by tissues or to pump some blood only under high filling pressure [1]. The main cause of all cardiovascular system diseases is HF. While the incidence of HF is %1 at a young age, this rate rises to 10% in people 70 years old and over [2]. In our country, the incidence of heart failure is between 0.3-2% at a young age and this rate is between 3-5% for people aged 65

and over and around 25% for those over 75 years old [3]. When the clinical findings of heart failure are evaluated, various factors come to the fore such as the patient's age, the rate and duration of deterioration of cardiac performance, the etiology of heart disease, the causes that trigger heart failure, and the first ventricle held during the disease [4].

HF is a clinical syndrome in which patients have typical symptoms (such as shortness of breath, ankle swelling, and weakness) and signs (such as increased jugular vein pressure, crepitation in the lung, and displacement of the apex of the heart) caused by a structural or functional disorder in the heart [5]. Most of the HF symptoms are not discriminatory, so, it has limited diagnostic value [6]. Most of the symptoms of HF are due to water and sodium retention and may be rapidly resolved with diuretic therapy, so patients on this therapy may not have symptoms. The underlying cardiac cause in the HF diagnosis is myocardial disease-causing systolic ventricular dysfunction [5]. However, ventricular diastolic dysfunction, heart valves, pericardium, endocardium, heart rhythm, and conduction abnormalities can also cause heart failure. Because heart failure is a multifactorial disease and a pathology with a known cause leads to definitive treatment detection of the problem is important to the treatment of heart failure.

1.3. CAUSES OF HEART FAILURE

HF is the most important disease. It is an important health problem and it affects a large part of the adult population in the world and causes high morbidity and mortality. HF causes limitations in the daily activities of patients due to symptoms and signs and it negatively affects the quality of life. HF is a multifactorial disease. Not only genetic factors but also environmental factors such as incorrect diets, increased consumption of fatty foods, obesity, stress, smoking, and alcohol consumption play a role in the development of HF.

The main cause of HF is left ventricular dysfunction. Disruption in the systolic or diastolic function of the left ventricle can lead to HF. The problem in diastolic dysfunction is related to left ventricular filling. Left ventricular size is normal, but end-diastolic pressure is increased. In systolic dysfunction, the left ventricle is usually dilated and wall movements are reduced.

Today, ischemic heart disease is the most common cause of HF in white women and men. However, heart failure due to coronary heart disease is observed less frequently in women than men, and HF due to valvular heart disease is observed more frequently (Table 1.1 and Table 1.2).

Table 1.1. Incidence of HF in Men and Women by Age [7-8].

AGE	FEMALE	MALE
50-59	2/1000	3/1000
80-89	22/1000	27/1000

Table 1.2. Prevalence of Heart Failure in Women and Men by age [7].

AGE	FEMALE	MALE
>44	25/1000	24/1000
>80	78/1000	66/1000

1.4. SYMPTOMS OF HEART FAILURE AND DIAGNOSIS

In HF; symptoms such as shortness of breath, weakness, fatigue, swelling of the ankles, cerebral symptoms such as confusion, decreased concentration, and gastrointestinal symptoms such as nausea and abdominal pain may occur during rest or exercise [9]. Among these, the most obvious symptoms of heart failure are shortness of breath, fatigue, and exercise intolerance [10-11]. In patients with HF, functional conditions should be included in the classification to improve symptoms [11]. The most commonly used practice in assessing the exercise capacities of patients with HF is the classification of the New York Heart Association (NYHA). Class four is the best exercise capacity while class four is the worst exercise capacity [5].

Table 1.3. Signs and results of HF

SIGNS	RESULTS
Typical Shortness of breath Weakness Tiredness Orthopnoea Decreased exercise tolerance	**Specific** S3 heart sound The murmur in the heart sounds
Less typical Loss of appetite Depression Cough Wheezing The feeling of swelling in the abdomen	**Less specific** Tachypnea Cachexia Hepatomegaly Tachycardia Edema in the periphery Irregular pulse

HF is determined as a result of clinical tests such as total blood count, brain natriuretic peptide (BNP), triglyceride, cholesterol, total bilirubin levels, blood urea nitrogen (BUN), creatine, and electrocardiogram (ECG), echocardiogram (ECO), chest X-ray, effort test, cardiac scintigraphy, Holter test, cardiac computed tomography, cardiac MRI (magnetic resonance imaging), coronary angiography and cardiac catheterization.

1.5. CLASSIFICATION OF HEART FAILURE

HF is classified as right/ left, acute/chronic, low beat/high beat, systolic/diastolic, ischemic/ non-ischemic heart failure [12].

1.5.1. Right/Left Heart Failure

In congestive HF, the theory is based on the fact that fluid accumulation occurs behind the affected region. Symptoms due to pulmonary congestion and pleural effusion are primarily associated with the left heart, while pretibial edema, hepatomegaly, and ascites are mostly the result of right HF. When the glomerular filtration rate and renin-angiotensin system activation decrease, fluid accumulation occurs. Decreased cardiac output decreases the glomerular filtration rate, increasing the release of renin and aldosterone. Hepatic insufficiency due to venous congestion and decreased blood flow affects aldosterone

metabolism, leading to a further increase in aldosterone. The result is water and salt retention.

1.5.2. Acute/ Chronic Heart Failure

The severity of clinical signs of HF and the frequency of symptom development depends on the availability of sufficient time for adaptive mechanisms to develop. For example, a sudden onset of anatomical or functional pathology (myocardial infarction, high ventricular response tachyarrhythmia, valve rupture secondary to infective endocarditis) in a previously perfectly normal person will produce a severe reduction in cardiac output, inadequate organ perfusion, or acute congestive symptoms behind the affected ventricle. However, when the same events occur over time, low cardiac output and anatomical anomalies will be tolerated for a long time with many adaptive mechanisms such as cardiac remodeling, and neurohormonal activation.

1.5.3 Low/High Beat Heart Failure

Low-beating HF at rest is a characteristic symptom of HF resulting from many cardiovascular diseases.

1.5.4. Systolic/ Diastolic Heart Failure

HF may occur as a result of impaired systolic function, which affects the pump function of the heart, or diastolic function, which affects its filling.

1.5.5. Ischemic/ Non-ischemic Heart Failure

HF is the most common cause of coronary artery disease in the population. Right-left, acute-chronic, systolic or diastolic heart failures may occur as a result of ischemia and infarction. The most important mechanism is myocardial necrosis resulting from myocardial infarction. In ischemic heart failure, it was evaluated that a history of myocardial infarction, chest pain, electrocardiographic findings of myocardial ischemia, and infarction and narrowing of epicardial vessels.

1.6. TREATMENT APPROACHES IN HEART FAILURE

Since blood cannot reach the tissues in the body of patients diagnosed with HF, oxygenation, delivery of nutrients, and the removal of waste materials from the existing tissue are interrupted. For this reason, diuretic drugs are given to the patients to remove the excess water and waste materials accumulated in the patients' bodies. Current therapies should focus not only on the symptomatic improvement of the disease but also on preventing HF progression and decreasing mortality. However, pure symptomatic treatments are generally more effective. Therefore, short-term and long-term goals should be determined for each patient. The goals of key treatment include cardiac remodeling, neuroendocrine and cytokine activation, fluid retention, and renal dysfunction.

Treatment options; general recommendations and measurements, exercise and exercise training, pharmacological treatment, tools, and surgery can be examined under Treatment options; general recommendations and measurements, exercise and exercise training, pharmacological treatment, tools, and surgery can be examined under non-pharmacological treatments, pharmacological therapy, devices, and surgery.

Treatment of Chronic HF with Drugs

Diuretics

- Thiazides
- Loop diuretics
- Potassium-sparing agents

Renin-angiotensin-aldosterone system inhibitors

- ACE inhibitors
- AT receptor blockers
- Aldosterone antagonists

Beta-adrenergic receptor blockers

- Cardioselective beta blockers
- Nonselective beta-blockers

Digital Glycosides

Direct Vasodilators

- Inorganic nitrates
- Hydralazine Calcium channel blockers
- Vasodilator prostaglandins

Natriuretic peptides

Neurohormonal inhibitors

Intravenous positive inotropic agents

- Dobutamine
- Phosphodiesterase inhibitors
- Dopamine

Cytokine Inhibitors

- Endothelin antagonists
- Neutral endopeptidase inhibitors
- TNF-alpha inhibitors

Supportive treatment

- Antithrombotic agents
- Antiarrhythmic drugs

Other

- Nesiritid
- Levosimendan

1.7. DIURETICS AND TYPES

Diuretics are among the drugs used for the treatment of patients with HF. Diuretics affect the reabsorption of sodium in the kidneys, and the internal balance of sodium and water, and thus blood volume and blood pressure are also affected. Because many HF patients have volume overload, the uses of diuretics are quite common.

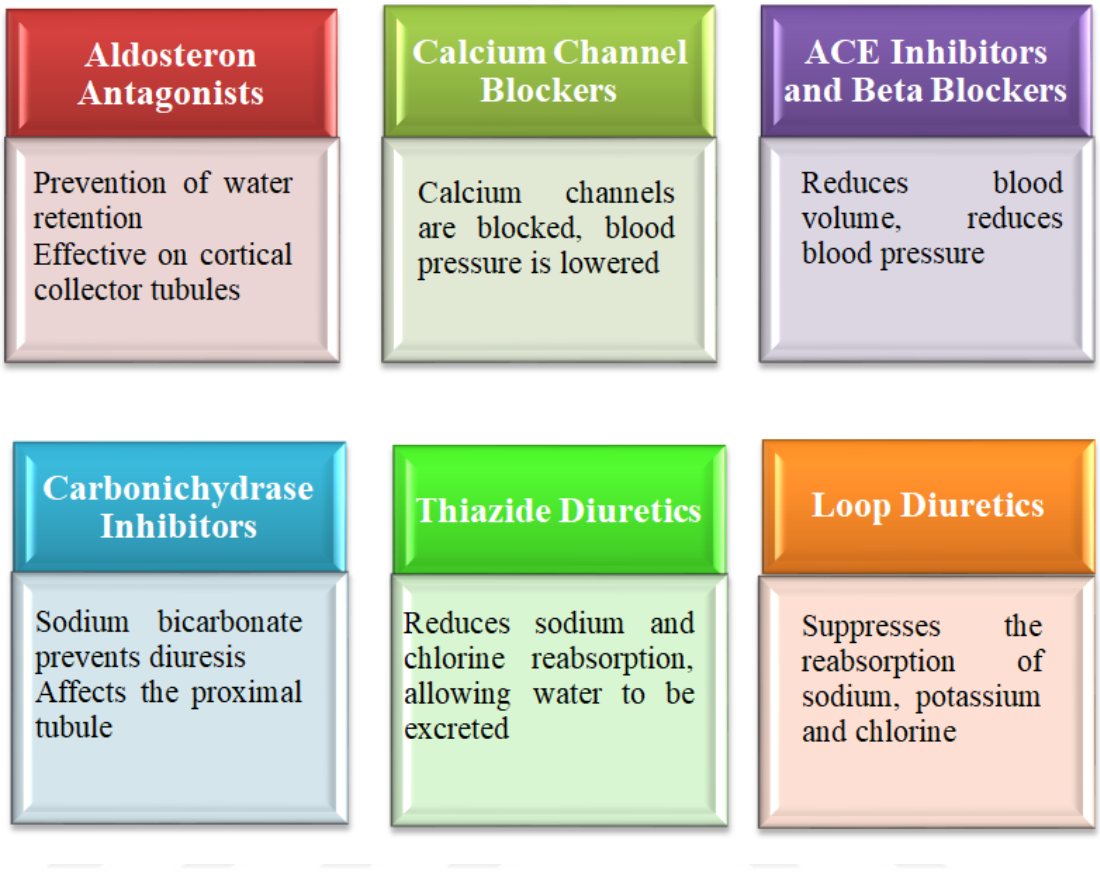


Figure 1. 1. Diuretic Types

Moderate HF can be treated with thiazide diuretics, but if HF worsens, loop diuretics are often required. All loop diuretics provide a similar increase in urine output when they use at equal doses. Doses of loop diuretics generally need to be increased in patients with severe HF.

1.7.1 Loop Diuretics

Loop diuretics reduce water and salt retention and decrease central venous pressure by 3-6 mmHg. Loop diuretics act on the ascending arm of the Henle handle in the nephrons. Loop diuretics bind to Na-K-2Cl (NKCC2) transport in this arm, blocking the reabsorption of sodium and chlorine across the apical membrane. 30% of the filtered sodium chloride is reabsorbed from the emerging thick arm of the Henle handle. The use of loop diuretics (eg. furosemide) is recommended in patients with HF. It is known that low-dose infusion therapy or oral therapy is preferable to intravenous high-dose bolus therapy [13]. Furosemide is a loop diuretic used to achieve normal plasma sodium concentration in patients with HF. Furosemide inhibits sodium and chloride reabsorption and Na + K + 2Cl

- co-transport mechanism in the rising part of the Henle handle. As a result, Na, Cl, and water excretion increases, as well as loss of Mg^{2+}, Ca^{2+}, and K^{+}. The cause of K^{+} loss is increased aldosterone and increased water and sodium flow in the distal tubule.

1.7.1.1. Furosemide

The accumulation of waste materials in the body causes water retention and consequently edema formation. The accumulation of waste materials prevents blood circulation in the body and this situation harms the heart. Thanks to its diuretic feature, furosemide allows the excess water and waste materials accumulated from the body to be removed from the body and relaxes circulation. For this reason, furosemide is among the diuretics frequently preferred in HF patients. As shown in Figure 1.2, the UGT enzyme encoded by the *UGT1A1* gene transfers glucuronic acid to furosemide and a glucuronidation reaction occurs. Thus, furosemide is metabolized in the body and then removed from the body [14].

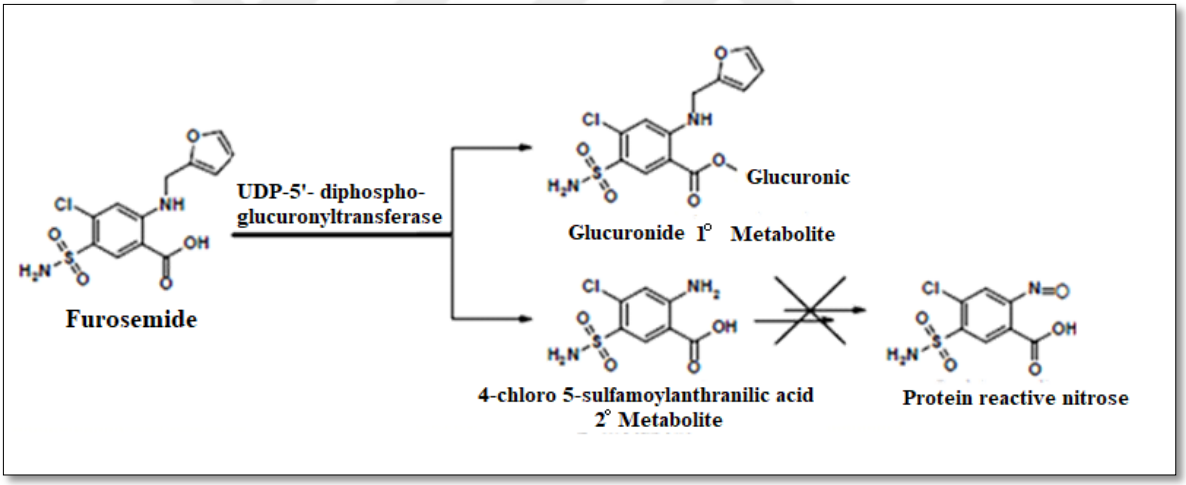


Figure 1. 2. Metabolites of Furosemide [14].

Furosemide is a loop diuretic used to achieve normal plasma sodium concentration in patients with HF. The effect of furosemide on the Henle Handle is shown in Figure 1. 2. Furosemide inhibits sodium and chloride reabsorption and the Na + K + 2Cl- co-transport mechanism in the rising part of the Henle handle. As a result, Na, Cl, and water excretion increases, as well as loss of Mg^{2+}, Ca^{2+}, and K^{+}. The cause of K^{+} loss is increased aldosterone and increased water and sodium flow in the distal tubule.

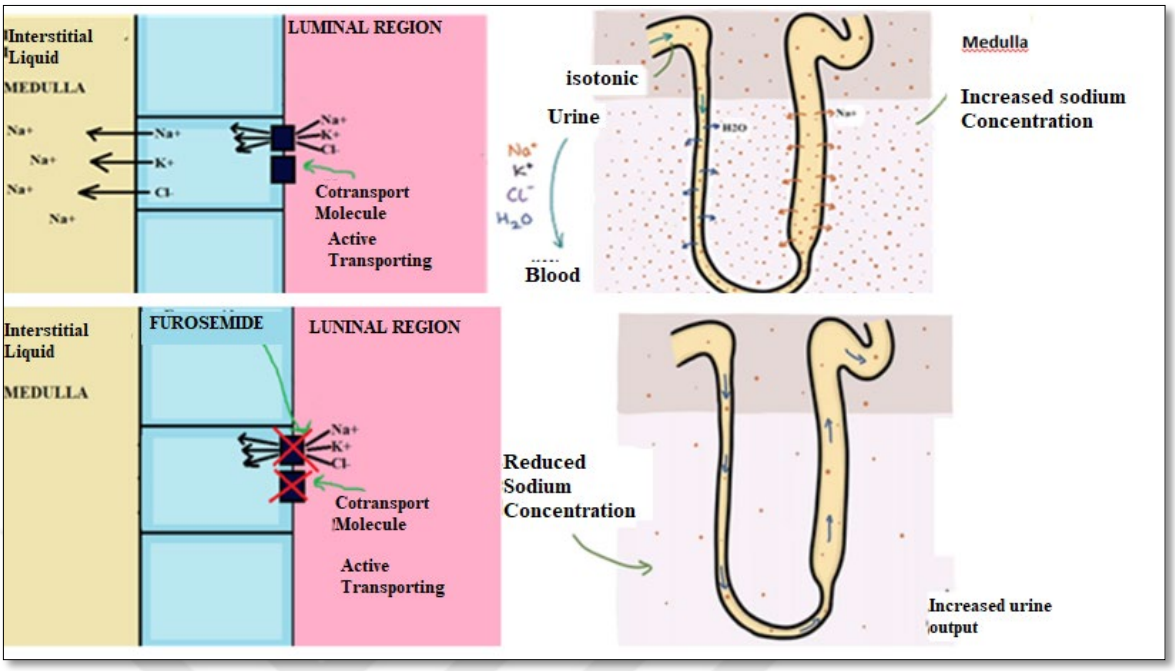


Figure 1. 3. Effect of Furosemide on Henle Handle Inspired [15].

Furosemide can cause hypokalaemia and decreased insulin activity in peripheral tissue and the release of insulin from pancreatic beta cells. When furosemide is administered orally, its effect begins 1-2 hours later and reaches its maximum effect within the next 1-2 hours. The effect of the drug lasts 6-8 hours. Oral bioavailability is up to 60% of the intravenous route [16].

1.8. DIURETIC RESISTANCE

Diuretic resistance is a condition in which the diuretic response is reduced or lost before the goal of edema treatment is achieved. Despite the liberal use of diuretics, the amount of extracellular fluid cannot be reduced. Decreased pharmacological response—decreased natriuresis against a given diuretic dose [17]. Diuretic resistance is the most common problem in the treatment of patients with HF. Individual differences in efficacy and toxicity of many drugs; drug-metabolizing enzymes, transport proteins, receptors, and gene mutations in other drug targets can cause diuretic resistance [16]. If a variation is seen more frequently than 1% in a normal population, it is called genetic polymorphism. Studies on such polymorphisms are among the study topics of the pharmacogenetic discipline. Pharmacogenetics is a science that investigates the interindividual changes which are

observed in drug response as a result of genetically controlled differences. Such studies help to administer drug doses at an optimal level based on the genetic structures of the patients [18]. It has been shown that diuretic resistance causes worsening of HF in patients, prolonged hospital stay, and increased mortality [19-20].

The development of diuretic resistance in the patient is decided by the clinician by evaluating the clinical status of the patient during routine check-ups or when a patient consults the physician with complaints. While the drug use is continued at a fixed-dose, the development of diuretic resistance can be detected in case the patient's HF condition worsens and the patient's symptoms increase, the need to increase the diuretic dose or the need to add different additional diuretic treatment alongside furosemide treatment. Furosemide dose is determined by the clinician according to the severity of symptoms of patients and response of patients to therapy. In patients with mild congestion findings, therapy is started with 40 mg of oral furosemide per day. If the severity of symptoms does not decrease during follow-up, the dose of the drug can be gradually increased up to a maximum of 400 mg daily. Diuretic therapies also can affect renal functions. Because of the side effects of diuretics on renal functions, renal function tests are performed on patients by clinicians. If an abnormality in renal function is detected, the diuretic dose is reduced. While the patients were stably followed up with previous doses of furosemide, in case of increased new-onset symptoms, it is thought that diuretic resistance developed by the clinician.

Valente MA. et al. and Testani JM. et al. have associated poor diuretic response or non-response to a diuretic with advanced (decompensated) HF [21-22]. In both studies, the poor diuretic response has been associated with renal dysfunction and higher blood urea nitrogen levels. In this case, the oral furosemide dose taken by the patient is increased in the first stage but there is often no response to the treatment due to widespread edema in the digestive system. In this situation, the furosemide dose is given intravenously way and if it is necessary, clinicians can be increased the dose to the maximum dose (400 mg). However, if it is not successful despite the increasing dose via intravenous way, different diuretic treatments are added (such as thiazide, spironolactone, and torsemide). When retreatment is not successful, dialysis treatments are applied to the patient in the clinic. Furosemide resistance development will be identified in the clinic not only with the patient not being treated with medication but also, with worsening of the disease during drug use,

Persistent congestion with >80 mg furosemide per day despite adequate and increasing diuretic doses [23] urinary sodium excretion <0.2% [24], failure to excrete at least 90 mmol sodium within 72 hours of an oral dose of 160 mg twice-daily furosemide [25], net fluid loss per milligram of furosemide (40 mg furosemide or equivalent) during hospitalization [22]. Clinicians will decide on furosemide resistance by urinalysis and evaluation of kidney functions in patients who participated in the study and are thought to develop diuretic resistance. Factors that are responsible for the development of diuretic resistance include neurohormonal activation, rebound absorption of sodium after volume loss, flow hypertrophy in the distal nephron, renal failure, decreased renal perfusion, incompatibility with drugs, and excess sodium. [26]. In some of the heart failure patients using furosemide, the fact that the drug does not work sufficiently (at the optimal dose), in other words, the formation of diuretic resistance is one of the most frequently encountered problems [27], [28], [29].

1.9. GENETIC POLYMORPHISMS

Genetic polymorphisms are changes in a gene or DNA sequence with a high frequency of being in society. The feature that distinguishes these changes in the DNA sequence from mutation is that it is present in at least 1 out of 100 people. The most common type of genetic polymorphism is single nucleotide polymorphisms containing single base pair variation [30]. Single nucleotide polymorphisms (SNP) are DNA sequence changes that occur when a single nucleotide (A, T, C, or G) is replaced by another in the genome. For this single nucleotide change to be expressed as SNP, it must be seen in at least 1% of the population. If the incidence is less than 1%, this variation is considered a mutation. SNPs include base changes such as transitions (changing of one purine base with another purine base (A → G, G → A) or one pyrimidine base with another pyrimidine base (C → T, T → C) and transversions (the replacement of a purine base with a pyrimidine base) [31].

1.9.1 Uridine 5'-diphospho-glucuronyltransferase (UGT)

Uridine 5'-diphospho-glucuronyltransferase (UDP-5'-diphospho glucuronyltransferase, UGT) is a phase II drug-metabolizing enzyme that catalyzes the glucuronidation of compounds by transferring glucuronic acid from UDP-glucuronic acid to substrates. The

Uridine 5'-diphospho glucuronyltransferase 1 (*UGT1A1*) gene encodes the UGT enzyme. The *UGT1A1* gene contains 5 exons and is located on 2q37 [32].

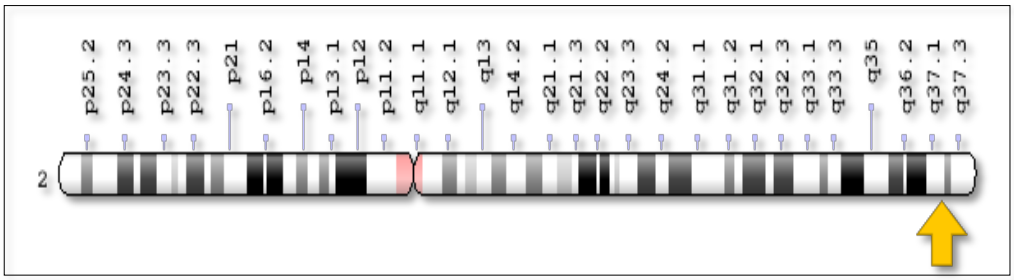


Figure 1. 4. *UGT1A1* Gene Location [33].

UGT1A1 is related to serum bilirubin levels [34]. The *UGT1A1* gene encodes a liver-specific glucuronosyltransferase (UGT) that converts bilirubin into a more water-soluble form. The *UGT1A1* gene encodes the UGT enzyme, performing a chemical reaction called glucuronidation. The *UGT1A1* gene prevents the accumulation of toxic waste in our body by providing the metabolism of drugs. Some genetic variations in *UGT1A1* reduce the enzymatic activity of UGT, which impairs the body's ability to detox and causes toxic substances to accumulate in the body. *UGT1A1*28* is one of the primary genetic variants responsible for reduced detoxification ability. These variations are rs4148323 and rs6742078. Since the mutations are found especially in the Exon 1 which is the largest region of the gene, we examined this region by direct sequencing in our study.

1.9.2. Interleukin- 6 (IL-6)

Intercellular active substances, some of which are formed by lymphocytes (lymphokines) and some of them are formed by monocytes and macrophages, are called interleukins. Interleukin-6 (IL-6) is a pleiotropic cytokine that is produced by lymphoid and non-lymphoid cells. [35]. *IL-6* starts leukocyte infiltration; however, extended inflammation can turn into a devastating response that causes tissue fibrosis. *IL-6* acts via the *IL-6* receptors *(IL-6R). IL-6R* is determined in some cell types such as hepatocytes or leukocytes. [36]. *IL-6* gene is located on the 7p15-p21 chromosome in humans [37-38], contains 5 exons and 4 introns [39]. Expression of the *IL-6* gene is particularly effective in autoimmune diseases, plasmacytoma-myeloma, and chronic proliferative inflammatory diseases. Irregularity in the production of *IL-6* is responsible for the formation of many diseases such as inflammation, autoimmune diseases, and malignity [40].

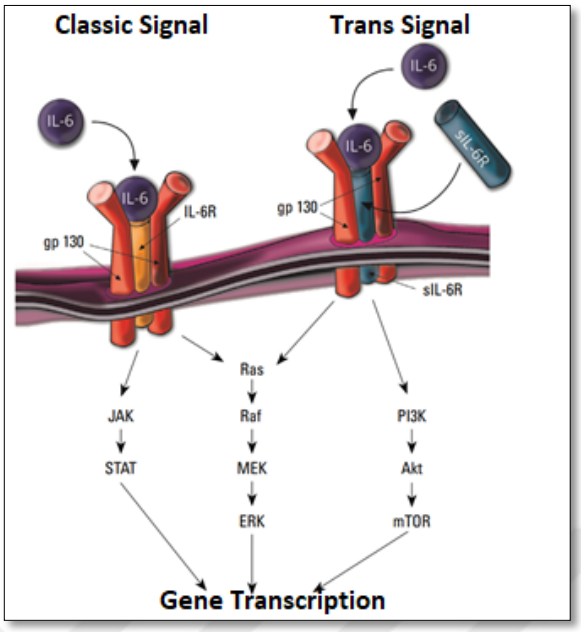


Figure 1. 5. *IL-6* signal pathway [41].

The classic signal pathway of *IL-6* is anti-inflammatory. In the classic signal pathway, *IL-6* binds to *IL-6R*. Following this connection, the gp130 subunit is connected to *IL-6 / IL-6R* to create a complex. Gp130 receptor subunit activates Janus kinase/signal transducer and transcription activators which are very important in the development of heart failure, 1/3 (JAK / STAT1 / 3) pathways, and Ras / mitogenic protein / extracellular signal regulator kinase (Ras / MEK / ERK1 / 2), phosphatidylinositol -3-kinase (PI3K) / Akt pathways [42]. Janus kinase is a family of tyrosine kinases associated with the cytoplasmic domain of growth factor receptors and cytokines. Upon activation, JAK proteins phosphorylate STAT proteins and incorporate STAT proteins whose phosphorylation causes homodimerization and migration to the mitochondria or nucleus into the JAK-STAT signal transduction pathway process [43]. STAT3 activates the Survivor Activation Factor Enhancement (SAFE) pathway which is a true protective signaling program that triggers ischemic post-condition that limits heart-induced cell death and prevents reperfusion injury from occurring. *IL-6* signal pathway and its " downstream" effector molecules are shown in Figure 1.5.

The Trans signal pathway is pro-inflammatory *IL-6* binds soluble *IL-6* receptors *IL(sIL)-6R* in this pathway. *IL-6/sIL-6R* binds to the *IL-6* non-expressing subunit of Gp130 [32]. *IL-6* inhibits the sarcoplasmic reticulum Ca^{+2} ATPase (SERCA2) associated with transcriptional

mechanisms and decreases the expression of alpha myosin light chain and cardiac alpha-actinin [44]. It is shown that *IL-6* and *sIL-6R* levels increase in patients with heart failure [45].

It was determined that the *IL-6R* level was higher in the high majority of patients than the *sIL-6R* level [46]. Studies have uncovered overexpression of genes in patients with ischemic and non-ischemic end-stage heart failure [47]. *IL-6* concentration depends on the etiology. In patients with HF caused by ischemic heart disease and cardiomyopathy, it has increased significantly compared to those affected by valvular heart disease or hypertensive heart disease [48].

Loss of gp130 causes vascular dilation caused by high-pressure loading [49]. However, overexpression of gp130 causes ventricular hypertrophy throughout the trans signal pathway process [50]. Activation of the "Survivor" Activation Factor Development (SAFE) pathway is required for pre and post-ischemic conditions. İschemic post-condition induces activation of JAK1, JAK2, STAT1, and STAT3, and inhibition of this pathway leads to the development of ischemic tolerance [51]. Loss of STAT3 results in neovascularization changes, the onset of fibroblast proliferation, development of heart failure, increased mortality, myocardial ischemia/reperfusion injury, increased sensitivity to infarction, higher apoptotic cell speed, and increased infarction size [52].

A similar effect was achieved in *IL-6*-silenced mice in which the ischemic post-conductive activation of the JAK / STAT was "downregulated" and the infarct size was not decreased [53]. Chronic activation of STAT3 along *IL-6* and gp130 in subacute vascular occlusion is responsible for inflammation, development of heart failure, and left ventricular ruptures [52]. The effect of *IL-6* can be both positive and negative depending on the duration of this cytokine activation. Synthesis of *IL-6* can protect myocardia for a short time after healing injury. Long-term synthesis and over-production of *IL-6* cause the development of ventricular dilatation and heart failure. While classical membrane-bound signal is associated with the anti-inflammatory effect of *IL-6,* the trans signal pathway mediates the path that causes harmful inflammation [52]. Heart failure is associated with the broken of the *IL-6* signal pathway. It's detected that variations of genes in the IL-6 signal pathway (*IL-6, IL-6R, IL-6ST*) are associated with coroner diseases and heart failure [54]. Hansen and coworkers studied to understand the *IL-6* pathway in patients with heart failure and analyzed *IL-6, IL-6R,* and *IL-6ST* varients. In the study, it has been shown that variations

occurring in the *IL-6* signal pathway may have a fatal effect in patients with severe HF [55].

Studies have shown that variations in the *IL-6* gene are effective in the development of cardiovascular diseases. There have been studies showing an association between heart failure, especially rs1800795 and rs1800796 polymorphisms. [54], [55]. GC nucleotide change in the promoter region of the *IL-6* gene at the -174 position and CG nucleotide change in the *IL-6* gene at the -572 position have a functional significance. Studies showed that there is a relation between IL-6 rs1800795, rs1800796 variations and cardiovascular diseases [54], [55].

In our study, we observed IL-6 polymorphisms and we showed that rs1800795 and rs1800796 caused the base change.

2. MATERIAL

2.1. MATERIALS

- Ethanol, Absolute, Sigma Aldrich, St. Louis, USA
- MicroAmp Fast Optical 96-Well Reaction Plate (0.1 mL), Applied Biosystems, Foster City, CA, USA
- Optical Adhesive Covers, Applied Biosystems, Foster City, CA, USA
- Sterile Filtered Pipette Tips 1000, 200, 10 µL, Capp, Nordhausen, Germany
- QIAamp DNA Blood Mini Kit, QIAGEN GmBH, Hilden, Germany
- Taqman SNP Genotyping Assays, Applied Biosystems, Foster City, CA, USA
- Taqman Universal Master Mix, Applied Biosystems, Foster City, CA, USA
- PureLinkTM Genomic DNA Mini Kit, 250 preps, Thermo Fisher, USA
- GeneRuler 100 bp DNA Ladder, Thermo Fisher, USA
- GeneRuler 50 bp DNA Ladder 50 µg, Thermo Fisher, USA
- PRIMER 100 NMOL 100 NMOL, Thermo Fisher, USA
- Taq DNA Polymerase, recombinant (5 U/µL) 500 units, Thermo Fisher, USA
- dNTP Set 100 mM Solutions 4 x 0.25 Ml, Thermo Fisher, USA
- 50X TAE ELECTROPHORESIS BUFFER EA, Thermo Fisher, USA
- Ethidium bromide, Sigma, USA
- Isopropanol, Sigma, USA
- Magnesium Chlorure Sigma, USA
- Proteinase K, Roche, Sweden
- Taq DNA Polymerase Enzyme Set, MBI Fermantas, Lithuania

2.2. INSTRUMENTS

- Real-Time PCR System 7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA
- Centrifuge, Spectrafuge 24D, Labnet International, Inc
- Heater, Bioer

- Nanodrop™ 2000/C Spectrophotometer, Thermo Scientific, Rockford, IL, USA
- Micro Pipettes (1000, 200, 20, 10 µL), Thermo Scientific, Rockford, IL, USA
- Vortex, IKA
- Water Bath, (Germany)
- Agarose Gel Electrophoresis Power Supply, EC105 Apparatus Corporation (USA)
- Agarose Gel Tank and Assembly EC105 Apparatus Corporation (USA)
- Thermal Cycler, BioRad, U.S.
- Refrigerator, Arcelik (Turkey)
- Freezer -20°C Bosch (Germany)
- Oven, Heraeus (Germany)
- Precision Balance, Mettler AT 261 (Switzerland)
- Heat Cycle Device, Techne (England)
- UV Source, Vilber Lourmat (France)
- Gel Documentation System, Biorad, U.S

2.3. PRIMERS USED IN STUDY

Primers were taken in lyophilized form and diluted with sterile distilled water to form the main stock and stored at -20°C. The solutions prepared from these stocks at a concentration of 10 pmol/µl were used in PCR reactions. PCR and sequence analysis primers are shown Table 2.1.

Table 2.1. Primers used for PCR and Sequence analysis

	DNA Sequence (5'-3')
***UGT1A1* Gene**	5'-primer 5'- TATATAAGTAGGAGAGGGCGAACCT -3′ 3'-primer 5'- AAAAGTCCCACTCCAATACACA -3′

3. METHODS

3.1. SELECTION OF STUDY POPULATION

Sixty heart failure patients using >40 mg furosemide/day and 30 healthy individuals were enrolled in this study as patient and control groups, respectively. Individuals who have heart failure symptoms and are diagnosed with low ejection fraction heart failure (systolic heart failure) were selected for the patient group. Patients are then divided into two sub-groups as Group 1 (non-responders) and Group 2 (responders). Group 1 patients are those who develop furosemide resistance and do not respond to drug therapy (n=30). Group 2 patients are those who respond to drug therapy (n=30). Healthy volunteers were enrolled to the control group (Group 3)(n=30).

For evaluating heart failure condition, the cardiologist performed a physical examination, electrocardiogram, blood gases level, laboratory (B-type natriuretic peptide (BNP), creatinine, sodium (Na), potassium (K), chest graphic, cardiac doppler- echocardiography, pulmonary angiography, thorax computed tomography, exercise test, heart scintigraphy, Holter test, cardiac catheterization, coronary angiography, and endomyocardial biopsy.

The inclusion and exclusion criteria are listed below.

Inclusion criteria of the study:

• Ninety volunteer patients diagnosed with HF and using furosemide will be included in the study.
• Thirty healthy volunteers will be included in the study.
• Individuals participating in the study will be selected from people between the ages of 18-90.

Exclusion criteria of the study:

• Persons not between the ages of 18-90 will not be included in the study.
• Patients who have not been diagnosed with HF will not be included in the study.
• Patients who do not use furosemide will not be included in the study.
• Patients who do not agree to participate in the study will not be included in the study.

3.1.1. Biochemical Parameters in Blood

Blood samples taken from patients who voluntarily participated in the study were examined in the laboratory. Blood Urea Nitrogen (BUN), creatinine, hematocrit (HTC), hemoglobin (HGB), sodium (Na), potassium (K), and ventricular natriuretic peptide (NT-proBNP) values of the patients were determined by autoanalyzer.

3.2. DNA ISOLATION FROM PERIPHERIC BLOOD

Whole blood samples were taken from all patients and volunteers. DNA was extracted from peripheric blood using commercially available kits (Qiagen, Germany) according to the manufacturer's instructions [56].

The stages of the method are;

- 200 µl of blood was put into 1.5 ml Eppendorf.
- 200 µl of Buffer AL and 20 µl of Proteinase K were placed in Eppendorf.
- Eppendorf was mixed for 15 seconds.
- Incubated for 10 minutes at 56ºC
- After incubation, the tube was centrifuged in a microcentrifuge.
- The mixture was taken from 1.5 ml of Eppendorf and transferred to the filtered column.
- Centrifuged at 6000g (8000 rpm).
- The filtered column was separated and the filtered part was placed in the new tube, and the separated tube was discarded.
- 500 µl of Buffer AW1 was added.
- It was centrifuged at 6000g (8000 rpm) for 1 minute.
- The filtered column was separated and the filtered part was placed in the new tube, and the separated tube was discarded.
- Added 500 µl of Buffer AW2.
- It was centrifuged at 20000g (14000 rpm) for 3 minutes.
- The filtered column was separated, the remaining liquid in the lower tube was poured, and the filtered part was placed in the same tube.

- It was centrifuged at 20000g (14000 rpm) for 1 minute.
- The filtered column was separated and the filtered part was placed in the new tube, and the separated tube was discarded.
- Added 100 µl of Buffer AE.
- Waited 2-3 minutes.
- Centrifuged at 6000g (8000 rpm).
- The filtered column was discarded and the lower tube was removed.
- Isolated DNA is in the bottom tube

NanoDrop spectrophotometer used for detection of DNA purity and concentrations (Thermo Scientific, Wilmington, USA). To detect the concentration of the isolated DNAs, 1 µl of isolated DNA was used. Measurements were made in the spectrophotometry at wavelengths of 260 and 280 nm. The solution was considered to contain 50 µg/ml of double-stranded DNA with an optical density (OD) value of 1 at a wavelength of 260 nm. Using the OD value at 260 nm, the concentrations of the samples were calculated using the following formula:

DNA Concentration = OD260 x 50 µg/ml x dilution rate

The purity of DNA samples was evaluated using the OD260/OD280 ratio. The OD260/OD280 value of well-purified DNA is considered to be approximately 1.8.

3.3. POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) was developed by Kary Mullis in 1988 [57]. A DNA sequence can be reproduced in desired quantities by this method with chemical reactions in in-vitro conditions. PCR reactions form 3 basic steps:

1. **Denaturation:** Double-stranded DNA is denatured and heated until it becomes single-stranded. This process takes approximately 5 minutes at 90-95^{o}C.
2. **Annealing:** Primers bind to single-standard DNA by reducing the temperature to 50- 70^{o}C. These primers are artificial oligonucleotides. Lengths of these are approximately 15-30 nucleotides and bind specifically to complementary sequences

at the ends of the DNA segment to be replicated. These primers serve as the starting point for the synthesis of template DNA.

3. **Extension:** Taq polymerase enzyme which obtains from "*Thermus aquaticus*" bacteria add the PCR mix. DNA synthesis performs at 70-75°C. Polymerase enzyme adds the nucleotides in 5' to 3' directions and provides primer extension. And a double-strand copy of the target DNA is formed.

Our PCR protocol was shown in Figure 3.1. At end of the these 3 steps, the cycle is completed. The number of DNA strands doubles with each cycle, and new strands act as templates for the next cycle. The PCR cycle is repeated many times. At the end of 25-30 cycles, the amount of DNA increases approximately 1,000,000 times. The process is carried out automatically in machines called heat cyclers, with programs that pre-set the number of cycles and temperatures. This process was carried out in 0.2 ml eppendorf tubes and the determined program was applied by placing the tubes in the thermal cycler (Figure 3.1.).

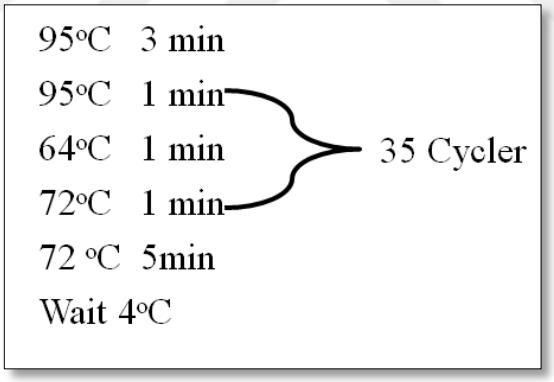


Figure 3.1. PCR Protocol

The first exon of *UGT1A1* was obtained after polymerase chain reaction, and amplicons which have 932 bp length were analyzed by agarose gel electrophoresis.

PCR mix was prepared for PCR. The volume of the mix is shown in Table 3. 1.

The mixture is calculated as the number of samples + negative control based on the 1 volume values given in the table. The mixture was prepared with the new values obtained by multiplying the total number of samples with the values in the table. The resulting

mixture was transferred to eppendorfs which were prepared for the samples in equal volumes of 45µl. And finally, 5µl of sample DNA was added to the eppendorfs.

Table 3.1. The volume of PCR mix

For 1X	Volume
Nuclease Free Water	33,6 µl
Buffer	5 µl
MgCl2	3µl
dNTP	1µl
F	1µl
R	1µl
Taq Pol.	0,4µl

3.4. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is used generally for the separation of DNA fragments, determination, and purification. DNA and RNA molecules between 200-50,000 bp sizes are determined with agarose gel electrophoresis. Agarose is a polysaccharide and it isolates from Agar-agar which is a type of red alga. The first agarose dissolves in hot water and when it is cold, a gel structure is formed by the formation of mutual hydrogen bonds in the polymer. The pore diameter can be adjusted by changing the agarose concentration between 0.5 and 1.5%. The running of DNA in the gel can be achieved by using a high concentration of agarose for small DNA fragments and low for large DNA fragments. The most effective agarose concentration is 0%.3-2 for the separation of nucleic acid. DNA is viewed on the gel by the fluorescent effect of ethidium bromide by binding between DNA bonds and absorbing light at 300-360 nm. DNA bands are detected under UV light. A marker that has a known size uses to detect the molecular size of DNA.

In this study, we used agarose gel electrophoresis to detect the PCR products. After PCR, we performed 2% agarose gel electrophoresis for imaging of amplified DNA (932 bp). 2% agarose gel was boiled in 1X TBE buffer and 0.25 µg/ml EtBr was added to the solution to view the DNA under the UV light. A gel pattern was prepared and the gel was kept in room temperature conditions until polymerization. After the polymerization, it was placed

in a gel tank which was filled with 1XTBE. 10 µl PCR products were mixed with loading dye and were loaded wells on the gel (Figure 3. 2.).

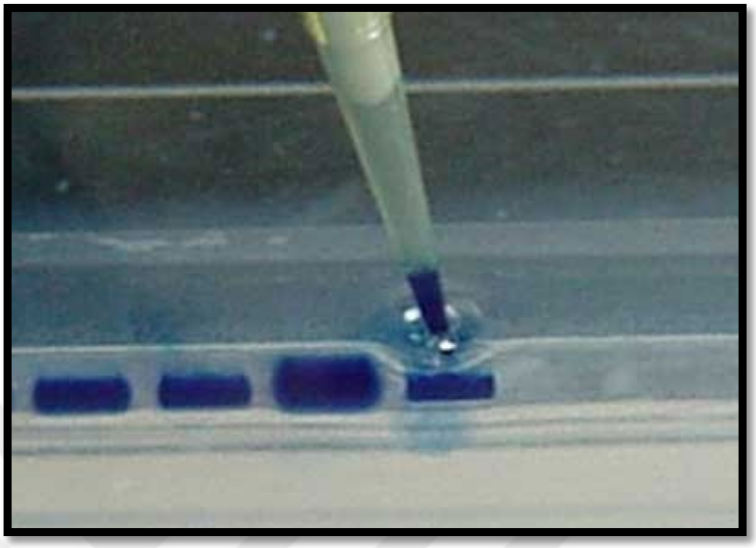


Figure 3.2. Agarose gel Loading

100 bp ladder (Promega) was loaded in the first well as a marker. Electrophoresis was applied as 90 V/45 mA. The gel was examined under the UV light after 45 min. PCR bands were viewed under UV light and were compared with the marker. And gel photos were taken by the gel imaging system (Figure 3.3.).

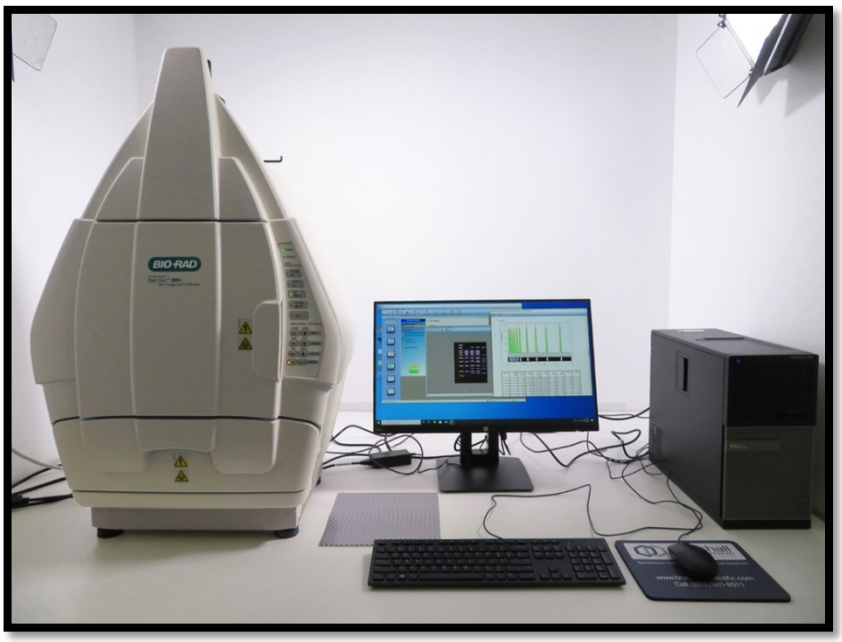


Figure 3.3. Gel imaging system

3.5. SANGER SEQUENCING

After obtaining accurate PCR results, DNA sequencing was performed with PCR products for mutation analysis. DNA sequence analysis is a method that uses for the detection of the nucleotide sequence of DNA. There are two classic DNA sequence analysis methods. While the Method of Allan Maxam ve Walter Gilbert is a chemical method that is about the breaking of DNA from certain sequences, the Method of Fred Sanger and coworkers perform the synthesis of a DNA strand that terminates at a certain base. Four different reactions are applied in both two methods. Products of four different reactions are decomposed into four different wells in the gel by electrophoresis and a sequence of DNA fragments can be read from bands in the gel.

In the Sanger sequence analysis method, the DNA chain is used as a pattern for the newly synthesized DNA chain. Synthesis is catalyzed by the DNA polymerase 1 enzyme. A sequence of DNA fragments is obtained by using modified dideoxynucleotide triphosphates in the method.

In the Sanger method, four different reaction mixes prepare in the experiment. Each of the reaction mixes includes DNA, a primer, four radioactive nucleoside triphosphates, and one of the dideoxyribonucleoside triphosphates. Because only a small amount of modified a nucleoside is used in each reaction, the new strand ends at random and a series of DNA fragments occurs. After synthesis, radioactive DNA fragments from the four reactions are run in four separate wells side by side in the electrophoresis gel. DNA bands are imaged with autoradiography and the nucleoid sequence is determined by reading the gel from the bottom up [58].

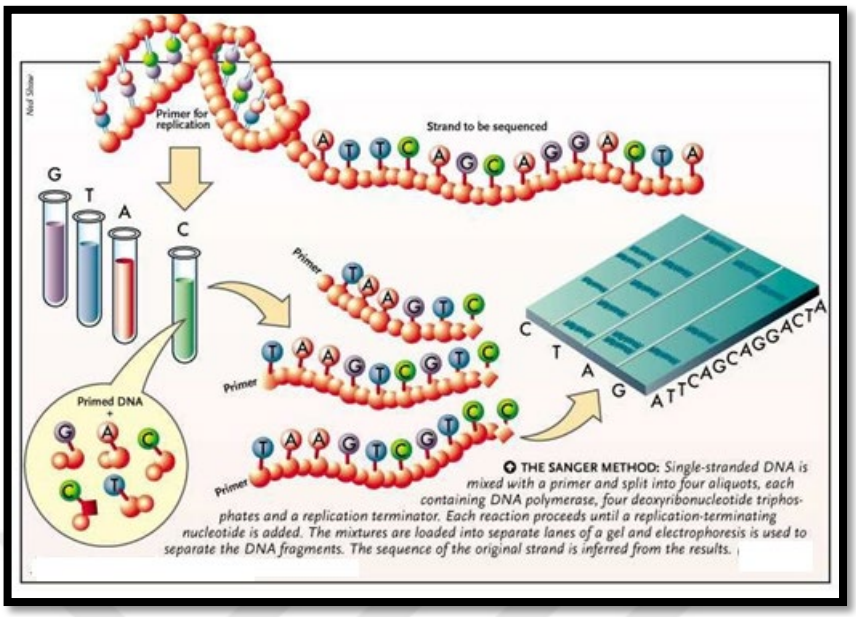


Figure 3.4. Sanger sequence analysis method [59].

In our study, 1st exon of the *UGT1A1* gene was investigated by the direct sequencing method [60]. Then direct sequencing results were compared with human genomic DNA sequence in the gene bank.

3.6. REAL-TIME POLYMERASE CHAIN REACTION

rs1800795 and rs1800796 variations in *IL-6* gene were analyzed by Applied Biosystems 7500 Fast Real-Time PCR (Applied Biosystems™, Foster City, CA, USA).

In this study, the detection of rs1800795, and rs1800796 variations in the *IL-6* gene were performed using a real-time polymerase chain reaction (RT-PCR) device, using "assays" specially designed for each mutation. The product codes of the variations to be studied are shown in Table 3.2.

Table 3.2. Codes of variations

Gene and variation names	Analyze Code
IL-6- rs1800795	C_1839697_20
IL-6- rs1800796	C__11326893_10

The protocol process is stated below.

1. Reaction mixes which included rs1800795 and rs1800796 probes were prepared for patients and control samples.
2. 9 µl reaction mixes were added to a 96-well plate for all wells.
3. DNA samples of patients and controls were added into the wells (1 µl DNA samples for each patient). Volumes of RT-PCR mixes are shown in Table 3.3.

Table 3.3. Preparation of Real-Time PCR Mix

Component	Volume (1X)	90X
Water	3,75 µl	337,5 µl
Master Mix	5 µl	450 µl
Assay	0,25 µl	22,5 µl
Template	1 µl	-

4. No template control (NTC) was added into a well.
5. The PCR program was set up according to the protocol.
   The protocol is shown in Table 3.4.

Table 3.4. Real-Time PCR program

Step	Temperature	Time
Holding stage	60°C	10 min
Denaturation	95°C	10 min
Annealing	95°C	15 min
Extension	60°C	1 min

The software of the system presented allelic discrimination of each polymorphism.

3.7. *IN SILICO* ANALYSIS

In the study *in silico* analysis was performed that C1, C15, and C26 individuals from the control group and D12, D15, D18, D22, and, D25 patients from the non-responders group. Y163C, S250C, L197P, V225G mutations were detected in C1, C15 and, C26 individuals from the control group respectively and, M213V, E283P, P270L, P270L, V81E mutations were detected in D12, D15, D18, D22, and D25 patients from non-responders group.

In silico means “performed on the computer or through computer simulation”. The design of drug molecules, homology modeling, and molecular docking studies are mostly performed *in silico* in today's technology [61].

3.7.1. Homology Modeling

Homology modeling is the three-dimensional (3D) modeling of amino acid sequences based on the structures and domains of proteins whose models have been demonstrated by experimental methods. Homology modeling benefits the reality that the evolutionarily related proteins which have similar sequences, as measured by sequence identity, have similar structures [62].

The Qualitative Model Energy Analysis (QMEAN) value determines the model quality in homology modeling. Qualitative Model Energy Analysis (QMEAN) is a compound calculation function that estimates model quality [63]. Statical calculations of QMEAN value are made by four different factors. The first factor is the C-β interaction distance term, the second factor is the all-atom interaction distance term, and the third factor is the torsion term which means that the torsion potential of 3 consecutive amino acids for analysis of the regional spine geometry of the structure, and the last factor is the solvation term that is necessary to evaluate residues levels. 3D models are selected according to the QMEAN values. QMEAN value of an ideal model is near the 1 [63].

SWISS-MODEL uses for homology modeling studies. The Swiss Institute of Bioinformatics (SIB) is a research institute in Switzerland. It developed a bioinformatic portal that is the Expert Protein Analysis System (ExPASy) for homology modeling [50]. ExPASy makes matches with sequences of models in its database to create the 3-dimensional structure of a protein and create estimate models at the end of the matching. SWISS-MODEL presents the report to the users about its results. Templates can download as a “.pdb” document [64], [65]. Rasmol [66], PyMOL [64] ve UCSF Chimera [65] programs are used for visualizing of “.pdb” files. UCSF Chimera is one of the most preferred programs due to its advantages. Automatic atom types determination, the hydrogen added, distance, angle, surface area, volume measurement, calculation of protein Ramachandran plots, calculation of root mean- square deviation (RMSD) value, protein theme map, superposition of proteins, demonstration of hydrophobicity, PDB and Mol2

representation of formats are made by Chimera. Model visualization can be done in three different ways as ribbon, hydrophobicity surface, and all atoms in the program. Ribon type is usually preferred to observe the differences. All atoms selection shows the whole atoms on the model and the hydrophobicity surface shows the hydrophobicity feature of the model [65].

Swiss-Model (www.swissmodel.expasy.org) database was used for the homology modeling study. Amino acid sequences of wild and mutant types loaded on the system and 3D structures were obtained. QMEAN values of models were calculated and among the 3D models, the model with the highest QMEAN value was selected and used as a model. Wild and mutant protein structures were visualized with ribbon selection by Chimera tools and structural differences of mutant proteins were shown.

3.7.2. Hydropathy

The hydrophobic image of the models was visualized by the Chimera program and it was interpreted according to the hydropathy index.

3.7.3. Physico-chemical Calculation

Physico-chemical properties are calculated for understanding the functional diversity of a protein. Therefore the ProtParam program was used for Physico-chemical calculations. In this study, wild and mutant types FASTA formats which were obtained on the GeneBank database were used for physico-chemical calculations on the ProtParam program. In the scope of the ProtParam program, amino acid number, molecular weight, theoretical pI value, the percent composition of amino acids, total number of negatively charged residues, the total number of positively charged residues, atomic composition, formula, total atomic number, molar damping coefficient, aliphatic index, and average hydropathy were calculated for each model.

3.7.4. Molecular Docking

Molecular docking (MD) is a computational procedure that efficiently predicts the receptor and ligand or non-covalent binding of macromolecules, starting from their unbound structures, structures which are got from MD simulations, or homology modeling. MD aims is to estimate the bound conformations and binding affinity. This information has used the discovery of new drug molecules [67]. Molecular docking studies can be performed between protein-ligand, protein-protein, protein-peptide, protein-nucleotide, and nucleotide-ligand structures. Before the docking studies, the molecular structure of the ligand molecule and protein/nucleotide structure should be detected by X-Ray Crystallography or NMR spectroscopy method and this structure should be found as data in the computer. Data files should be found in the computer environment with ".pdb" or ".pdbqt" extensions [67].

The HDOCK (http://hdock.phys.hust.edu.cn/) is a macromolecular docking, protein-protein docking, and homology modeling server. HDOCK server is automatically predicted receptor and ligand molecule interactions. This server uses amino acid sequences. Wild and mutant type FASTA sequences and ligand molecules are loaded on the server. Wild and mutant type protein structures and ligand molecules also can be uploaded to servers as ".pdb" files. HDOCK server gives templates and docking-based binding models. Model interactions are visualized. This server allows free download. HDOCK completes a docking study within 30 min.

3.8. STATISTICAL ANALYSIS

Statistical analyses were performed using IBM SPSS (Statistical Package of Social Sciences, Version 25.0. Armonk, NY: IBM Corp. Variables were expressed using median (min-max), percentage, and frequency values. Variables were evaluated after checking the prerequisites of normality and homogeneity of variance (Shapiro-Wilk and Kolmogorov Smirnov tests). While performing data analysis, the independent samples t-test (Student's t-test) was used for the comparison of two groups, and the Mann Whitney-U test when the prerequisites were not met. Kruskal-Wallis tests were conducted to compare the three independent groups. The Chi-Square and Fisher's exact test, where appropriate, was used

to compare the proportions of the groups. Logistic regression analysis was performed for calculating risk values (Odd ratio) between responders/non-responders groups and patient/control groups. Independent sample t-test and Mann-Whitney U test were used to evaluate the relationship between risk factors and genotypes. p values less than 0.05 were considered as statistically significant.

4. RESULTS

4.1. CLINIC DATA

Baseline characteristics are shown in Table 4.1.

Table 4.1. Baseline characteristics of study population

Sample name	Age	Gender	BUN	NT-proBNP	Na	K	HBG	HCT	Creatinine
N1	63	M	31	1494	134	4,6	15,2	43,5	1,07
N2	44	M	17	2290	137	4,8	15,7	47,7	0,91
N3	75	M	21	2911	136	4	16,3	50	1,14
N4	73	M	18	392,1	141	4,4	10,5	32,5	0,64
N5	71	F	22	6054	141	4,7	11,9	38,1	1,38
N6	44	M	11	1180	138	3,8	13,6	38	0,83
N7	79	M	45	1020	134	4,9	12,4	37,4	2.22
N8	55	M	35	432,1	134	5,4	11,3	33,5	0,72
N9	78	M	30	530,8	136	5,3	10,2	36,2	0,86
N10	62	F	15	1100	142	4,8	12,2	38	1,24
N11	69	M	36	6187	153	4,3	12,4	30,6	2,38
N12	71	F	28	14432	137	3,6	9,4	35,4	1,07
N13	75	F	20	241	136	3,7	12,1	35	1,15
N14	82	F	20	6277	137	5	12,3	40,2	1,4
N15	36	F	13	250	142	4,4	13,2	48	0,71
N16	54	M	18	5698	141	4,5	16,4	40	0,97
N17	62	F	7	352	136	5,1	10,12	32,3	0,75
N18	65	F	10	5203	141	4,7	9,4	23,1	0,62
N19	62	F	7	5038	137	2,7	10,18	33,3	0,75
N20	78	F	33	464,4	137	4,4	9,9	29,3	0,98
N21	75	M	26	527,3	138	3,1	14,4	29,4	0,88
N22	61	M	14	1482	139	4,3	15,3	44,9	0,86
N23	27	M	18	7887	138	4,8	13,5	43,5	1,04
N24	77	M	46	24881	141	4,4	12,4	39,2	1,31
N25	83	F	12	5350	140	4,7	9,5	28,8	0,73
N26	73	F	20	4983	141	5,3	13,7	40,2	0,75
N27	55	F	18	4569	137	3,4	13,2	45,3	0,82
N28	57	M	13	1678	125	5,7	13,5	38,3	1,9
N29	44	M	21	3570	138	8,6	13,4	41	1,21
N30	51	M	57	1485	128	4	14,8	41,5	2,16
D1	65	M	20	2105	152	4	12,6	44,1	2,2
D2	72	M	47	3500	135	4,3	11,7	34,1	1,15
D3	74	M	19	2927	138	4,5	10,1	28,4	0,9
D4	67	M	35	3520	134	3,9	9,5	28,4	1,61
D5	65	M	17	2844	137	3,9	13,7	40,9	1,28
D6	58	M	32	2915	138	3,4	12,5	41	1,07

D7	79	M	43	5453	129	3,8	11	32	1,07
D8	68	F	70	8592	134	4,9	10,2	30,7	2,55
D9	62	M	38	5623	131	4,5	12	35,5	0,97
D10	55	F	29	8455	137	4,6	10,1	32,1	0,87
D11	33	M	35	7463	132	4,8	11,3	36,5	0,85
D12	76	M	21	2910	139	4,6	16,7	52,9	1,35
D13	68	M	44	13807	142	3,5	12,1	38,1	1,81
D14	82	M	41	7077	138	5,7	13	40,9	1,47
D15	69	F	33	8604	128	5	11,9	35,2	0,95
D16	67	M	35	803,5	140	5	11,8	57	1,12
D17	58	F	39	808,4	136	4,3	14	28,3	0,86
D18	67	M	21	1256	137	5,4	14,6	45,2	1,43
D19	55	M	13	3500	141	4,3	15,4	45,3	0,81
D20	88	F	42	1326	140	5,2	15,6	44,8	0,7
D21	59	F	19	2753	141	4,8	11,7	35,5	0,81
D22	79	M	33	2864	133	4,3	12,4	45	1,65
D23	53	M	28	5623	142	5	12,8	36,8	1,25
D24	52	M	17	1261	131	3,4	13,1	37,4	0,90
D25	81	F	35	3257	134	4,9	13,3	40,2	1,11
D26	48	F	20	3265	132	4,4	16,8	35,3	0,86
D27	57	F	35	986,3	139	5,1	15,3	41,4	1,92
D28	67	M	39	1513	140	5,3	13,9	40,7	1,96
D29	72	F	22	329,8	138	4,3	11,5	34,8	0,52
D30	65	F	17	974,9	142	4,7	13,4	40,5	0,87

***F:** Female, **M:** Male, **BUN:** Blood Urea Nitrogen, **NT- proBNP:** Ventricular natriuretic peptide, **Na:** Sodium, **K:** Potassium, **HBG:** Hemoglobin, **HCT:** Hemotacryte

4.2. DNA ISOLATION

The results of the nanodrop measurement of the DNA samples of the patients after DNA isolation from the blood is shown in Table 4.2.

Table 4.2. Measurement results of DNA samples

Sample name	**260/280 ratio**	**Concentration (ng/µl)**
Control group		
C1	1,63	33
C2	1,7	39,5
C3	1,61	32,8
C4	1,5	31,9
C5	1,55	32,5
C6	1,55	26,9
C7	1,56	34

C8	1,63	39,2
C9	1,62	35,6
C10	1,61	33,4
C11	1,63	36,1
C12	1,63	33,4
C13	1,51	30,6
C14	1,66	30,9
C15	1,57	30,9
C16	1,66	28,1
C17	1,7	32,6
C18	1,7	32
C19	1,69	54,1
C20	1,52	33,4
C21	1,6	29,1
C22	1,63	36,8
C23	1,6	31,9
C24	1,52	33.8
C25	1,8	39,1
C26	1,73	43,5
C27	1,61	36,2
C28	1,8	29,9
C29	1,85	30,2
C30	1,74	29,1
Non-responders group		
D1	1,92	14,9
D2	1,97	10,8
D3	1,75	26
D4	1,85	16,6
D5	1,8	15
D6	1,9	9,7
D7	1,93	46,4
D8	1,89	13,7
D9	1,92	33,8
D10	1,98	16,8
D11	1,95	49,5
D12	1,77	29,3
D13	1,87	15,5
D14	1,95	20,7
D15	1,95	26

D16	1,95	26,2
D17	1,89	43,6
D18	1,97	18,9
D19	1,85	24,9
D20	1,85	30,2
D21	1,88	32,7
D22	1,79	28,1
D23	1,68	28,7
D24	1,53	30,1
D25	1,73	17,2
D26	1,73	11,4
D27	1,84	14,5
D28	1,76	20,6
D29	1,82	11,7
D30	1,69	29,4
Responders group		
N1	1,79	32
N2	1,76	28,5
N3	1,76	70
N4	1,81	27,5
N5	1,9	17,7
N6	1,8	18,2
N7	1,78	19,1
N8	1,87	23,5
N9	1,95	18
N10	1,87	13,1
N11	1,86	21
N12	1,84	15,5
N13	1,75	21,2
N14	1,83	23,4
N15	1,67	34,2
N16	1,88	33,2
N17	1,71	19,2
N18	1,78	15,3
N19	1,77	19,2
N20	1,75	15,7
N21	1,66	12,6
N22	1,82	12,7
N23	1,81	24

N24	1,6	16,2
N25	1,68	14,7
N26	1,6	12,6
N27	1,76	26,4
N28	1,73	32,5
N29	1,67	17,4
N30	1,65	15,4

4.3. POLYMERASE CHAIN REACTION (PCR) RESULTS

The 1st exon region of the *UGT1A1* gene was amplified by PCR. 932 bp PCR product was analyzed on 2% agarose gel.

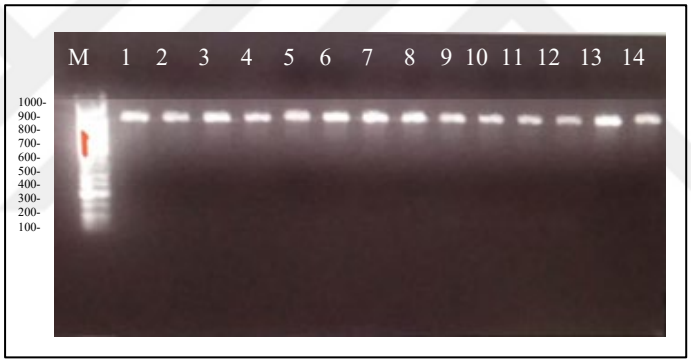
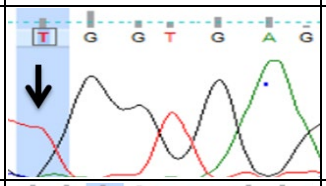
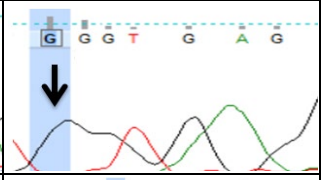
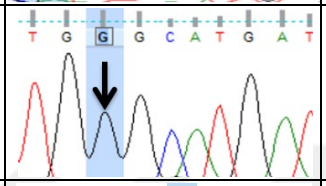
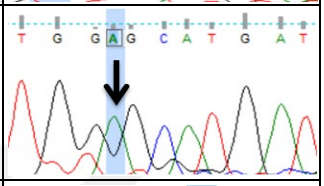
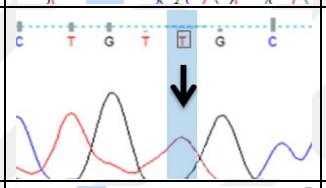
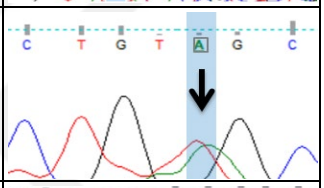
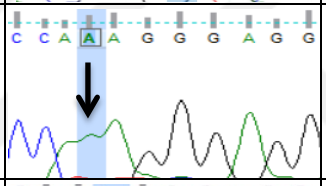
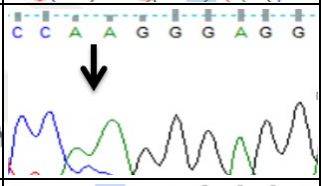
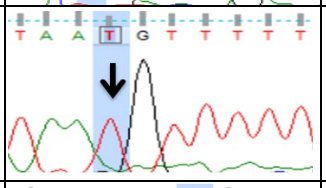
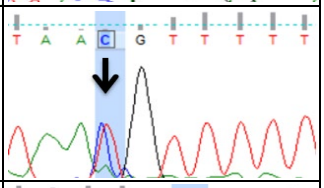
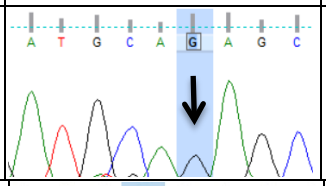
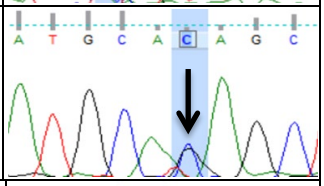
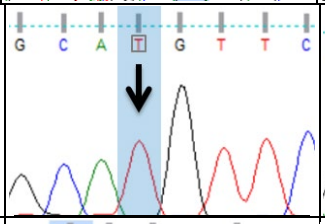
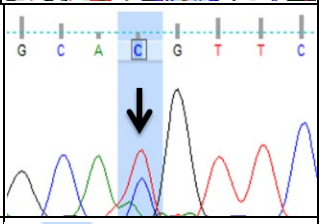
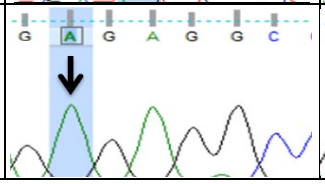
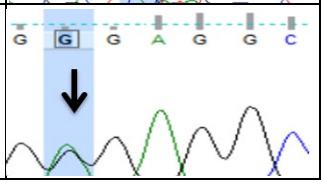


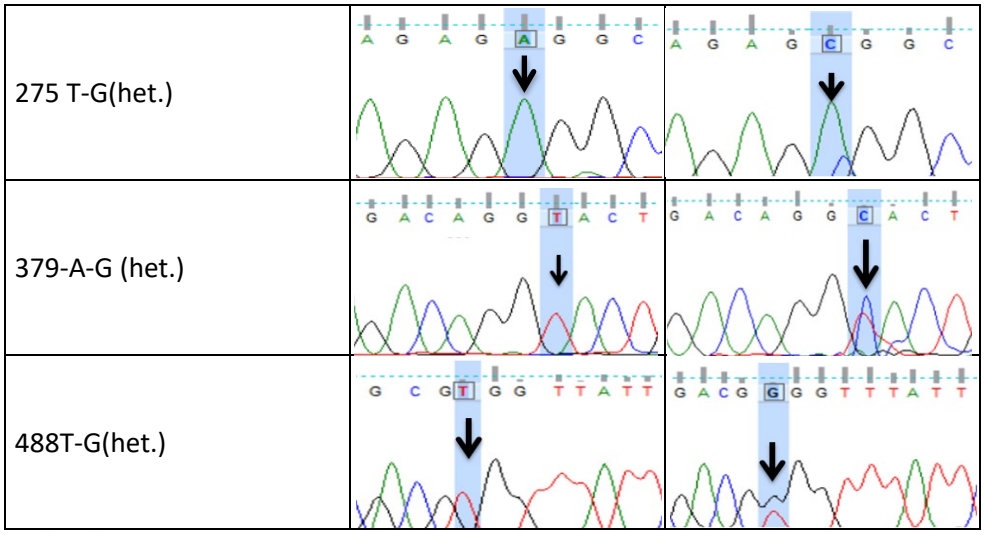
Figure 4.1. *UGT1A1* 1st exon region amplification sample. (M (first well) contains 100 bp ladder, other wells (from 1 to 14) also contain pcr products which are 932bp length.)

4.4. SANGER SEQUENCING RESULTS

The Pubmed database was used for the detection of mutations [68]. Sequence analysis results of detected mutations are shown in Table 4.3.

Table 4.3. *UGT1A1* Sequence Analysis Results

Nucleotides changes in *UGT1A1* gene	**Normal**	**Mutant**
19 A-C (hom.)		
55 T-ins.		
59 T-A (het.)		
63 A-del.		
110 T-C (het.)		
115 C-G(het.)		
226 A-G (het.)		
274 T-C (het.)		



*het:heterozygte, hom: homozygote

The location of the mutations/polymorphisms determined by sequence analysis in the *UGT1A1* gene and the changes caused are summarized in Table 4.4.

Table 4.4. *UGT1A1* gene mutations

Sample Name	Base substitution	Aminoacid change	Contig Number	Mutation Type
D15	19 A-C (hom.)	Gln-Pro CAA>CCA Codon 283	233761135	*New mutation In contig reference (rs755308142), base substitution is G, in our study it is C
D18, D22	55 T-ins.	Pro-leu CCC>CTC Codon 270	233761096	*New mutation missense mutation
D25	59 T-A (het.)	Val-Glu GTG>GAG Codon 81	233760529	*New mutation missense mutation
N6	63 A-del.	Gln-Gln CAA>CAA Codon 21	233760539	*New mutation silent mutation
N2	110 T-C (het.)	Asn-Asn AAT> AAC Codon 98	233760581	Known mut. silent mutation rs138183896 uncertain significance
C15	115 C-G(het.)	Ser>Cys TCT>TGT Codon 250	233761036	*New mutation missense mutation

D12	226 A-G (het.)	Met-Val ATG>CTG Codon 213	233760924	*New mutation missense mutation
C15	274 T-C (het.)	Leu-Pro CTC>CCC Codon 197	233760877	*New mutation missense mutation
D12	275 T-G(het.)	Pro-Pro CCT>CCG	233760875	*New mutation silent mutation
C1	379-A-G (het.)	Tyr-Cys TAC>TGC Codon 163	233760775	*New mutation missense mutation
C26	488T-G(het.)	Val-Gly GTG> GGG Codon 225	233760961	Known mut. silent mutation rs35003977 Pathogenic

*het:heterozygte, hom: homozygote

4.5. REAL-TIME POLYMERASE CHAIN REACTION RESULTS

Real-time PCR results of rs1800795 are shown in Table 4.5.

Table 4.5. Real-Time PCR Results of rs1800795 in *IL-6*

Rs1800795	
Patient Name	**RT-PCR Results**
D1	Wild-Type
D2	Wild-Type
D3	Heterozygot Mutation
D4	Wild-Type
D5	Heterozygot Mutation
D6	Wild-Type
D7	Heterozygot Mutation
D8	Heterozygot Mutation
D9	Wild-Type
D10	Heterozygot Mutation
D11	Wild-Type
D12	Wild-Type
D13	Wild-Type
D14	Wild-Type

D15	Wild-Type
D16	Heterozygot Mutation
D17	Heterozygot Mutation
D18	Wild-Type
D19	Wild-Type
D20	Heterozygot Mutation
D21	Wild-Type
D22	Heterozygot Mutation
D23	Wild-Type
D24	Heterozygot Mutation
D25	Wild-Type
D26	Heterozygot Mutation
D27	Wild-Type
D28	Heterozygot Mutation
D29	Heterozygot Mutation
D30	Heterozygot Mutation
N1	Heterozygot Mutation
N2	Wild-Type
N3	Heterozygot Mutation
N4	Wild-Type
N5	Wild-Type
N6	Wild-Type
N7	Wild-Type
N8	Wild-Type
N9	Wild-Type
N10	Heterozygot Mutation
N11	Heterozygot Mutation
N12	Wild-Type
N13	Wild-Type
N14	Heterozygot Mutation
N15	Wild-Type
N16	Heterozygot Mutation
N17	Wild-Type
N18	Heterozygot Mutation
N19	Wild-Type
N20	Wild-Type
N21	Wild-Type
N22	Heterozygot Mutation
N23	Heterozygot Mutation
N24	Wild-Type
N25	Wild-Type
N26	Wild-Type
N27	Wild-Type
N28	Wild-Type
N29	Heterozygot Mutation
N30	Wild-Type

Real-time PCR results of rs1800795 are shown in Table 4.6.

Table 4.6. Real-Time PCR Results of rs1800796 in *IL-6*

Rs1800796	
Patient Name	**RT-PCR Results**
D1	Wild-Type
D2	Wild-Type
D3	Wild-Type
D4	Wild-Type
D5	Wild-Type
D6	Wild-Type
D7	Wild-Type
D8	Wild-Type
D9	Wild-Type
D10	Wild-Type
D11	Heterozygot Mutation
D12	Heterozygot Mutation
D13	Wild-Type
D14	Wild-Type
D15	Wild-Type
D16	Wild-Type
D17	Wild-Type
D18	Heterozygot Mutation
D19	Wild-Type
D20	Wild-Type
D21	Heterozygot Mutation
D22	Wild-Type
D23	Heterozygot Mutation
D24	Wild-Type
D25	Wild-Type
D26	Wild-Type
D27	Heterozygot Mutation
D28	Wild-Type
D29	Wild-Type
D30	Wild-Type
N1	Wild-Type
N2	Wild-Type
N3	Wild-Type
N4	Wild-Type
N5	Wild-Type
N6	Wild-Type
N7	Wild-Type
N8	Wild-Type
N9	Heterozygot Mutation
N10	Wild-Type

N11	Wild-Type
N12	Heterozygot Mutation
N13	Wild-Type
N14	Wild-Type
N15	Wild-Type
N16	Wild-Type
N17	Wild-Type
N18	Wild-Type
N19	Wild-Type
N20	Wild-Type
N21	Heterozygot Mutation
N22	Wild-Type
N23	Wild-Type
N24	Wild-Type
N25	Wild-Type
N26	Wild-Type
N27	Wild-Type
N28	Heterozygot Mutation
N29	Wild-Type
N30	Wild-Type

4.6. *IN SILICO* ANALYSIS

4.6.1. Homology Modeling Study

C1, C15, and C16 individuals from the control group and D12, D15, D18, D22, and D25 patients from the non-responders group were selected for *in silico* analysis. The amino acid changes detected in these patients were demonstrated by a homology modeling study. A homology modeling study was made by the Swiss-Model database [69]. Wild and mutant type sequences were loaded into the system. 3D structures which have high Qualitative Model Energy Analysis (QMEAN) values were used as a model. In our study, the model number is 5gl5.1.A for wild type.

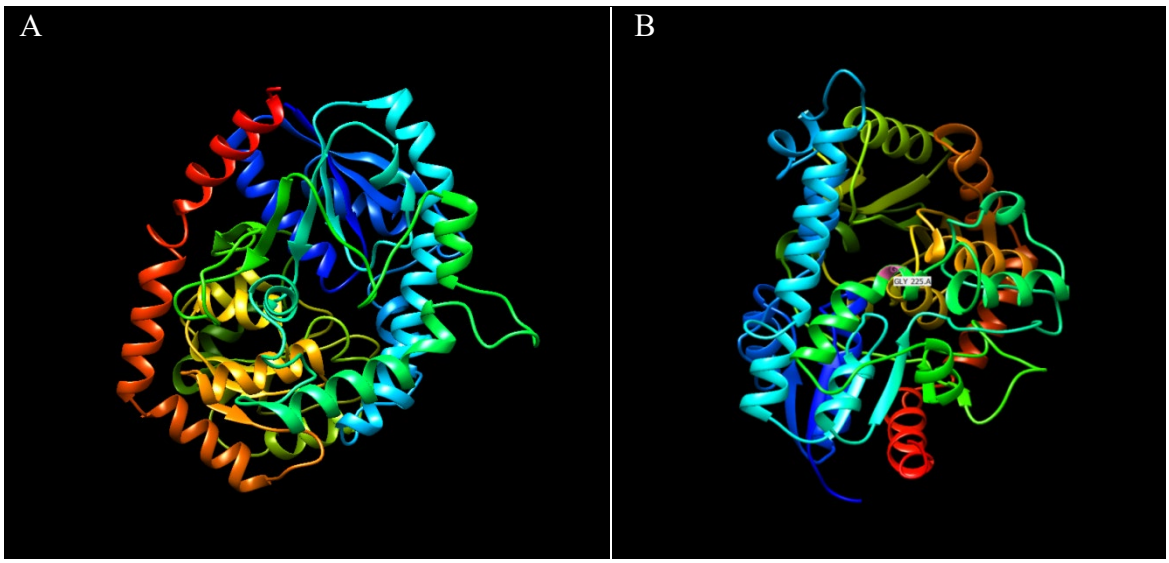
Table 4.7. QMEAN values of models for which homology modeling has been created by Swiss-Model.

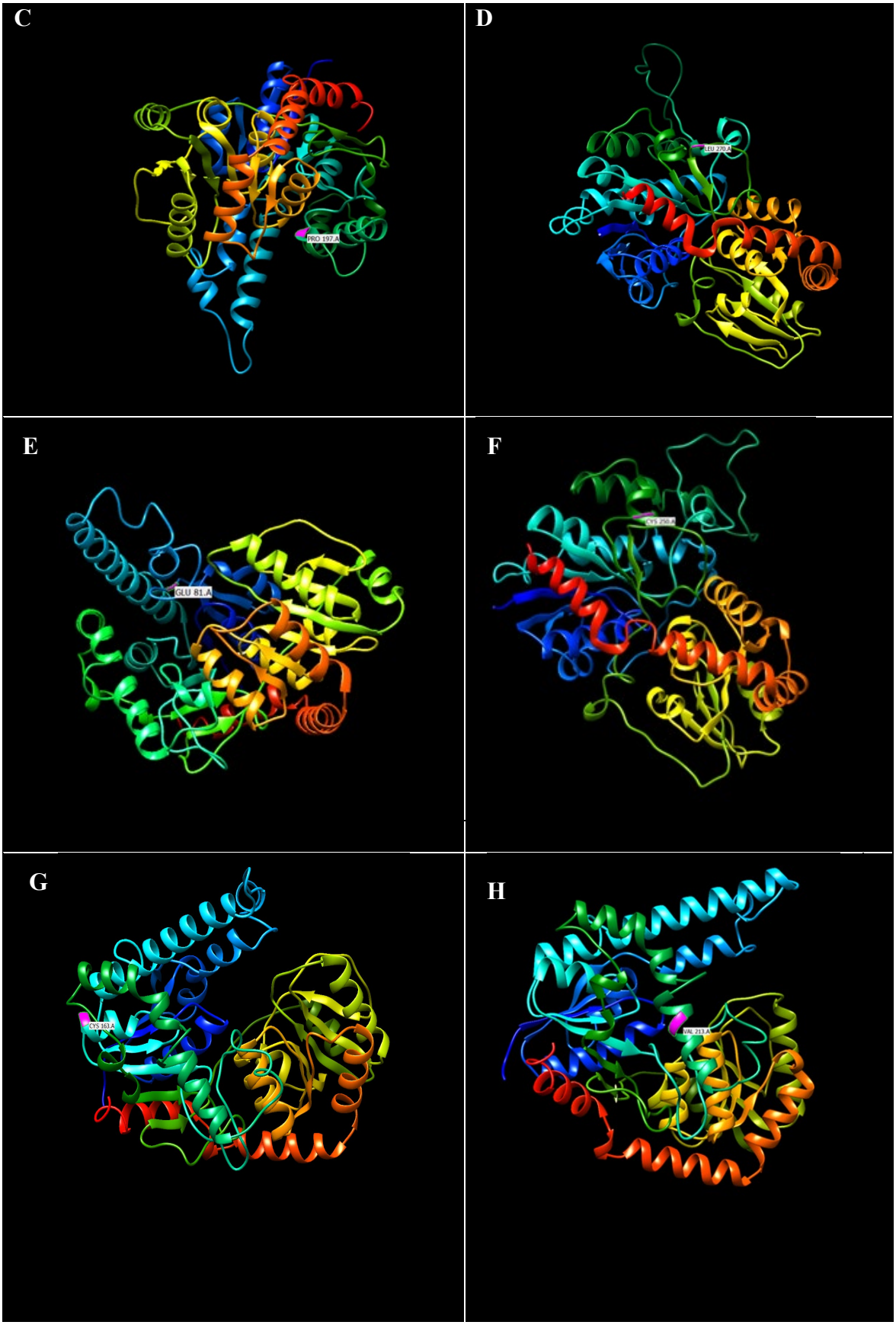
MODEL	QMEAN
Wild Type	-3.75
C26	-3.43
D18	-4.11
D25	-4.70
C1	-3.85
D12	-3.50
C15	-4.72
C15	-3.64
D15	-3.46
D22	-4.11

4.6.2. Visualization Study of Three-Dimensional Structures

All proteins were matched with each other using the UCSF Chimera program devices. Structural differences in mutant proteins were observed by visualizing wild-type and mutant protein structures with a ribbon display.

Ribbon forms of V225G, L197P, P270L, V81E, S250C, Y163C, M213V, E283P, and P270L mutations were detected and analyzed in the *UG1A1* gene and the wild type protein structure of the *UGT1A1* gene are shown in Figure 4.1.




C
PRO 197.A
D
LEU 270.A
E
GLU 81.A
F
G
CYS 163.A
H
VAL 213.A

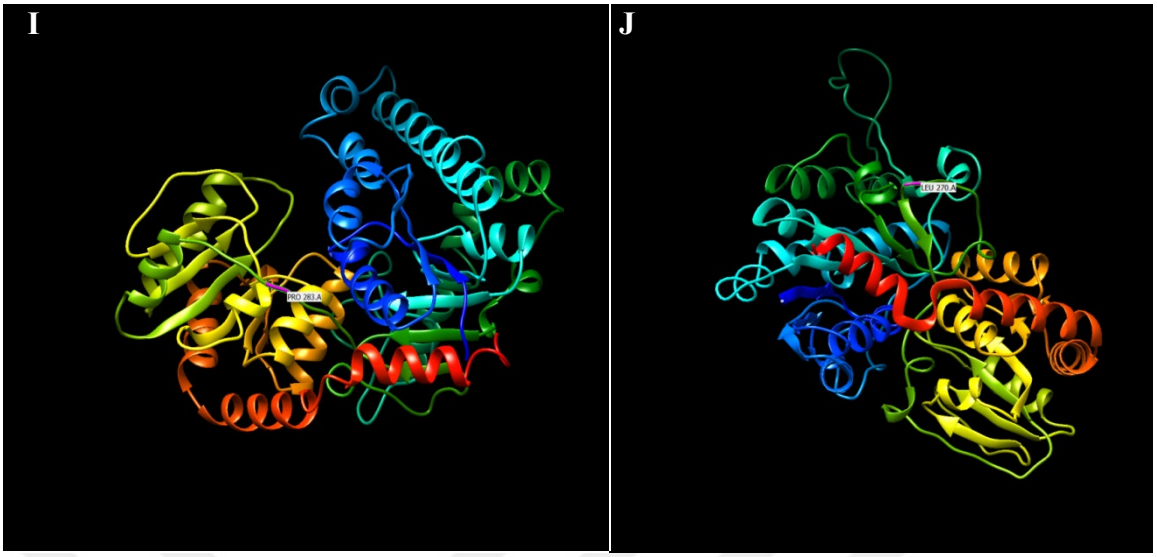


Figure 4.2. Homology Models of *UGT1A1* wild and mutant type proteins A: *UGT1A1* wild type ribbon structure **B:** C26 patient Val- Gly mutant type ribbon structure, **C:** C15 patient Leu-Pro mutant type ribbon structure, **D:** D18 patient Pro-Leu mutant type ribbon structure, **E:** D25 patient Val-Glu mutant type ribbon structure, **F:** C15 patient Ser-Cys mutant type ribbon structure, **G:** C1 patient Tyr-Cys mutant type ribbon structure, **H:** D12 patient Met-Val mutant type ribbon structure, **I:** D15 patient Gln-Pro mutant type ribbon structure, **J:** D22 patient Pro-Leu mutant type ribbon structure Hydropathy

The hydrophobicity structure of mutant types created with the Chimera program is shown in Figure 3.2. The program prefers red-white-and-blue colors to visualize these structures. The hydropathy index changes from negative to positive, from blue to white, then to red. The amino acid with the lowest hydropathy index is shown in blue, the amino acids close to zero are shown in white, and the amino acids with the highest hydropathy index are shown in red. The change in the hydrophobic structure that occurs in the mutant type affects the folding of proteins [70-71]. This change may cause conformational changes in the models.

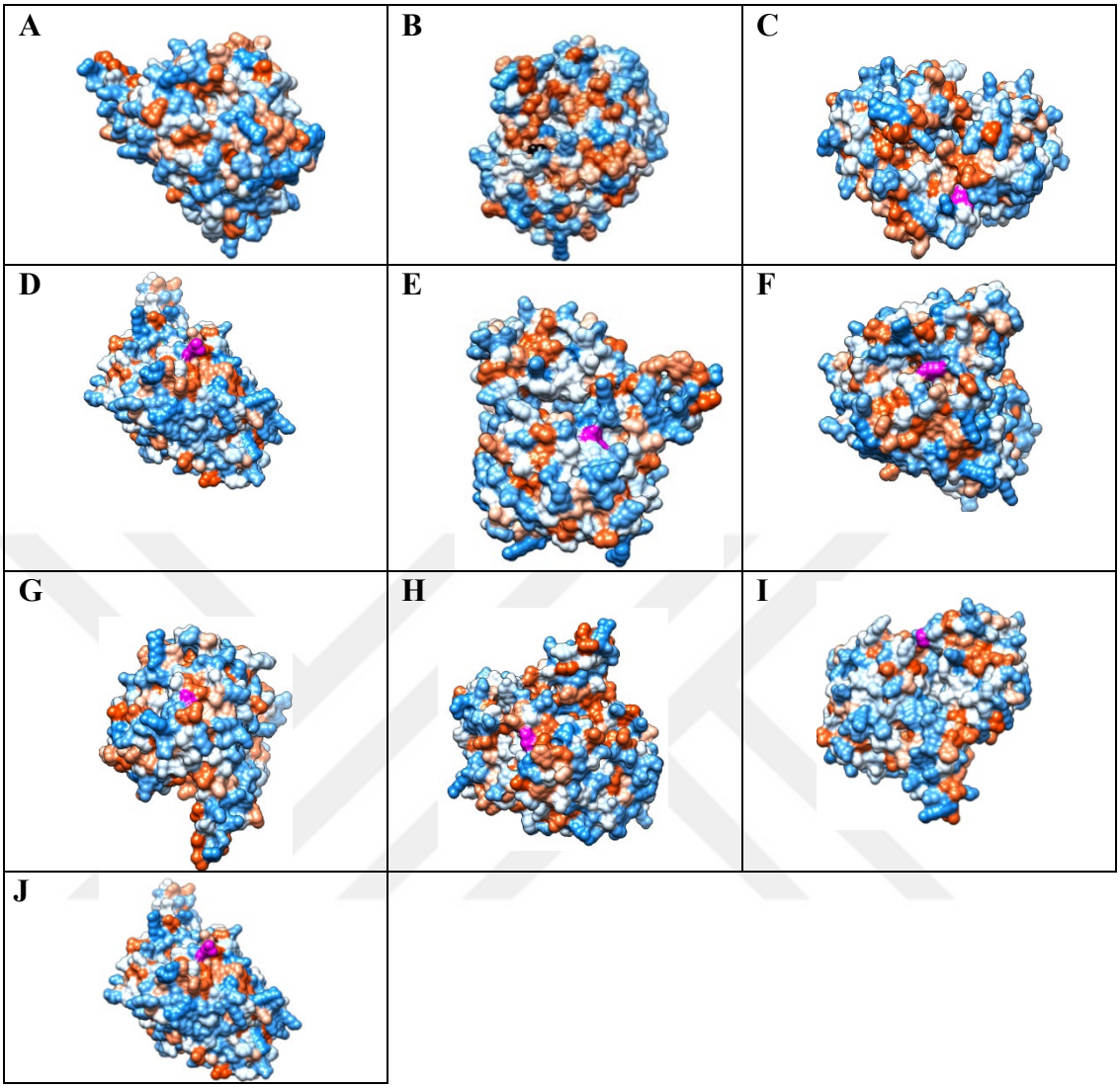


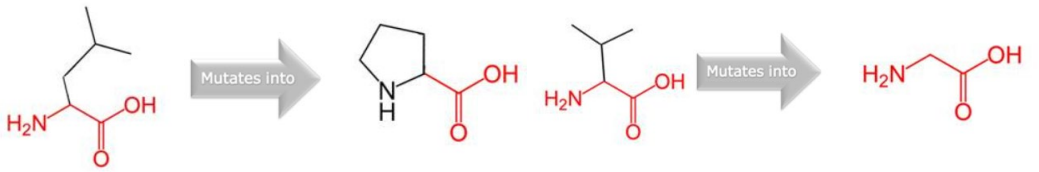
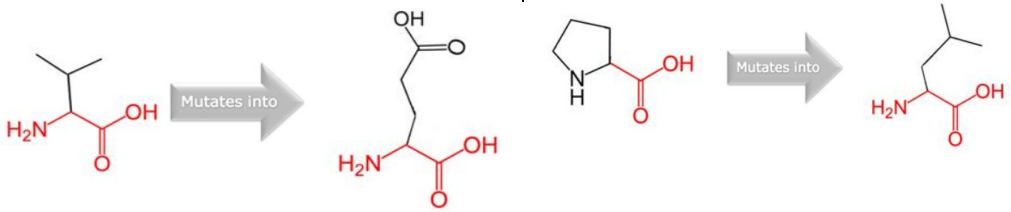
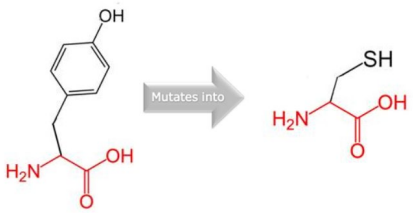
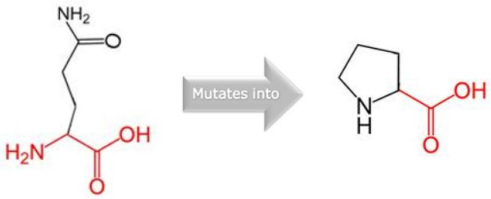
Figure 4.3. Hydrophobic structures of UGT1A1 wild and mutant type proteins **A:** UGT1A1 wild type hydrophobic structure, **B:** C26 patient Val- Gly mutant type hydrophobic structure, **C:** C15 patient Leu-Pro mutant hydrophobic structure, **D:** D18 patient Pro-Leu mutant type hydrophobic structure, **E:** D25 patient Val-Glu mutant type hydrophobic structure, **F:** C15 patient Ser-Cys mutant type hydrophobic structure, **G:** C1 patient Tyr-Cys mutant type hydrophobic structure, **H:** D12 patient Met-Val mutant type ribbon structure, **I:** D15 patient Gln-Pro mutant type ribbon structure, **J:** D22 patient Pro-Leu mutant type hydrophobic structure

When the wild type and mutant types were compared in hydrophobicity surface structure, it was seen that the results matched with the Hydropathy average (GRAVY) mean. Hydropathy average (GRAVY) is bigger than “0” in all models. It shows that the overall

model is hydrophobic. Increased hydrophobicity affects the folding and access of proteins to the cell membrane. GRAVY calculation was shown in the Physico-chemical property tables (Table 3.9-3.18). The GRAVY values were calculated as *UGT1A1* wild type 0.064, C26 patient 0.056, D18 patient 0.074, C1 patient 0.071, D25 patient 0.050, D11 patient 0.069 D15 patient 0.068, C15 (Leu-Pro) 0.054 and C15 (Ser-Cys) 0.071, respectively. Different hydropathy indices in the wild and mutant models affect the hydrophobic structure. Hydrophobic structure changing affects the folding of the proteins [70-71-72-73]. It is thought that this change may cause conformational changes in the models.

We observed the mutations of proteins in the patient groups. The structures of the wild-type amino acid (left) and the mutant (right) amino acid shows in Table 4.8. The backbone, which is the same for each amino acid, is colored red. The unique side chain is black.

Table 4. 8. Amino acid changes in patients [74].

C15 Leu-Pro 	C26 Val-Gly
D25 Val-Glu 	D18 Pro-Leu
C1 Tyr-Cys 	C15 Ser-Cys 
D12 Met-Val 	D15 Gln-Pro 

D22 Pro-Leu

C26 patient has a mutation of Valine into Glycine at position 225. The wild-type residue is bigger and more hydrophobic than the mutant residue. Glycines are flexible amino acids and they can disturb the rigidity of the protein at this position. Mutant residue localized in the domain. This position is important for the main activity of the protein. Mutation of the residue can disturb this function.

Leucine changes to the Proline in C15 patient. The wild-type residue is bigger than the mutant residue. The wild-type residue is not conserved at this position. In this case, the mutation is deleterious.

C15 patient has a mutation of Serine into Cysteine at position 250. The mutant residue is a more hydrophobic residue when compared with the wild-type residue. This mutation may cause damage to protein structure.

The mutation of a Valine into a Gluytmic acid at position 81 in D25 patient. The wild-type residue is a small residue when compared with the mutant residue. The wild-type residue is not conserved at this position. In this case, the mutation is possibly damaging to the protein.

C1 patient has a mutation of Tyrosine into Cysteine at position 163. The wild-type residue is a bigger residue when compared with mutant residue. This situation may cause a loss of interactions. The mutant residue which is located in the domain is important for the main activity of the protein. Mutation might disturb protein function.

D12 patient has a mutation of Methionine into Valine at position 213. The wild-type residue is smaller when compared with mutant residue. The mutant residue is located near a highly conserved position. The mutation is deleterious.

D15 patient has a mutation of Glycine into proline at position 283. The mutant residue is smaller and more hydrophobic when compared with wild-type residue. The wild-type residue is not conserved at this position. The mutation is not deleterious. The mutation is probably not damaging to the protein.

Proline changes to the leucine in the D18 patient. The wild-type residue is small. Proline is a very rigid amino acid and it is a wild-type residue. It induces a special backbone conformation which may be required at this position. This mutation can disturb this special conformation. This mutation is damaging to the protein.

D22 patient has a mutation of Proline into Leucine. The mutant residue is located near the highly conserved position. This mutation is probably damaging to the protein and its special conformation.

4.6.3. Calculation of Physico-chemical Properties of Models

Physico-chemical properties were calculated by using the ProtParam program of ExPASy. *UGT1A1* Wild Type (Table 4.9), C26 patient (Table 4.10), D18 patient (Table 4.11), D25 patient (Table 4.12), C1 patient (Table 4.13), D12 patient (Table 4.14), C15 patient (Leu-Pro) (Table 4.15), C15 patient (Ser-Cys) (Table 4.16), D15 patient (Table 4.17) and D22 patient (Table 4.18) are shown in the tables respectively.

The total number of negatively charged residues is 47 in wild type, C26, D12, D18, C1, C15, D22, and D15, and there are 48 amino acids for the D25 patient. The total number of positively charged residues is 50 amino acids. Electrical properties play an important role in the folding of proteins [75]. Therefore, the change of the total positive charge may cause an electrical change in the whole of the models. Since this electrical change will affect the folding of the models, it is thought that conformational change may occur [75-76]. The theoretical pI (isoelectronic point) of all models is above seven, which means that all proteins are positively charged.

Table 4. 9. The physicochemical property of UGT1A1 wild type

Number of amino acids: 533 **Molecular weight:** 59591.39 **Theoretical pI:** 8.19 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 11 2.1% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 64 12.0% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 31 5.8% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 46 8.6% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2700 Hydrogen H 4222 Nitrogen N 710 Oxygen O 748 Sulfur S 31 **Formula:** $C_{2700}H_{4222}N_{710}O_{748}S_{31}$ **Total number of atoms:** 8411 **Aliphatic index:** 95.63 **Grand average of hydropathicity (GRAVY):** 0.064

Table 4.10. The Physico-chemical property of patient C26

Number of amino acids: 533 **Molecular weight:** 59549.31 **Theoretical pI:** 8.19 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 11 2.1% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 31 5.8% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 64 12.0% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 31 5.8% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 45 8.4% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2697 Hydrogen H 4216 Nitrogen N 710 Oxygen O 748 Sulfur S 31 **Formula:** C2697H4216N710O748S31 **Total number of atoms:** 8402 **Aliphatic index:** 95.08 **Grand average of hydropathicity (GRAVY):** 0.056

Table 4.11. The Physico-chemical property of patient D18

Number of amino acids: 533 **Molecular weight:** 59607.44 **Theoretical pI:** 8.19 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 11 2.1% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 65 12.2% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 30 5.6% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 46 8.6% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2701 Hydrogen H 4226 Nitrogen N 710 Oxygen O 748 Sulfur S 31 **Formula:** C2701H4226N710O748S31 **Total number of atoms:** 8416 **Aliphatic index:** 96.36 **Grand average of hydropathicity (GRAVY):** 0.074

Table 4.12. The Physico-chemical property of patient D25

Number of amino acids: 533 **Molecular weight:** 59607.35 **Theoretical pI:** 7.95 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 24 4.5% Cys (C) 11 2.1% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 64 12.0% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 31 5.8% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 45 8.4% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 48 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2699 Hydrogen H 4218 Nitrogen N 710 Oxygen O 750 Sulfur S 31 **Formula:** C2699H4218N710O750S31 **Total number of atoms:** 8408 **Aliphatic index:** 95.08 **Grand average of hydropathicity (GRAVY):** 0.050

Table 4.13. The Physico-chemical property of C1

Number of amino acids: 533 **Molecular weight:** 59531.36 **Theoretical pI:** 8.17 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 12 2.3% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 64 12.0% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 31 5.8% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 16 3.0% Val (V) 46 8.6% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2694 Hydrogen H 4218 Nitrogen N 710 Oxygen O 747 Sulfur S 32 **Formula:** C2694H4218N710O747S32 **Total number of atoms:** 8401 **Aliphatic index:** 95.63 **Grand average of hydropathicity (GRAVY):** 0.071

Table 4.14. The Physico-chemical property of patient D12

Number of amino acids: 533 **Molecular weight:** 59559.33 **Theoretical pI:** 8.19 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 11 2.1% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 64 12.0% Lys (K) 30 5.6% Met (M) 19 3.6% Phe (F) 27 5.1% Pro (P) 31 5.8% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 47 8.8% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2700 Hydrogen H 4222 Nitrogen N 710 Oxygen O 748 Sulfur S 30 **Formula:** C2700H4222N710O748S30 **Total number of atoms:** 8410 **Aliphatic index:** 96.17 **Grand average of hydropathicity (GRAVY):** 0.069

Table 4.15. The Physico-chemical property of C15 (Leu-Pro)

Number of amino acids: 533 **Molecular weight:** 59575.35 **Theoretical pI:** 8.19 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 11 2.1% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 63 11.8% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 32 6.0% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 46 8.6% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2699 Hydrogen H 4218 Nitrogen N 710 Oxygen O 748 Sulfur S 31 **Formula:** C2699H4218N710O748S31 **Total number of atoms:** 8406 **Aliphatic index:** 94.90 **Grand average of hydropathicity (GRAVY):** 0.054

Table 4.16. The Physico-chemical property of C15 (Ser-Cys)

Number of amino acids: 533 **Molecular weight:** 59607.45 **Theoretical pI:** 8.16 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 12 2.3% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 64 12.0% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 31 5.8% Ser (S) 41 7.7% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 46 8.6% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2700 Hydrogen H 4222 Nitrogen N 710 Oxygen O 747 Sulfur S 32 **Formula:** C2700H4222N710O747S32 **Total number of atoms:** 8411 **Aliphatic index:** 95.63 **Grand average of hydropathicity (GRAVY):** 0.071

Table 4.17. The Physico-chemical property of patient D15

Number of amino acids: 533 **Molecular weight:** 59560.38 **Theoretical pI:** 8.19 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 11 2.1% Gln (Q) 18 3.4% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 64 12.0% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 32 6.0% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 46 8.6% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2700 Hydrogen H 4221 Nitrogen N 709 Oxygen O 747 Sulfur S 31 **Formula:** C2700H4221N709O747S31 **Total number of atoms:** 8408 **Aliphatic index:** 95.63 **Grand average of hydropathicity (GRAVY):** 0.068

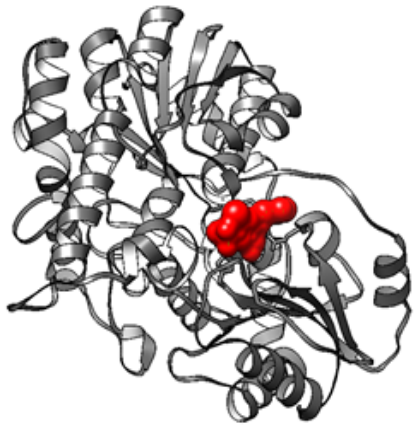
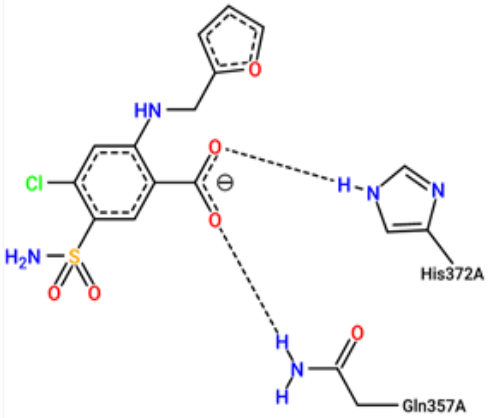
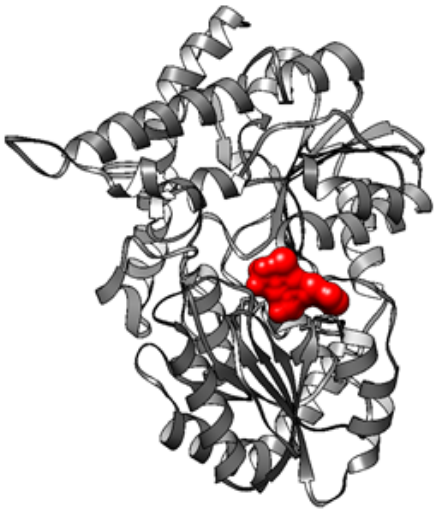
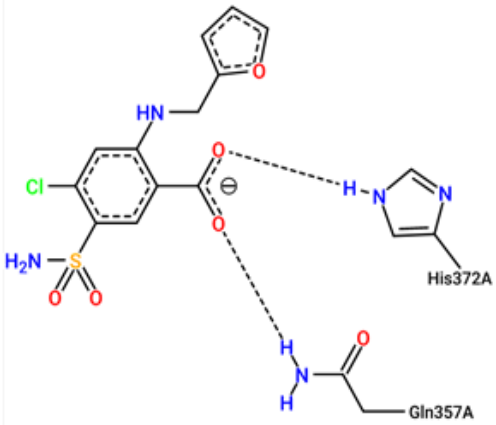
Table 4.18. The Physico-chemical property of patient D22

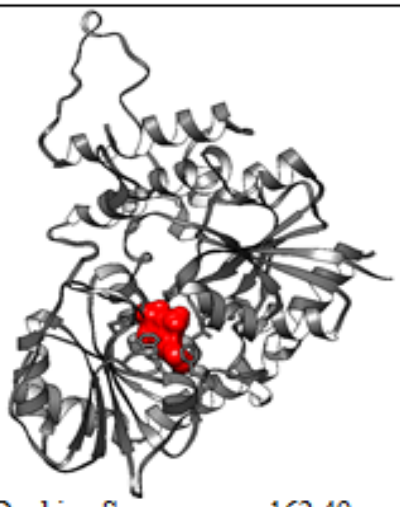
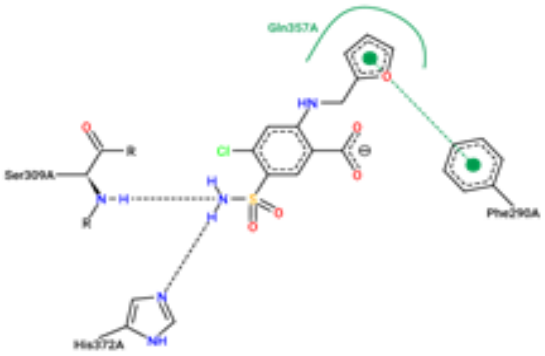
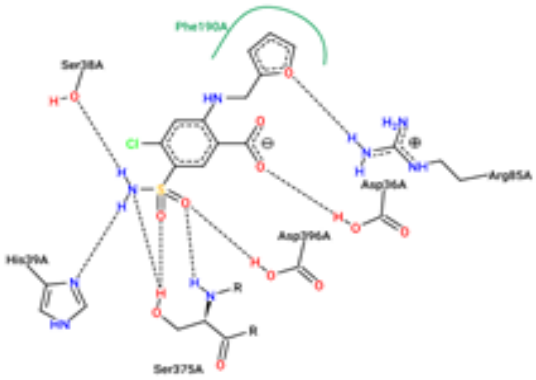
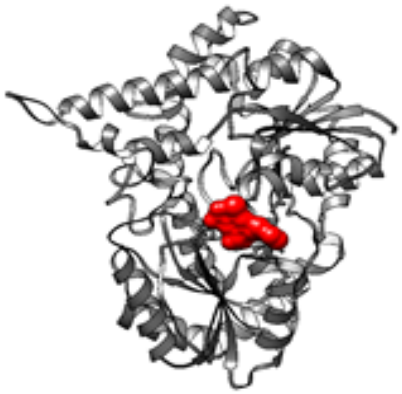
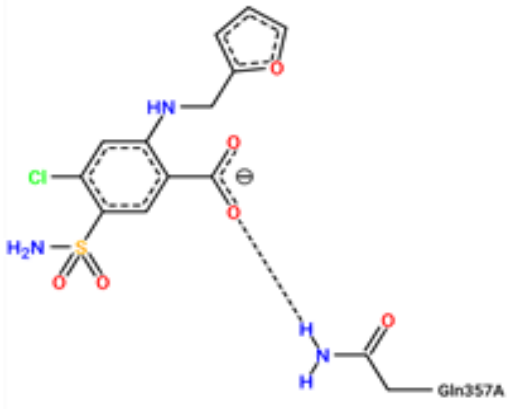
Number of amino acids: 533 **Molecular weight:** 59607.44 **Theoretical pI:** 8.19 **Amino acid composition:** Ala (A) 37 6.9% Arg (R) 20 3.8% Asn (N) 20 3.8% Asp (D) 23 4.3% Cys (C) 11 2.1% Gln (Q) 19 3.6% Glu (E) 24 4.5% Gly (G) 30 5.6% His (H) 21 3.9% Ile (I) 23 4.3% Leu (L) 65 12.2% Lys (K) 30 5.6% Met (M) 20 3.8% Phe (F) 27 5.1% Pro (P) 30 5.6% Ser (S) 42 7.9% Thr (T) 22 4.1% Trp (W) 6 1.1% Tyr (Y) 17 3.2% Val (V) 46 8.6% Pyl (O) 0 0.0% Sec (U) 0 0.0% (B) 0 0.0% (Z) 0 0.0% (X) 0 0.0%	**Total number of negatively charged residues (Asp + Glu):** 47 **Total number of positively charged residues (Arg + Lys):** 50 **Atomic composition:** Carbon C 2701 Hydrogen H 4226 Nitrogen N 710 Oxygen O 748 Sulfur S 31 **Formula:** C2701H4226N710O748S31 **Total number of atoms:** 8416 **Aliphatic index:** 96.36 **Grand average of hydropathicity (GRAVY):** 0.074

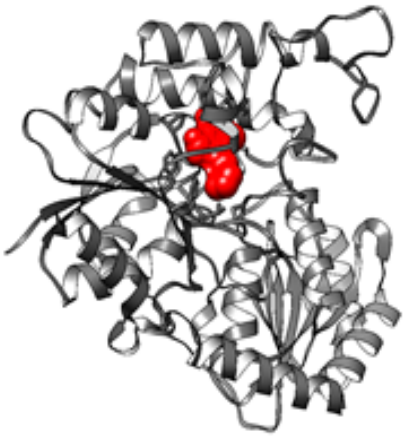
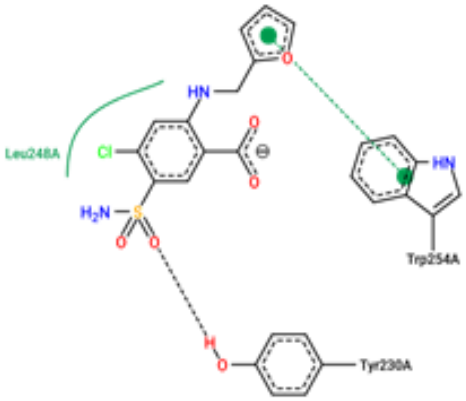
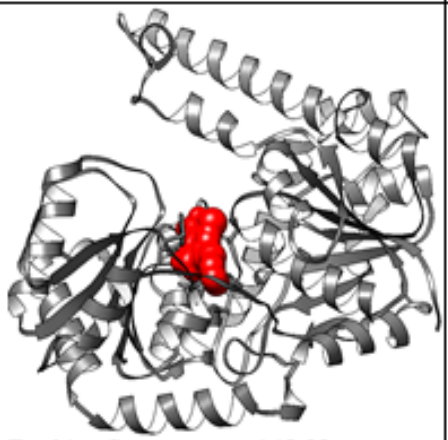
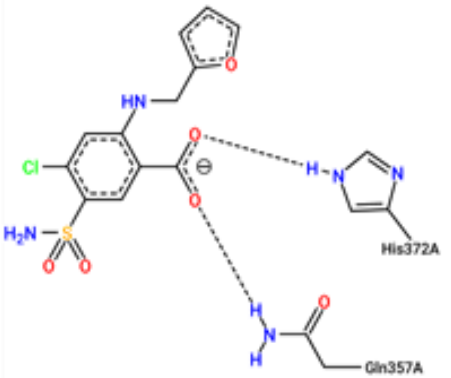
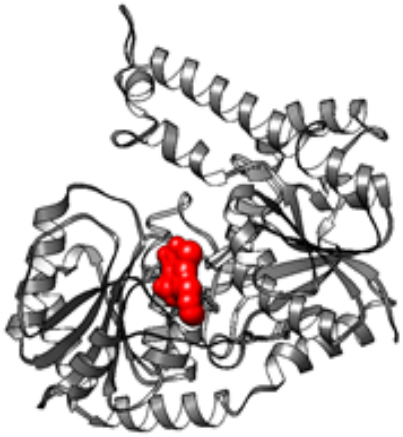
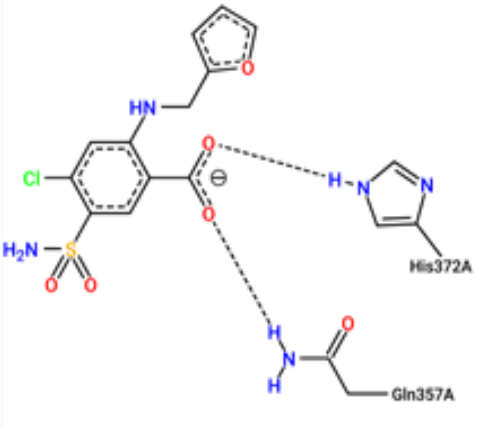
4.6.4. Molecular Docking Study

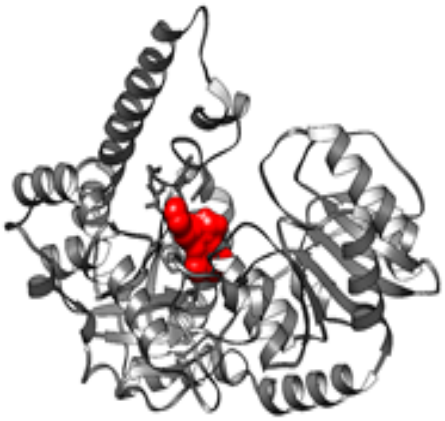
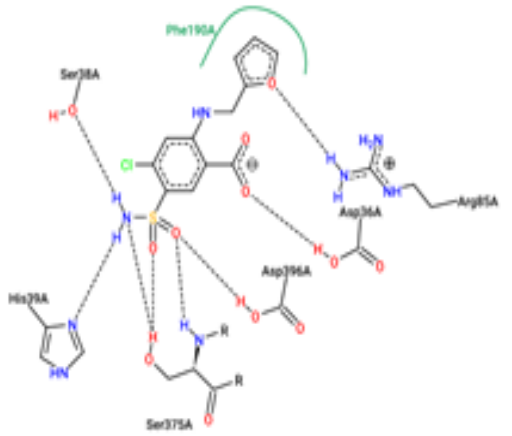
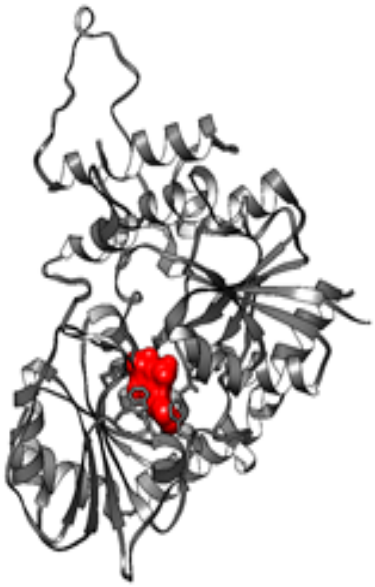
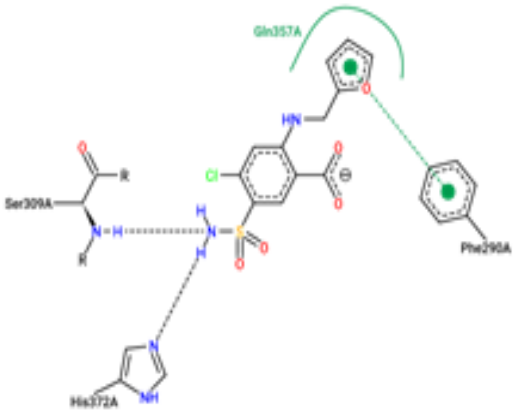
Molecular docking analysis was performed by the HDOCK server. Docking results, docking score, and ligand rmsd values were shown in Table 4.19.

Table 4.19. Docking results of *UGT1A1* gene for wild and mutant types.

C15 Leu-Pro	 Docking Score -149.64 Ligand rmsd (Å) 48.73	
C26 Val-Gly	 Docking Score -149.46 Ligand rmsd (Å) 48.74	

D18 Pro-Leu	 Docking Score -162.49 Ligand rmsd (Å) 62.66	
D25 Val-Asp	 Docking Score -149.31 Ligand rmsd (Å) 40.03	
C1 Tyr-Cys	 Docking Score -149.90 Ligand rmsd (Å) 48.73	

C15 Ser-Cys	 Docking Score -150.90 Ligand rmsd (Å) 73.40	
D12 Met-Val	 Docking Score -149.89 Ligand rmsd (Å) 48.73	
D15 Gln-Pro	 Docking Score -149.46 Ligand rmsd (Å) 48.70	

D25 Val-Glu	 Docking Score -149.31 Ligand rmsd (Å) 40.03	
D22 Pro-Leu	 Docking Score -162.49 Ligand rmsd (Å) 62.66	

4.7. STATISTICAL ANALYSIS RESULTS

4.7.1. Baseline Characteristics Analysis

The baseline characteristics of the study population of two groups (patient and control group) are shown in Table 4.20. When baseline characteristics were compared between groups; hematocrit (HCT), hemoglobin (HBG), and sodium (Na) levels were found statistically high in the control group whereas creatinine, blood urea nitrogen (BUN), and N-terminal proB-type Natriuretic Peptide (NT-proBNP) levels were found statistically high in the patient group ($p<0,05$).

Table 4.20. Baseline characteristics of the study population of two groups

Baseline Characteristics		**Groups (number of participants)**		**p-values**
		Patient (n=60)	**Control (n=30)**	
Gender	Female	24 (40%)	18 (60%)	0,073
	Male	36 (60%)	12 (40%)	
Age		67 (27-88)	62 (50-67)	**0,03***
HCT		38,05 (23,1-57)	42,5 (38-54)	**0,000$^{\lambda}$**
HBG		12,45 (9,4-16,80)	14,05 (12,7-17)	**0,000$^{\lambda}$**
K		4,55 (2,7-8,6)	4,65 (3,5-5,5)	0,722
Na		137,5 (125-153)	139,5 (135-145)	**0,009***
Creatinine		1,055 (0,52-2,55)	0,8 (0,5-1,1)	**0,000$^{\lambda}$**
BUN		22 (7-70)	15 (10-20)	**0,000$^{\lambda}$**
NT-proBNP		2798,5 (241-8604)	112,5 (90-124)	**0,000$^{\lambda}$**

HTC: Hematocrit, **HBG:** Hemoglobin, **K:** Potassium, **Na:** Sodium, **BUN:** Blood Urea Nitrogen, **NT-proBNP:** N-terminal proB-type Natriuretic Peptide *$p<0,05$, ,$^{\lambda}$$p<0,001$

The baseline characteristics of the study population of three groups (responders, non-responders, and control group) are shown in Table 4.21. When baseline characteristics were compared between groups; hematocrit (HCT), hemoglobin (HBG) and sodium (Na) levels were found statistically high in control group whereas creatinine, blood urea nitrogen (BUN) and N-terminal proB-type Natriuretic Peptide (NT-proBNP) levels were found statistically high in non-responders as well as responders group ($p<0,05$).

Table 4.21. Baseline characteristics of the study population of three groups

Baseline Characteristics		**Non-responders (n=30)**	**Responders (n=30)**	**Control (n=30)**	**p-values**
Gender	Female	0,175	13 (43,3%)	18 (60%)	0,175
	Male	19 (63,3%).	17 (56,7%)	12 (40%)	
Age		67 (33-88)	64 (27-83)	62 (50-67)	0,081
HCT		37,75 (28,3-57)**	38 (23,1-50)*	42,5 (38-54)*,**	**0,001*/ 0,003****
HBG		12,55 (9,5-16,80)**	12,4 (9,4-16,4)*	14,05 (12,7-17) *,**	**0,001*,** ***
K		4,55 (3,4-5,7)	4,55 (2,7-8,6)	4,65 (3,5-5,5)	0,937
Na		137,5 (128-152)*	137,5 (125-153)	139,5 (135-145)*	**0,028***
Creatinine		1,09 (0,52-2,55) $^{\lambda}$	0,975 (0,62-2,38)*	0,8 (0,5-1,1)*, $^{\lambda}$	**0,002*/ 0,000$^{\lambda}$**
BUN		33 (13-70) **,$^{\lambda}$	20(7-57)*,**	15 (10-20) *,$^{\lambda}$	**0,014*/ 0,012** /0,000$^{\lambda}$**
NT-proBNP		2912,5 (329-8604) $^{\lambda\lambda}$	1586 (241-7887)$^{\lambda}$	112,5 (90-124) $^{\lambda,\lambda\lambda}$	**0,000$^{\lambda,\lambda\lambda}$**

HTC: Hematocrit, **HBG:** Hemoglobin, **K:** Potassium, **Na:** Sodium, **BUN:** Blood Urea Nitrogen, **NT-proBNP:** N-terminal proB-type Natriuretic Peptide *,**p<0,05,$^{\lambda,\lambda\lambda}$p<0,001

4.7.2. Distribution of nucleotide variations in *UGT1A1* & *IL-6*

The distribution of nucleotide variations in two groups (patient and control group) is shown in Table 4.22. When groups were compared with each other heterozygous genotype of rs1800796 variation in the *IL-6* gene was found statistically high in the patient group (p=0.027).

Table 4.22. Distribution of nucleotide variations in UGT1A1 & IL-6 in two groups

Nucleotide position	**Nucleotide changes**	**Gene**	**Groups**						**p-values**
			Patient Group (n=60)			**Control Group (n=30)**			
			Wild	**Heterozygote**	**Homozygote mutant**	**Wild**	**Heterozygote**	**Homozygote mutant**	
rs1800795	**G>C**	*IL-6*	36 (%60)	24 (40%)	0 (0%)	20 (66,7%)	10 (33,3%)	0 (0%)	0.539
rs1800796	**G>C**	*IL-6*	50 (%83,3)	10 (%16,7)*	0 (0%)	30 (100%)	0 (0%)	0 (0%)	**0.027***
19	**A>C**	*UGT1A1*	59 (98,3%)	0 (0%)	1 (1,7%)	30 (100%)	0 (0%)	0 (0%)	1
55	**T-ins**	*UGT1A1*	58 (96,7%)	0 (0%)	2 (3,3%)	30 (100%)	0 (0%)	0 (0%)	0.551
59	**T>A**	*UGT1A1*	59 (98,3%)	1 (1,7%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	1
63	**A-del**	*UGT1A1*	59 (98,3%)	0 (0%)	1 (1,7%)	30 (100%)	0 (0%)	0 (0%)	1
110	**T>C**	*UGT1A1*	59 (98,3%)	1 (1,7%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	1
115	**C>G**	*UGT1A1*	60 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.333
226	**A>G**	*UGT1A1*	59	1 (1,7%)	0 (0%)	30	0 (0%)	0 (0%)	1

			(98,3%)			(100%)			
274	**T>C**	*UGT1A1*	60 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.333
275	**T>G**	*UGT1A1*	59 (98,3%)	1 (1,7%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	1
379	**A>G**	*UGT1A1*	60 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.333
488	**T>G**	*UGT1A1*	60 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.333

*p<0.05

The distribution of nucleotide variations in three groups (responders, non-responders and control group) is shown in Table 4.23. When groups were compared with each other heterozygous genotype of rs1800796 variation in the *IL-6* gene was found statistically high in the non-responders group (p=0.043).

Table 4.23. Distribution of nucleotide variations in UGT1A1 & IL-6 in three groups

Nucleotide position	Nucleotide changes	Gene	Percentage of Nucleotide Changes								p-Values
			Non-responders Group (n=30)			Responders Group (n=30)			Control Group (n=30)		
			Wild	Heterozygote	Homozygote mutant	Wild	Heterozygote	Homozygote mutant	Wild	Heterozygote	Homozygote mutant	
rs1800795	G>C	*IL-6*	16 (%53,3)	14 (%46,7)	0 (0%)	20 (%66.7)	10 (%33,3)	0 (0%)	20 (%66,7)	10 (%33,3)	0 (0%)	0.469
rs1800796	G>C	*IL-6*	24 (%80)	6 (%20)	0 (0%)	26 (%86,7)	4 (%13.3)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	**0.043 ***
19	A>C	*UGT1A1*	29 (96,7%)	0 (0%)	1 (3,3%)	0 (0%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	0.364
55	T-ins	*UGT1A1*	28 (93,3%)	0 (0%)	2 (6,7%)	0 (0%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	0.129
59	T>A	*UGT1A1*	29 (96,7%)	1 (3,3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	0.364
63	A-del	*UGT1A1*	30 (100%)	0 (0%)	0 (0%)	29 (96,7%)	0 (0%)	1 (3,3%)	30 (100%)	0 (0%)	0 (0%)	0.364
110	T>C	*UGT1A1*	30 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	0.364
115	C>G	*UGT1A1*	30 (100%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.364
226	A>G	*UGT1A1*	29 (96,7%)	1 (3,3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	0.364
274	T>C	*UGT1A1*	30 (100%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.364
275	T>G	*UGT1A1*	29 (96,7%)	1 (3,3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	0.364
379	A>G	*UGT1A1*	30 (100%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.364
488	T>G	*UGT1A1*	30 (100%)	0 (0%)	0 (0%)	30 (100%)	0 (0%)	0 (0%)	29 (96,7%)	1 (3,3%)	0 (0%)	0.364

*p<0.05

4.7.3. Allelic Discrimination

The allelic discrimination in two groups (patient and control group) is shown in Table 4.24.

Table 4.24. Allelic discrimination in UGT1A1 & IL-6 in two groups

	Case Group (n=60)	**Control Group (n=30)**	**p-values**
rs1800795			
G	96 (80%)	50 (83,3%)	0,590
C	24 (20%)	10 (16,7%)	
rs1800796			
G	110 (91,7%)	60 (100%)	**0,032***
C	10 (10 8,3%)	0 (0%)	
379			
A	120 (100%)	59 (98,3%)	0,333
G	0 (0%)	1 (1,7%)	
274			
T	120 (100%)	59 (98,3%)	0,333
C	0 (0%)	1 (1,7%)	
115			
C	120 (100%)	59 (98,3%)	0,333
G	0 (0%)	1 (1,7%)	
488			
T	120 (100%)	59 (98,3%)	0,333
G	0 (0%)	1 (1,7%)	
110			
T	119 (99,2%)	60 (100%)	1,000
C	1 (0,8%)	0 (0%)	
63			
-	118 (98,3%)	60 (100%)	0,553
T	2 (1,7%)	0 (0%)	
226			
A	119 (99,2%)	60 (100%)	1,000
G	1 (0,8%)	0 (0%)	
19			
A	118 (98,3%)	60 (100%)	0,553
C	2 (1,7%)	0 (0%)	
55			
-	118 (98,3%)	60 (100%)	0,553
T	2 (1,7%)	0 (0%)	
59			
T	119 (99,2%)	60 (100%)	1,000
A	1 (0,8%)	0 (0%)	
275			
T	119 (99,2%)	60 (100%)	1,000
G	1 (0,8%)	0 (0%)	

*p<0,05

When two groups were compared with each other rs1800796 variation in **the** *IL-6* gene was found statistically high in the patient group (p=0,032).

The allelic discrimination in three groups (responders, non-responders, and control group) is shown in Table 4.25.

Table 4.25. Allelic discrimination in *UGT1A1* & *IL-6* in three groups

rs1800795	**Non-responders (n=30)**	**Responders (n=30)**	**Control Group (n=30)**	**p-values**
G	46 (76,7%)	50 (83,3%)	50 (83,3%)	0,56
C	14 (23,3%)	10 (16,7%)	10 (16,7%)	
rs1800796				
G	54 (90%)	56 (93,3%)	60 (100%)	0,052
C	6 (10%)	4 (6,7%)	0 (0%)	
379				
A	60 (100%)	60 (100%)	59 (98,3%)	0,366
G	0 (0%)	0 (0%)	1 (1,7%)	
274				
T	60 (100%)	60 (100%)	59 (98,3%)	0,366
C	0 (0%)	0 (0%)	1 (1,7%)	
115				
C	60 (100%)	60 (100%)	59 (98,3%)	0,366
G	0 (0%)	0 (0%)	1 (1,7%)	
488				
T	60 (100%)	60 (100%)	59 (98,3%)	0,366
G	0 (0%)	0 (0%)	1 (1,7%)	
110				
T	60 (100%)	59 (98,3%)	60 (100%)	0,366
C	0 (0%)	1 (1,7%)	1 (1,7%)	
63				
-	60 (100%)	58 (96,7%)	60 (100%)	0,132
T	0 (0%)	2 (3,3%)	1 (1,7%)	
226				
A	59 (98,3%)	60 (100%)	60 (100%)	0,366
G	1 (1,7%)	0 (0%)	0 (0%)	
19				
A	58 (96,7%)	60 (100%)	60 (100%)	0,132
C	2 (3,3%)	0 (0%)	0 (0%)	
55				
-	56 (93,3%)	60 (100%)	60 (100%)	**0,017***
T	4 (6,7%)*	0 (0%)	0 (0%)	
59				
T	59 (98,3%)	60 (100%)	60 (100%)	0,366
A	1 (1,7%)	0 (0%)	0 (0%)	

275				
T	59 (98,3%)	60 (100%)	60 (100%)	0,366
G	1 (1,7%)	0 (0%)	0 (0%)	

p<0,05

When three groups were compared with each other 55-T insertion in the *UGT1A1* gene was found statistically high in the non-responders group (p=0.017).

4.7.4. The relationship between risk factors and mutations

We did not detect any significant difference between risk factors and genotypes.

5. DISCUSSION

Diuretic drugs help to reduce shortness of breath by removing the edema accumulated in the body due to heart failure through the kidneys. Before the goal of edema treatment is achieved, diuretic resistance may develop in cases where the diuretic response is reduced or lost. In this study, we investigated the effect of *IL-6* and *UGT1A1* mutations on furosemide resistance.

IL-6 levels were found to be decreased in patients using furosemide [77]. The G-C change (rs1800795) defined at position –174 in the promoter region of the *IL-6* gene has functional significance. It's determined that there is a relationship between the - 174G / C mutation (rs1800795) occurring in the *IL-6* gene [78], with systemic-onset juvenile chronic arthritis [79], Alzheimer's disease [80], ischemic stroke [81] and coronary heart diseases, [82-83], cancer [84], diabetes [85], periodontitis [86], and rheumatoid arthritis [87]. In a study which is conducted by Phulukdaree et al., the G allele of rs1800795 on the *IL-6* gene was evaluated in terms of the development of cardiovascular diseases, it was determined that individuals with mutations in this region have an increased risk for the development of cardiovascular disease, especially heart failure [88].

-572 G/C (rs1800796) is a polymorphism identified in the promoter region of IL-6 [89]. It's determined that there is a relationship between the -572 G/C mutation occurring in the IL-6 gene with cardiovascular diseases, coronary heart diseases, and abdominal aortic aneurysms [90], [91], [92], [93].

In a meta-analysis study, it is shown that rs1800795 and rs1800796 variations were detected in a relationship with an increased risk of cardiovascular diseases [94]. In our study, when two groups (patient-control) were compared with each other heterozygous genotype of rs1800796 variation in the *IL-6* gene was found statistically high in the case group (p=0,027). Additionally, when three groups (responders-nonresponders-control) were compared with each other heterozygous genotype of rs1800796 variation in the *IL-6* gene was found statistically high in the non-responders group (p=0,043). When allele distributions are compared between two groups, similarly C allele of rs1800796 was found statistically high in the patient group (p=0,032).

The *UGT1A1* gene encodes the UGT enzyme and performs a chemical reaction called glucuronidation, preventing the accumulation of toxic waste in our bodies. Some genetic variations in *UGT1A1* reduce the enzymatic activity of UGT, which impairs the body's ability to detox and causes toxic substances to accumulate in the body. *UGT1A1* is one of the primary genetic variants responsible for reduced detox ability. Studies on the subject show that mutations in the *UGT1A1* gene play a role in the development of heart failure [95-96].

In a study, it was determined that the rs6742078 variations in the *UGT1A1* gene plays an effective role in the development of HF and cardiac disorders, and increases the risk of developing HF and cardiac disorders [97]. In another study, it was determined that rs887829 and rs4148323 variations in the *UGT1A1* gene triggered the development of HF [98]. In another study, it was determined that rs4148323, rs887829, and rs6742078 variations in the *UGT1A1* gene cause the development of HF [97]. In their study, Sook-Hee An et al. showed that rs887829 and rs4148323 variations in the *UGT1A1* gene trigger the development of HF and cause an increase in heart failure complaints because they cause drug resistance during HF treatment [97].

In our study, we investigated the 1st exon of the *UGT1A1* gene by direct sequencing method. Totally eleven mutations were detected in *UGT1A1*, of which nine of them are novel and nine of them cause amino acid change. Three mutations were found deleterious and six mutations were detected as probably damaging to protein functions. The binding angles of furosemide with wild type and mutant types were determined. Changes in binding sites and bond structures were detected in six of the mutant types. Additionally, when allele distributions of *UGT1A1* mutations were compared between three groups, 55 T-insertion was found statistically high in the non-responders group (p=0,017).

Potassium (K+) is the most important cation for humans. While %98 of K+ is in the intracellular region, %2 of K+ is in the extracellular. K+ is responsible for the contraction and relaxation of the heart. Low potassium level negatively affects the work of the heart. Diuretic drugs used for therapeutic purposes, especially in HF patients, may cause potassium loss in the urine. Potassium retention may also occur with diuretic drugs that retain potassium in the body. The normal value of potassium in the body is 3.6-5.2 mmol/l. [99]. Thomsen RW et al. found that the potassium level increased in patients with HF[100]. Legrand M. et al. was shown that serum potassium level >4,6 mmol/L was

related to increased mortality risk in HF patients [101]. Aldahl M. et al were shown that serum potassium level >4.8 mmol/L was associated with elevated mortality risk [102]. In our study when the K^+ level of the groups was compared with each other, no significant difference was detected (p>0,05).

Sodium is a mineral that has important functions in the organism, such as maintaining electrolytes, fluid, acid-base balance, maintaining normal muscle movements, stimulating nerves, and regulating blood pressure. Its excessive consumption is associated with many diseases such as cardiovascular diseases, cancer, osteoporosis, and especially hypertension. The ideal sodium level is 135-145mEq/L.Gheorghiade M. et al. found a significant association between mortality risk in heart failure and sodium level (138mmol/l or less) [103]. In our study, when two groups (patient-control) were compared with each other sodium level was found statistically high in the control group (p=0,009). When three groups (responders-nonresponders-control) were compared with each other similarly sodium level was found statistically high in control group (p<0,05).

One of the most common comorbid conditions in heart failure is iron deficiency and anemia (HBG<12g/dl) [104]. Lupon J. et al found that HBG levels were inversely proportional to mortality and hospitalization due to HF. While the amount of plasma HBG is 13.5-17.5 g/dL in adult men, this rate is 12.5-15.5 g/dL in adult women [105]. In our study, when two groups (patient-control) were compared with each other HBG level was found statistically high in the control group (p<0,001). When three groups (responders-nonresponders-control) were compared with each other similarly HBG level was found statistically high in control group (p=0,001)

Creatinine has an important role in heart contraction and energy metabolism. Increased serum creatinine level is associated with an increased risk of heart failure. In heart failure expression of the creatine transporter reduces and this causes the development of mortality [106]. While the normal value for serum creatinine is 0.50 mg/dL to 1.40 mg/dL in adults men, this value is 0.50 mg/dL to 1.30 mg/dL in adults women [107]. In a study conducted with hospitalized heart failure patients, the increase in creatinine value was found to be significant. It was determined that the creatinine level should be >0.3 mg/dl [108]. In our study, when two groups (patient-control) were compared with each other creatinine level was found statistically high in the patient group (p<0,001). When three groups (responders-

nonresponders-control) were compared with each other creatinine level was found statistically high in non-responders as well as responders group.

NT-proBNP is mainly synthesized in the ventricles (mainly the left ventricle), stored there, and secreted from there. BNP and NT-proBNP are secreted from myocytes and some from fibroblasts in the perimyocardial region into the circulation via the coronary sinuses. NT-proBNP is a parameter used to help diagnose and monitor heart failure. The NT-proBNP level in the blood can be considered as NT-proBNP <125 pg/ml (<450 pg/ml over 75 years) for healthy individuals [109]. In our study, when two groups (patient-control) were compared with each other NT-proBNP level was found statistically high in the patient group ($p<0,001$). When three groups (responders-nonresponders-control) were compared with each other NT-proBNP level was found statistically high in non-responders as well as responders group ($p<0,001$).

Blood urea nitrogen (BUN) is a nitrogenous end product of protein metabolism [110]. The BUN test, or in other words, blood urea nitrogen, is a type of laboratory test used to measure the urea nitrogen that is found in the blood. The reference range is 10 mg/dL to 20 mg/dL in adults. In children, 5 mg/dL to 18 mg/dL is accepted as the reference value [111]. A result above the reference values of the BUN test may be a precursor of acute or chronic kidney diseases, while a result above the critical value may be a sign of much more serious disorders such as kidney failure [112]. The decrease in blood flow to the kidneys due to these diseases may also cause serious heart diseases such as congestive heart failure [113]. In our study, when two groups (patient-control) were compared with each other BUN level was found statistically high in the patient group ($p<0,001$). When three groups (responders-nonresponders-control) were compared with each other BUN level was found statistically high in the non-responders as well as responders group ($p<0,05$).

Hematocrit is the ratio of the volume of red blood cells to the total blood volume. The normal values of hematocrit vary depending on age and gender, 42-52% in an adult male and 36-46% in a female [114]. In our study, when two groups (patient-control) were compared with each other HCT level was found statistically high in the control group ($p<0,001$). When three groups (responders-nonresponders-control) were compared with each other HCT level was found statistically high in the control group ($p<0,05$).

6. CONCLUSION

In this present study, a possible relation between genetic and environmental factors which play a role in furosemide resistance metabolism in HF patients was investigated. It was determined that rs1800796 variation in *IL-6* and 55-T insertion in the *UGT1A1* gene may play role in furosemide resistance.

This study showed that *UGT1A1* and *IL-6* parameters could be used for the detection of furosemide resistance risk in heart failure patients. However, clinical studies with large cohorts will answer whether it is significant to incorporate polymorphisms in the treatment process of HF.

The changing drug interactions have been detected in the mutant *UGT1A1* gene by the docking study. We thought that effective treatment can be provided for patients with common mutations by performing new drug molecule synthesis in different conformations. Our study can be a guide for effective treatment for HF patients in future studies.

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APPENDIX A: ETHICAL APPROVAL FORM

Marmara Üniversitesi Tıp Fakültesi
Klinik Araştırmalar Etik Kurulu

BAŞVURU BİLGİLERİ	PROTOKOL KODU	09.2019.860
	PROJE ADI	Interlokin-6 (IL-6) ve Urinidin 5'- difosfo-glukuronoiltransferaz 1 (UGT1A1) Mutasyonlarının Kalp Yetmezliği ve Diüretik Direnci Oluşumuna Etkisinin Moleküler Düzeyde Araştırılması
	SORUMLU ARAŞTIRICI ÜNVANI/ADI	Gizem KÖPRÜLÜLÜ KÜÇÜK

KARAR BİLGİLERİ	**Tarih : 04. 10. 2019** **Yukarıda** başvuru bilgileri verilen araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri **dikkate alınarak** incelenmiş ve gerçekleştirilmesinde sakınca bulunmadığı **için Kurulumuzca** onaylanmasına oy birliği ile karar verilmiştir. **Onay sonrasında** yapılacak her türlü proje değişiklikleri (katılımcılar, başlık vb.) veya protokol değişikliklerinin Etik Kurula bildirilerek **proje onayının yenilenmesi gerekmektedir.**

ÜYELER

Unvanı / Adı / Soyadı	Uzmanlık Dalı	Kurumu / EK Üyeliği	Onaylanan Proje ile İlişkisi	Toplantıya katılım	İmza
Prof.Dr. Haner DİRESKENELİ	Romatoloji	M.Ü Tıp Fakültesi/ Başkan	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Prof.Dr. Tülin ERGUN	Dermatoloji	M.Ü Tıp Fakültesi/Başkan Yrd.	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Prof.Dr. Atila KARAALP	Farmakoloji	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ EVET ☐ HAYIR	
Prof. Dr. Şefik GÖRKEY	Tıp Tarihi ve Etik	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Prof.Dr. Handan KAYA	Patoloji	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Prof.Dr. M.Bahadır GÜLLÜOĞLU	Genel Cerrahi	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Prof.Dr. Semra SARDAŞ	Eczacı	M.Ü Eczacılık Fak./Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Prof.Dr. Başak DOĞAN	Diş Hekimi	M.Ü Diş Hekimliği Fak./Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Prof. Dr. Beste Melek AYASOY	Radyasyon Onkolojisi	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Doç. Dr. Elif KARAKOÇ AYDINER	Çocuk Sağlığı ve Hastalıkları	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Doç.Dr. Meltem KORAY	Diş Hekimi	İstanbul Üniv. Diş Hekimliği Fak./Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Doç. Dr. Gürkan SERT	Hukukçu	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Doç.Dr. Figen DEMİR	Halk Sağlığı	Acıbadem Üniv. Tıp Fak.	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Doç.Dr. Pınar Mega TİBER	Biyofizik	M.Ü Tıp Fakültesi/Üye	☐ Var ☐ Yok	☐ Evet ☐ Hayır	
Gözde Aynur MİRZA	Sağlık Mensubu olmayan kişi	Serbest	☐ Var ☐ Yok	☐ Evet ☐ Hayır	