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ENERGY DRINK EFFECT ON ADORA3 GENE EXPRESSION AND STUDY OF ITS GENETIC POLYMORPHISMS ON SOME PHYSIOLOGICAL PARAMETERS AMONG DRINKERS

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May 2023

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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Basil Nassri Hasan HASAN

ABSTRACT

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The prevalence and popularity of energy drinks raises concerns about their effects on human health. Energy drink consumption is predominantly a public health concern among male adolescents and young adults. It is associated with an increase in substance abuse and risky behavior. The most common adverse events affect the cardiovascular, liver damage, kidney dysfunction and neurological systems. Commercial energy drinks are composed primarily of caffeine and numerous other ingredients, many of which have not been adequately studied and are poorly understood, particularly when consumed in excess. The stimulating impact of these beverages are related to their high quantity of caffeine, the most frequently used CNS stimulant in the world, as well as vitamins, taurine, guarana, and other components. Caffeine is responsible for the majority of the biological effects it causes in blocking the activity of all types of adenosine receptors, such as A1, A2A, and A3, that lead to exerts effects in some times on neurons and glial cells of all brain areas. The aim of study to detection the effect of consuming energy drinks on some physiological parameters, such as serum caffeine levels, liver enzymes, urea and creatinine levels, also to the investigate of effect the energy drinks on ADORA3 gene expression and the genotypes on some physiological parameters among the drinks. Totally 100 samples were collected from energy drink users and 20 control samples for comparison, for the period from April 2022 to July 2022. Several physiological and chemical tests (Caffeine, GOT, GPT, ALP, BU and SCR) were conducted to study the effect of energy drink consumption. Also, the genotypes rs923 and rs1544223 were investigated, the gene expression of the ADORa3 gene was studied and the relationship of the genotypes with the gene expression of energy drink consumers. The study found a substantial ($P<0.05$) rise in (Caffeine, GOT, GPT, ALP, BU and SCR) levels among energy drink drinkers as compared to control samples. The research revealed that both case and control samples have the highest prevalence of the rs923 SNP's GG genotype, followed by the GA genotype. Statistical analysis did not show any significant differences in the prevalence of rs923 SNP genotypes. As for the rs1544223 SNP, the study showed that the highest prevalence was for the CC genotype, followed by the CT genotype. Statistical analysis also did not show any significant differences in the prevalence of genotypes between case and control samples.

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Keywords: Energy drink, Molecular, ADORA3, Caffeine

ÖZET

ENERJİ İÇECEĞİNİN ADORA3 GEN EKSPRESYONUNA ETKİSİ VE İÇENLERDE GENETİK POLİMORFİZMİNİN BAZI FİZYOLOJİK PARAMETRELER ÜZERİNE ETKİSİNİN ARAŞTIRILMASI

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Enerji içeceklerinin yaygınlığı ve popülaritesi, insan sağlığı üzerindeki etkileri konusunda endişelere yol açmaktadır. Enerji içeceği tüketimi ağırlıklı olarak erkek ergenler ve genç yetişkinler arasında bir halk sağlığı sorunudur. Madde bağımlılığı ve riskli davranışlarda artış ile ilişkilidir. En sık görülen yan etkiler kardiyovasküler, karaciğer hasarı, böbrek fonksiyon bozukluğu ve nörolojik sistemleri etkiler. Ticari enerji içecekleri öncelikle kafein ve birçoğu yeterince çalışılmamış ve özellikle aşırı tüketildiğinde yeterince anlaşılmamış olan diğer birçok bileşenden oluşur. Bu içeceklerin uyarıcı etkisi, dünyada en sık kullanılan CNS uyarıcısı olan yüksek miktarda kafeinin yanı sıra vitaminler, taurin, guarana ve diğer bileşenlerle ilgilidir. Kafein, A1, A2A ve A3 gibi her türlü adenosin reseptörünün aktivitesini bloke etmede neden olduğu biyolojik etkilerin çoğundan sorumludur ve bu da bazen tüm beyin bölgelerinin nöronları ve glial hücreleri üzerinde etkili etkilere yol açar. Çalışma, enerji içecekleri tüketmenin serum kafein düzeyleri, karaciğer enzimleri, üre ve kreatinin düzeyleri gibi bazı fizyolojik parametreler üzerindeki etkisini tespit etmeyi ve ayrıca enerji içeceklerinin ADORA3 gen ekspresyonu ve genotipleri üzerindeki etkisinin bardaklardaki bazı fizyolojik parametreler üzerindeki etkisinin araştırılmasını amaçlamaktadır. Nisan 2022'den Temmuz 2022'ye kadar karşılaştırma için enerji içeceği kullanıcılarından yüz örnek ve 20 kontrol örneği toplanmıştır. Enerji içeceği tüketiminin etkisini incelemek için çeşitli fizyolojik ve kimyasal testler (Kafein, GOT, GPT, ALP, BU ve SCR) yapılmıştır. Ayrıca, rs923 ve rs1544223 genotipleri araştırılmış, ADORa3 geninin gen ekspresyonu ve genotiplerin enerji içeceği tüketicilerinin gen ekspresyonu ile ilişkisi incelenmiştir. Çalışma, kontrol örneklerine kıyasla enerji içeceği içicileri arasında (Kafein, GOT, GPT, ALP, BU ve SCR) seviyelerinde önemli bir (P <0.05) artış bulmuştur. Araştırma, vaka ve kontrol örneklerinin rs923 SNP'nin GG genotipinin en yüksek prevalansına sahip olduğunu ve bunu GA genotipinin izlediğini ortaya koymuştur. İstatistiksel analiz, rs923 SNP genotiplerinin prevalansında anlamlı bir farklılık göstermedi. Rs 1544223 SNP'ye gelince, çalışma en yüksek prevalansı CC genotipi için ve ardından BT genotipi için olduğunu göstermiştir. İstatistiksel analizler ayrıca vaka ve kontrol örnekleri arasındaki genotiplerin prevalansında anlamlı bir farklılık göstermedi.

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Anahtar Kelimeler: Enerji içeceği, Moleküler, ADORA3, Kafein

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CONTENTS

LIST OF SYMBOLS

bp	Base pair
°C	Celsius degree
cm	Centimeter
dL	Deciliter
g	Gram
h	Hour
μL	Microliter
mL	Mililiter
mg	Milligram
min	Minute
nm	Nanometer
%	Percentage
pmol	Picomole
U	Unit
V	Volt

LIST OF ABBREVIATIONS

ADORA3	Adenosine A3 receptor
AF	Atrial fibrillation
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ARMS	Amplification refractory mutation system
ARs	Adenosine receptors
AST	Aspartate aminotransferase
AT	Annealing temperatures
DNA	Deoxyribonucleic acid
DW	Distilled water
ELISA	Enzyme-linked immunosorbent assay
GGT	Gamma-glutamyl transferase
GOT	Glutamic-oxaloacetic transaminase
NCBI	National center for biotechnology information website
NHANES	National health and nutrition examination survey
OD	Optical density
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription PCR
RBL	Rat basophilic leukaemia cells
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphisms
UTR	Untranslated region
WHO	World health organization

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1. INTRODUCTION

Energy drinks and carbonated soft drinks are being taken by adults and young people worldwide on various occasions, becoming a part of the youth culture (Dwaidy *et al.* 2018, Malinauskas *et al.* 2007). The first energy drink was introduced in the United States in 1949 under the brand name "Dr. Enuf" (Alsunni 2015). In 1987, they were introduced for the first time in Europe. Subsequently, the market extended throughout much of the world, and they were extremely well-known after the introduction of Red Bull in 1997 (Zucconi *et al.* 2013). There is an abundance of energy drinks on the market, and their consumption has increased in prevalence, especially among younger populations, with 42.3% of adolescents (aged 11 to 18) consuming them (Joachim 2019). The consumption of these beverages has been linked to high-risk behaviors, mortality among young people, and negative health impacts, particularly those connected to an increased risk of cardiovascular disease (Higgins and Ortiz 2014; Williams 2022). Sugar and caffeine are the primary ingredients in energy drinks, which gives them their characteristic effects. Caffeine, on the other hand, has been linked to a number of negative health effects, including malfunction in the cardiovascular system, the gastrointestinal tract, the central nervous system and renal dysfunction (Malinauskas *et al.* 2007).

However, various study studies revealed several harmful impacts of energy and soft drink usage. Customers report that these beverages, particularly energy drinks, provide them with the energy they need to deal with difficult circumstances or stay awake for extended periods (Nowak and Jasionowski 2015). A review of the toxicity of energy drinks reported in the National Poison Data from 2010-2011 revealed that major adverse effects included three cases of seizure, two cases of non-ventricular dysrhythmia, one case of ventricular dysrhythmia, and one case of tachypnea. All of these were reported as having occurred as a direct result of consumption of energy drinks (Seifert *et al.* 2013). The stimulating impact of these beverages is related to their high quantity of caffeine, the most frequently used CNS stimulant in the world, as well as vitamins, taurine, guarana, and other components. Caffeine is an adenosine antagonist that stimulates neurotransmitter release and a phosphodiesterase inhibitor that modifies intracellular cAMP levels while also promoting glucose metabolism and lipolysis (Eltahir *et al.* 2020). Caffeine is responsible for the majority of the biological effects it causes in blocking the activity of all types of adenosine receptors (ARs), including A1, A2A, A3, and A2B; then it exerts effects on neurons and glial cells of all brain areas (Ribeiro and Sebastião 2010).

ADORA3 is a gene found on human chromosome 1p21-p13. This gene involves 318 amino acids. The ADORA3 gene contains two exons and one intron of size 2.2 kb separating the two exons (Jacobson *et al.* 2018). The promoter region of the ADORA3 gene contains potential binding sites for many transcription factors and consensus binding sites for transcription factors that are critical to immune function. Numerous studies have linked alopecia variants in the ADORA3 gene to several diseases, including coronary heart disease, autism spectrum disorder and urticaria. Aspirin (Campbell *et al.* 2013, Peculis *et al.* 2011). The testes, spinal cord, different brain areas, the bladder, whole blood, adipose tissue and lung have the greatest amounts of ADORA3 gene expression according to genomic study. The greatest level of expression was 12.4 RPKM. The activation of the region in these tissues lends credence to the potential significance of ADORA3 ligands in the treatment of nervous system disorders and pain (Jacobson *et al.* 2018). A large number of phenotypic variations across and within species are caused by variations in gene expression. Gene expression is a quantitative trait that is susceptible to both natural selection and random drift. The knowledge of human evolution depends on a greater comprehension of how natural selection affects gene expression in primates. This understanding may also provide light on the molecular underpinnings of diversity in quantitative features and illness risk within a species (Fair *et al.* 2020).

On the other hand, clinically highly toxic amounts of caffeine have not been associated to clinically obvious liver injury, despite the fact that caffeine can have severe effects on the function of the brain, heart, kidneys, and muscles (NIDKD 2012). Where a study demonstrated that drinking a lot of energy drinks affects the development of liver damage and increased transaminase levels (Vivekanandarajah and Waked 2011). In contrast, there have been multiple instances of liver damage connected to the consumption of energy drinks that are high in caffeine (NIDKD 2012). These reports do not seem to be very persuasive, and the most of them do not have sufficient documentation.

1.1 Aim of Study

- Studying the effect of consuming energy drinks on some physiological parameters, such as serum caffeine levels, liver enzymes, urea and creatinine levels.
- The influence of inherited variants in the gene roles on the action of energy drinks.
- Comparison of gene expression of ADORA3 gene in different genotypes.

2. LITERATURE REVIEW

2.1 Energy Drinks

Energy drinks are commonly accessible beverages that include caffeine and taurine as well as glucose and various substances that are supposed to boost either physical or mental performance (Higgins *et al.* 2018). Throughout the 1960s, energy drinks initially appeared in Europe and Asia. Following Red Bull's 1987 launch on the European market, an exponential increase in sales was noticed, which was also witnessed ten years later in the United States. Since the advent of bottled water, Energy drinks are the fastest-growing beverage category product. Red Bull Energy Drink is one of the most consumed energy drinks (Heckman *et al.* 2010, Zucconi *et al.* 2013).

Studies in sixteen European countries found that the prevalence of energy drink use among adolescents aged 10 to 18 years was 68%. And 12% of these consumers were "chronic" drinkers. They averaged 7 liters of energy drinks per month. As for the rate of consumption among adults, the previous study showed that 30% of adults consume energy drinks, while only 18% of young people aged between 12-19 years drink one beverage per month (Erdmann *et al.* 2021) (Table 2.1).

Table 2.1 Energy drink ingredients (Al Yacoub *et al.* 2020)

Each portion	**(8 ounces. or 240 milliliters)**
Taurine	1000 mg
Guarana seed extract	100 mg
Caffeine	80 mg
Glucuronolactone	50 mg
L-carnitine	25 mg
B8 (inositol)	25 mg
B3 (niacin)	20 mg
B6	2 mg
B12 (cyanocobalamin)	2 micrograms
B2 (riboflavin)	3.4 mg
B5 (pantothenic acid)	10 mg

2.1.1 Energy drinks ingredients-caffeine and taurine contribution

Caffeine contribution:

ED formulations vary greatly, and producers sometimes do not reveal the exact amounts of specific chemicals. Furthermore, energy drink businesses make no distinction between whatever component, either alone or in combination, determines the drink's supposed good advantages (Heckman *et al.* 2010, Zucconi *et al.* 2013). However, it appears that Energy drinks mostly include caffeine, which could be mixed with additional components, for instance, artificial or natural sweeteners, glucuronolactone, amino acids, B-vitamins, and herbal supplements (Higgins *et al.* 2018, Nowak and Jasionowski 2016). Caffeine can also be found in other chemicals listed on energy drink labels (Nowak and Jasionowski 2016). Vigor juices typically contain between 75 to 240 mg of caffeine (Attipoe *et al.* 2016, Nowak and Jasionowski 2015).

The ergogenic effects of caffeine are related to its biological effect on peripheral organs. Also, CNS. Caffeine motivates the central nervous system by inhibiting the sleep-promoting adenosine action. This causes the brain's serotonin, norepinephrine receptors, and dopamine to release (Ferré 2008). The production of neurotransmitters leads to psychomotor coordination, tiredness delay, and alertness (Barcelos *et al.* 2020, Ferré 2008). Caffeine can also affect proinflammatory cytokine production in the brain via the adenosine receptor subtypes A1R and A2aR. When cytokines, particularly TNF-, IL-1, IL-6, and IL-10, are raised as a result of physical exercise, this trait has the potential to interfere with the inflammatory process. (Barcelos *et al.* 2020). Caffeine's effect on peripheral tissues is more extensive. Caffeine can increase the release of intracellular Ca2+ in muscle cells. Caffeine may exert this action by acting as an opponent of the A1 subtype of the adenosine receptor on the skeletal muscle membrane (Barcelos *et al.* 2020, Tallis *et al.* 2015). In addition to enhanced Ca2+ release, higher myofibrillar Ca2+ sensitivity, better SR Ca2+ permeability, and reduced activity of the SR Ca2+ pump are achieved, resulting in altered muscle function (Domaszewski *et al.* 2021, Tallis *et al.* 2015). Furthermore, investigations have revealed that caffeine treatment increases norepinephrine, circulating adrenaline, cortisol, and plasma lactate while increasing plasma beta-endorphins about two-fold (Laurent *et al.* 2000). These effects may have an influence on endurance by reducing the sensation of pain, influencing how efficiently energy substrates are utilized, and making the body less sensitive to insulin (Fredholm *et al.* 2011, Laurent *et al.* 2000). However, the nature of these metabolic reactions and their influence on the muscles are unknown. Caffeine doses of 40 to 60 mg have been shown to boost cognitive functioning, but a level of 3 mg/kg is necessary to improve performance (McLellan and Lieberman 2012). Caffeine administration (3-6 mg/kg) has an ergogenic impact in exercise tests such as isokinetic peak torque, 1 repetition maximum strength, power output, aerobic vertical jump height, and muscular endurance, according to systematic reviews and meta-analyses (Pickering and Grgic 2019). Some studies found that using caffeine extended the amount of time spent running overall, the distance traveled at sprint energy drinks, and the number of sprints (Salinero *et al.* 2019). Moreover, some studies did not find significant variances among men and women in fatigue index and aerobic performance after consuming caffeine (Mielgo-Ayuso *et al.* 2019). Male athletes, however, created greater power when lifting and sprinting than female athletes. Aside from the effect that caffeine has on a person's physical body, it has been demonstrated through research that a low to moderate intake of caffeine can improve a person's energy level, mood, and other cognitive processes such as focus (Lorenzo Calvo *et al.* 2021).

Taurine contribution:

Taurine a sulfur-containing amino acid that is synthesized automatically by the human body and distributed widely in tissues, stays the second most important part of energy drinks after caffeine (Chepkova *et al.* 2002). Taurine is found naturally in meat and fish. It is also found industrially in nutritional supplements, while it is rarely found in plants, so vegetarians have 20% less muscle structure than those who eat meat. People consume an average of 40 to 400 mg of taurine per day (McCarty 2004). Taurine consumption for humans is thought to range between 40 and 400 mg per day (Finnegan 2003). The amount of taurine is not often specified in the ingredients list of many brands of energy drinks, but a search of 10 brands of energy drinks showed the average taurine content to be 342.28

mg/100 mL (Rai *et al.* 2016). This confirms that the source of taurine in energy drinks is several times higher than its source in food.

Taurine has a widespread series of functional activities, including control of mitochondrial function, control of calcium levels in cells, bile acid conjugation and cytoprotective effects (Ripps and Shen 2012). This amino acid shows a chief part in regulating neurotransmission and contractile function, so it is abundant in skeletal muscles and the head (Chepkova *et al.* 2002, Dutka *et al.* 2014). The effects of taurine appear to be related to various hormones such as growth hormone and norepinephrine, as well as its association with dopamine, but the mechanism of the direct effect on hormone secretion is still unknown (Caine and Geracioti 2016). According to research, taurine may modulate muscle contractile function via a variety of biochemical processes (Dutka *et al.* 2014). The effect of caffeine on physical performance has been widely documented and a systematic evaluation found that an isolated dose of taurine at varying levels (1-6 g) has the potential to increase overall endurance performance (Waldron *et al.* 2018).

There is some evidence to support the hypothesis that combining caffeine and taurine may result in the production of synergistic effects. An isolated dose of 3-6 mg/kg of caffeine supports this theory (McLellan and Lieberman 2012, Pickering and Grgic 2019, Salinero *et al.* 2019) or 1-6 g taurine intake (Waldron *et al.* 2018). stay appropriate to cause an ergogenic effect in individuals (Erdmann *et al.* 2021).

2.1.2 Effects of energy drink consumption that are alarming

The incidence of energy drinks, especially between teen-agers and grown-ups has led to many concerns regarding their safety and health. Companies resort to using tempting phrases to market shot drinks by targeting physical and electronic sports without addressing their harmful effects (Buchanan *et al.* 2018, Pollack *et al.* 2020, Thomas *et al.* 2019). The side effects of consuming energy drinks vary from one person to another, from minor and non-hazardous effects to fatal effects. Energy drinks are usually consumed when exercising sleep deprivation or neurological conditions, and therefore the side effects resulting from the consumption of these drinks are more obvious. Teenagers are more vulnerable to the special effects of caffeine than other constituents in energy drinks because of their smaller body sizes (Azagba *et al.* 2014, Degirmenci *et al.* 2018, Zucconi *et al.* 2013).

Energy drinks have short and long-term effects, and the short-term effects are usually more obvious and manifested in their effect on the nervous system and the circulatory system (Ali *et al.* 2015). According to some studies, there are several effects of consuming energy drinks among adolescents, as studies indicated that (23.1%) suffer from depressive mood, while 23.1% and 35.4% suffer from stress and insomnia, respectively. For consumption of energy drinks among adults, they are: insomnia, nervousness and digestive system disorder, as the prevalence of these symptoms was 24.7% 29.8% and 21.6% respectively. This study found that participants in both groups experienced a number of symptoms that are linked to the consumption of energy drinks. These symptoms included tachycardia, headache, sleeplessness, and increased urine (Nadeem *et al.* 2021). Although modest side effects may be tolerated by energy drink patients, major problems are undesirable.

According to numerous accounts, energy drinks can cause a variety of arrhythmias, the most common of which is atrial fibrillation (AF) (Özde *et al.* 2020). Consuming a large amount of energy drinks may lead to sudden death in healthy people as a result of aortic dissection or sudden coronary vasospasm (Piccioni *et al.* 2021, Somers and Svatikova 2020, Ullah *et al.* 2018). The long-term consumption of energy drinks can lead to weak heart activity as a result of abnormalities in the heart muscle (Munteanu *et al.* 2018).

The metabolic effect of energy drinks has been observed to be conflicting (Shearer *et al.* 2020). they reported that caffeinated drinks might produce acute transitory insulin resistance in healthy teenagers (Shearer *et al.* 2020). Moreover, energy drink consumers were more likely to gain weight due to their consumption of large amounts of junk food as well as their prolonged exposure to screens (Almulla and Faris 2020). A study also found that regular consumption of energy drinks may lead to tooth erosion and obesity due to the high sugar content of their ingredients and the low pH (Clapp *et al.* 2019).

Aside from the previously noted serious consequences of energy drink use, athletes have additional issues. Energy drinks cause diuresis and impede fluid absorption, which can have a detrimental effect, especially in races that need intense resistance, such as marathons, and in hot weather (McLellan and Lieberman 2012, Piccioni *et al.* 2021, Simulescu *et al.* 2019). One of the most prevalent adverse effects is shaky hands (Nadeem *et al.* 2021). It may have a detrimental impact on the result in sports where accuracy is necessary. A study that looked at the relationship between energy drink usage and mental disorders found that young individuals who swallowed energy drinks and engaged in physical exercise showed fewer symptoms of depression (Tóth *et al.* 2020). Similar outcomes were seen in prior trials where no individuals had any negative mental effects (Erdmann *et al.* 2021).

2.2 A3 Adenosine Receptor

Adenosine is a purine nucleoside. It acts as a functional tissue regulator, which was dubbed a 'revenge metabolite' in the 1980s (Fredholm 2014, Fredholm *et al.* 2011). Adenosine is found throughout the body. Where all cells secrete it and it is produced outside the cell by a group of external enzymes (Zimmermann 2000) (Table 2.2).

Table 2.2 Exon appearance used for the ADORA3 gene in several human tissues (Jacobson *et al.* 2018)

Tissue	RPKM[b]
Testes	12.401
Brain (spinal cord, cervical C-1)	5.612
Brain (substantia nigari)	4.268
Adrenal gland	3.884
Spleen	3.495
Small intestine (terminal ileum)	2.778
Brain (amygdala)	2.405
Brain (hypothalamus)	2.201
Nerve (tibial)	2.102
Brain (hippocampus)	1.99
Bladder	1.764
Lung	1.747
Adipose (subcutaneous)	1.73
Whole blood	1.709
Colon (transverse)	1.604
Artery (coronary)	1.517

Under physiologically unfavorable situations, adenosine concentrations rise. Tissue hypoxia, for example, causes increased ATP breakdown and adenosine production. Hypoxia-dependent regulation of the salvage enzyme adenosine kinase, which rephosphorylates the nucleoside to AMP, may also help with adenosine release. Adenosine acts through the A1, A2A, and A2B adenosine receptors (ARs) to produce its effects (Borea *et al.* 2015).

2.2.1 Identification of the A3 adenosine receptor

The ADORA3 was proposed around three decades ago to identify the kind of ARs involved in adenosine's inhibitory activity at the frog neuromuscular junction. It was suggested that the brain included a separate AR that was linked to Ca2+ metabolism. However, this was not the same ADORA3 that was eventually cloned. Another step toward defining ADORA3 was the discovering trendy antigen-stimulated RBL-2H3 cells. The ligand was initially unnamed, but further study gave it the name ADORA3 (Borea *et al.* 2015).

2.2.2 Adenosine receptor A3 molecular characterization

ADORA3 has been cloned in humans, sheep, and mice. Differences in the structure of ADORA3 have emerged between species. Mice are similar to humans and sheep in only 74% of their sequences, while ADORA3 showed 85% similarity to the sequence between sheep and humans. This is mirrored in the species homologs' widely diverse pharmacologic profiles, particularly in terms of antagonist binding, making identification of this AR subtype problematic (Brandon *et al.* 2006).

ADORA3, which comprises 318 amino acid residues, takes stayed found on humanoid chromosomes 1p21-p13. The ADORA3 gene has been shown to have twofold exons divided via a particular intron of roughly 2.2 kb (Borea *et al.* 2015).

2.2.3 Receptor adenosine ligands: medicinal chemistry and pharmacology

Agonists:

Numerous substitutions of the maternal nucleoside, adenosine, resulted in potent and selective ADORA3 agonists. The structural changes involve N6-, C2-, and 59-substitutions as well as ribose moiety alteration (Coppi *et al.* 2022).

Antagonists:

A vast numeral of heterocyclic composites stood produced besides tested by way of ADORA3 antagonists in an initial effort (Jacobson *et al.* 2009).

2.2.4 Distribution of the A3 adenosine receptor

The ability to identify the tissue distribution of the A3 adenosine receptor has been made possible by the creation of cDNA for ADORA3. ADORA3 is extensively expressed in rats, sheep, and humans, with mRNA, is popular in the testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon, and eye (Burnett *et al.* 2010). However, there is significant variability in fashionable manifestation levels within and between species. However, there is major changeability in expression ranks, in particular, containing significant quantities of A3 mRNA, whereas most other rat tissues have modest levels. The human lung and liver contain high levels of A3 mRNA expression, but levels in the brain and aorta are minimal. In sheep, the spleen, lung, pineal gland, and pars tubercles have the greatest quantities of A3 mRNA (Nishat *et al.* 2016).

The presence of the ADORA3 protein has been tested in a range of tissues and cell lines using radioligand binding, immune, and functional tests. ADORA3 binding sites have been detected in the brains of mice, rats, gerbils, and rabbits at a broad, but relatively low level (Borea *et al.* 2015). Because of this low expression, several investigators have reported that in situ hybridization assays cannot discover the ADORA3 gene or binding site in the CNS (Rivkees *et al.* 2000). ADORA3 expression in the thalamus and hypothalamus has been described by others (Yaar *et al.* 2002). biochemical data and electrophysiologic on the other hand, put forward the existence of ADORA3 cutting-edge rat cortex (Brand *et al.* 2001, Lopes *et al.* 2003). Its occurrence in the retinal ganglion cells and brain has also been demonstrated in functional tests (Zhang *et al.* 2010). Pharmacologic and immunohistochemical investigations have recently confirmed the presence of ADORA3 by mechanical nerve terminals (Cinalli *et al.* 2013). ADORA3 expression has been identified at the cellular level of voguish microglia and astrocytes, the CNS's local immune cells (Gessi *et al.* 2013, Ohsawa *et al.* 2012).

There isn't any concrete proof that ADORA3 is present in cardiomyocytes (Peart and Headrick 2007). However, several studies have connected it to cardio protection in a variety of species and models, including isolated cardiomyocytes and myocardial preparations (Xu *et al.* 2006). ADORA3 expression was found to be highly expressed in human coronary and carotid arteries (Janes *et al.* 2016).

The ADORA3 initially detected hip enteric neurons and epithelial cells by immunohistochemistry and then measured in intestinal mucosa by radio ligand binding tests (Gessi *et al.* 2004, Ren *et al.* 2011). Through radioligand binding and immunohistochemistry experiments, the presence of ADORA3 has been established in human lung parenchyma and bronchi (Calzetta *et al.* 2011).

ADORA3 was found in a wide range of primary cells implicated in inflammatory reactions. Binding assays with rat basophilic leukemia cells (RBL-2H3) revealed a high ADORA3 density, and many investigations have suggested that ADORA3 contributes to the decomposition of mast cells in rats (Borea *et al.* 2015, Gomez *et al.* 2011). Finally, ADORA3 protein expression was shown to be extremely high voguish range of cancer cell lines and tissues (Madi *et al.* 2013, Nagaya *et al.* 2013, Varani *et al.* 2011).

2.2.5 A3 adenosine receptor's therapeutic applications

Central nervous system:

The significance of ADORA3s in a variety of illnesses is frequently debatable, depending on acute and chronic agonist treatment. It has been proposed that ADORA3s protect neurons during the early stage of ischemia by reducing synaptic transmission (Rivera-Oliver and Díaz-Ríos 2014). However, when ADORA3 is stimulated for an extended period, the paraphernalia shift from protective to harmful (Pugliese *et al.* 2007).

ADORA3s have been shown to have a helpful effect on brain development (Boison 2007). Adenosine antagonists may offer therapeutic prospects in many types of epilepsy in humans, as ADORA3s destabilize GABA currents in many epileptic tissues (Roseti *et al.* 2009).

Cardiovascular system:

ADORA3 activation appears to have direct cardioprotective effects in newborn rat cardiomyocytes, according to many studies (McIntosh and Lasley 2012). Even though ADORA3 expression is low in cardiac material, a numeral of the readings have shown that severe management with agonists elicits beneficial "anti-ischemic" effects (Chanyshev *et al.* 2012). These findings were similarly verified in ADORA3-overexpressing mice, where infarct size was less following in vivo regional IR in wild-type animals (Shneyvays *et al.* 2004).

Pulmonary system:

Adenosine is important in the regulation of the respiratory system, and high amounts of this nucleoside have been identified in the bronchi of illness with asthma and chronic obstructive pulmonary disease (Salvatore *et al.* 2000).

Immune system and inflammation:

ADORA3s show a role in the physiological regulator of inflammatory and immune processes (Haskó and Cronstein 2013). ADORA3, which mediates suppression of chemotaxis and oxidative burst with anti-inflammatory action, has a profound influence on neutrophil behavior (Van Der Hoeven *et al.* 2008).

Osteoarthritis and rheumatoid arthritis:

Rheumatoid arthritis is a disease that affects 1% of the world's population and is a chronic autoimmune condition of unknown origin. Rheumatoid arthritis must be treated early with appropriate medications to avoid negative effects (Flögel *et al.* 2012).

ADORA3s increase popular rheumatoid arthritis, psoriasis, and Crohn's disease. Indications propose that ARs may take a significant character in the treatment of rheumatoid arthritis, as adenosine synthesis has emerged as a cellular mechanism to regulate inflammation (Ochaion *et al.* 2009).

Muscle system:

Because skeletal muscle is the most sensitive tissue in the extremities, it is wellknown that IR may cause substantial harm (Blaisdell 2002). As a result, preserving skeletal muscle against IR assault is a critical goal of treatment to alleviate muscle and organ harm (Tsuchida *et al.* 2003).

Eye diseases:

ADORA3s has been linked to a variety of eye disorders, including dry eye, uveitis, and glaucoma. In glaucoma-associated pseudo exfoliation syndrome, levels of ADORA3 mRNA and protein are higher compared to normal eyes (Schlötzer-Schrehardt *et al.* 2005).

2.3 Tetra Arms PCR

Because of their prevalence in the genome and relative ease of analysis, single-nucleotide polymorphisms (SNPs) have been regarded as one of the most important genetic markers then the conclusion of the Human Genome Project in 2003 (Collins *et al.* 2003). As also the International HapMap Project in 2005 (Altshuler *et al.* 2005). The most fundamental form of polymorphism that can occur between two genomes at random is called an SNP. When we say that something is a mutation, we mean that at a certain spot, one nucleotide is changed for another. By definition, this must happen in more than 1% of the population (Shastry 2002). It occurs with a frequency of approximately 800 base pairs (bp) per genome, which contains approximately 9 million SNPs (Rocha *et al.* 2006).

Genome-wide association studies, which examine the relations among drawn SNPs and their incidence in common complex illnesses in large patient populations have revolutionized the study of many diseases in recent years (Manolio *et al.* 2008). The increase of high-throughput genotyping techniques has ushered in a new age for genetic and genomic research (Bui and Liu 2009). More than ever, scientists are generating genomic data weekly and increasing the number of well-validated gene-disease connections (Feero *et al.* 2010).

The polymerase chain reaction (PCR) is now the method of choice for low- and medium-throughput SNP genotyping (Chuang *et al.* 2008). There are many available PCR techniques. Choosing the optimal PCR technique is the key to the success of any experiment. Therefore, when choosing several, things must be taken into account, including the availability of equipment, the number of samples, the type of polymorphism and the accuracy of genotyping (Hamajima *et al.* 2000).

Tetra-primer amplification refractory mutation system-PCR (ARMS-PCR) is a simple and inexpensive genotyping approach in which genotyping can be obtained at a lower cost and requires only a particular PCR reaction followed by gel electrophoresis (Ye *et al.* 2001). This technique depends on the use of special primers that are not identical at the ends, which makes the specific primer to one allele only and does not produce a result at the other allele. In this case, DNA polymerase can only elongate the first primer if its 3' end is fully opposite the template. A PCR amplicon is generated when this condition occurs. Target DNA can be genotyped by determining whether or not an amplicon is a component (Kwok 2001). The mismatch in a tetra-primer is positioned amid the allele-specific primers and four primers are used for the reaction. In the ARMS method, mismatches are located at the 3' ends of the allele-specific primers, and five primers are utilized. Another contrast is that tetra-primer genotyping is performed in a single reaction with two unique annealing temperatures (AT), one higher in the early cycles and one lower in succeeding cycles. This allows the genotyping process to be completed more quickly (Ye *et al.* 2001).

In a tetra-primer ARMS-PCR, four primers are used simultaneously. Two of the primers (called inner primers) amplify the entire target region. The allele-specific fragments will be generated by following the pattern laid forth by the fragment of the external primer, which acts as a template for the two allele-specific primers (inner primers) that will be utilized to construct them (Rubio *et al.* 2008). The two allele-specific fragments on an agarose gel may be differentiated by inserting the external primers at varying distances from the polymorphic nucleotide (Ye *et al.* 2001) (Figure. 2.1).

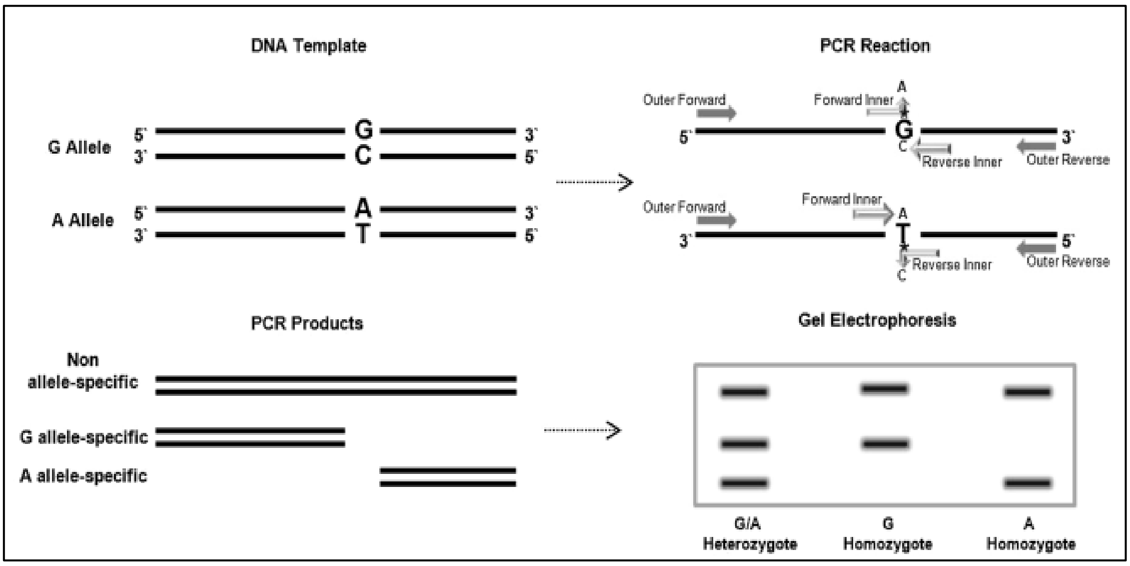

Figure 2.1 Tetra-primer ARMS–PCR assay for SNP genotyping (Medrano and De Oliveira 2014)

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment and devices

The devices and tools used in the study are shown in Table 3.1.

Table 3.1 Devices and tools used in the study

No	Equipment	Origin	Processing company
1	AIA- 360 Tosoh	Japan	Tosoh
2	AURA TM PCR Cabinet	Italy	Sigair
3	Autoclave	England	Arnoldand
4	Balance	Germany	Kernpfb
5	Centrifuge	Taiwan	GEMMY
6	Combi-spin	Lative	Biosan
7	Cool box	India	Blow kings
8	Deep freezer -20℃	Korea	Kelon
9	Digital Camera	Japan	Sony
10	Document system	USA	Labnet
11	Gel electrophoresis	USA	CBS, Scien.
12	Mini-centrifuge	Germany	Bio San
13	Incubator	China	Jrad
14	Micropipette	Germany	Slamed
15	Micro spin	Lativa	Biosan
16	Microwave	China	Gosonic
17	Power Supply 300V, 2200V	China	Wenzhou
18	Gradient Thermal Cycler	USA	Labnet
19	Nano drope	USA	Thermo
20	Refrigerator (2-10℃)	Korea	Kelon
21	Spectrophotometer	Japan	APEL
22	UV. Trans illuminator	France	Vilber lour.
23	Water bath	China	HH-2

3.1.2 Glassware

Table 3.2 indicate the glass and plastic tools and instruments used in the study, as well as their origin and the company that supplied them.

Table 3.2 The glass and plastic instruments used in the study

No	Equipment	Origin	Processing company
1	Disposable syringe	U.A. E	Medeco
2	EDTA tube	Jordan	AFMA
3	Gel tubes	Jordan	AFCO
4	Graduated Cylinders	England	Volac
5	Flasks	England	Volac
6	Plastic disposable	Denmark	Nunclon
7	Microcentrifuge tubes	Italy	LP Italiana SPA
8	Can tubes	China	Plastech
9	Tornica	China	BSmed
10	Medical Cotton	China	BSmed
11	Wound tape	China	Flmed
12	Tips (Yellow, Blue, white)	China	CNWTC
13	Eppendorf Tube 1.5 mL	Jordan	AFCO

3.1.3 Chemicals and laboratory supplies

Lists the chemicals and laboratory equipment used in this study was showing in Table 3.3 along with an explanation of their origin and the company that manufactures them.

Table 3.3 The chemicals utilized in the study

No	Equipment	Origin	Processing company
1	Urea Kit	Spain	Linear Chemicals
2	GOT (AST) Kit	France	BIOLABO
3	GPT (ALT) Kit	France	BIOLABO
4	ALP Kit	France	BIOLABO
5	LDH Kit	Spain	Linear Chemicals
6	S. Creatinine Kit	Spain	Linear Chemicals
7	DNA Genomic DNA Mini Kit	USA	Geneaid
8	Agarose	USA	Conda
9	Ethidium bromide	Korea	Intron
10	Loading dye 6X	Korea	Intron
11	DNA Markers Sizer (100bp)	Korea	Intron
12	Premix pcr	Korea	Intron
13	TBE buffer 10 X	USA	Conda
14	Specific Primer	USA	Bioneer
15	Distal Water	France	Panpharma
16	Absolute Ethanol	England	BDH Chemical
17	Medical alcohol	Iraq	Samara

3.2 Methods

3.2.1 Collection of study samples

Samples for the study were collected from April 2022 to July 2022. The study involved gathering 100 blood samples. from males consuming energy drinks (Redbull, Tigerm Powerhorse and Bison) with an age group ranging from (20-40) years and 20 males not consuming energy drinks as a control group. Samples were collected from Al-Ramadi Teaching Hospital and the main blood bank in Al-Anbar Governorate, Iraq. The study was done with their consent as donors and their phone numbers were taken to inform them of the results and to keep the information confidential.

For this, a questionnaire with questions about name, age and personal information was created in advance.

- How long have you been drinking energy drinks?
- What is the kind of energy drink use?
- Do you have any chronic illnesses? And what types of medicines were used.

After obtaining the consent of all participants in the study and accordance with the rules of ethics for scientific research.

3.2.1.1 Method for collecting and preserving blood samples

After sanitizing the site of the withdrawal with ethanol by the side of concentration of 70%, 5 mL of venous blood from the study subjects was drawn with a disposable syringe. A plastic tube containing EDTA K2 anticoagulant was then filled with 2.5 mL of the withdrawn blood and gently shaken for 5 minutes. The full blood picture was then examined, and the final sample was put into a gel tube that did not contain EDTA. The gel tubes were then put into a centrifuge at 3000 rpm for 10 minutes. Other blood components were separated from the serum and put into 1.5 mL Eppendorf tubes.

3.2.2 Biochemical tests

Automated devices were used to perform the examinations. The method of work was mentioned according to the manual method to show the principle of each examination.

3.2.2.1 Kidney functions

Measuring of serum urea level:

Measuring serum urea by using a LINEAR CHEMICALS, S.L.U SPAIN kit (Tietz 1999, Kind and King 1954).

The basic principle:

Ammonia and CO_2 are produced when urea is hydrolyzed and the ammonia then reacts with salicylate and hypochlorite to produce green Indophenol. The following Equation (3.1) shows a color intensity comparative to the amount of urea in the blood serum.

$$\text{Urea} + H_2\text{O} \xrightarrow{Urease} CO_2 + 2NH_3 \quad (3.1)$$

Solutions needed to determine urea concentration:

1. Reagent solution (R1)

- Enzyme reagent (Urease) / 500 U/mL

2. Reagent solution (R2)

- Buffered chromogen
- Phosphate buffer (PH 6.9) / 20 mmol/L

- EDTA / 2 mmol/L
- Sodium Salycilate / 60 mmol/L
- Sodium nitroprusside / 3.4 mmol/L

3. Reagent solution (R3)

- Alkaline hypochlorite
- Sodium hypochlorite / 10 mmol/L
- NaOH / 150 mmol/L

4. Standard

- Urea/50 mg/dL

Preparing working reagent:

R1 was mixed with R2 and kept for 4 weeks at 2-8°C or for 1 week at room temperature. R3 is ready to work until the permeability date

Procedure:

For each of the three tubes, 1 mL of the working reagent (R1+R2) was used. Then, 10 mL of distilled water, 10 mL of blood serum, and 10 mL of the standard solution were each added, thoroughly combined.

Then incubated for 10 minutes at a temperature of after 10 minutes of mixing and incubation at a temperature of 15 to 25°C, 1 mL of R3 was added to the three tubes. The absorbance was then measured at a wavelength of 580 nm, as displayed in the resulting (Table 3.4).

Table 3.4 Procedure to the determination of serum urea level

Tube	Blank	Sample	CAL. Standard
In work reagent	1.0 mL	1.0 mL	1.0 mL
Taster	-	10 µL	-
CAL. Standard	-	-	10 µL
R3	1.0 mL	1.0 mL	1.0 mL

Quantification:

Quantification is calculated by Equation (3.2).

$$\text{Urea Concentration (mg/dL)} = \frac{\text{A.Sample}}{\text{A.Standard}} \times \text{Standard Conc. (mg/dL)} \qquad (3.2)$$

Where (A) Absorbance

Serum creatinine test:

The serum creatinine concentration was measured using a kit manufactured by Linear Chemicals (Tietz 1999) which consisted of the following materials (Table 3.5):

Table 3.5 Procedure to the determination of serum Creatinine level

Reagent	Contents	Concentration
R1	Picric acid	25 mmol/L
R2 Alkaline buffer	Phosphate buffer SDS	300 mmol/L 2.0 g/L (w/v)
CAL. Standard		2mg/dL

The method of work:

- Equal quantities of solutions R1 and R2 were mixed to form a working reagent solution.

- Two clean test tubes were prepared and the solutions and samples were added to them as shown in Table 3.6.

Table 3.6 Reagent used in the tests

Reagent	Test	Standard
Working reagent	1.0 mL	1.0 mL
Sample	100 µL	-
Standard	-	100 µL

- Solutions were mixed well, then after 30 seconds the absorbance was read for each of the samples and the standard solution, then after 90 seconds the absorbance was read again at a wavelength of 510 nm.

The accounts:

Creatinine concentration = (second reading - first reading) for the sample/(second reading -first reading) for the standard solution x the concentration of the standard solution.

As: the first reading represents the absorbance after 30 seconds.

The second reading represents the second absorbance after 90 seconds.

3.2.2.2 Liver functions

Determination of alanine aminotransferase activity (ALT):

The analysis kit (Kit) from the French company (Biolabs) was used, and it employed the enzymatic method. The ALT enzyme works on the base substances alanine and alpha-ketoglutaric acid and converts the amino acid directly to pyruvate, which then readily transforms the complex resulting from hydrazine into a reddish-brown color. By incorporating the chemical (2-4 dinitrophenylhydrazine), whose color intensity is gauged at a 505 nm wavelength (Tietz 1999, Reitman and Frankel 1957).

As in the following Equation (3.3).

$$L - \text{Alanine} + 2 - \text{Oxaglutarate} \xrightarrow{ALT} \text{Pyruvate} + L - \text{glutamate} \quad (3.3)$$

Procedure:

0.5 mL of Reagent No. 1 (R1) solution was poured into a test tube. The tube was then kept in the incubator for five minutes at 37°C. It was then taken out of the incubator and given 0.1 mL of serum. The tube was thoroughly mixed before being put back in the incubator for another 30 minutes. After this time, it was taken out of the incubator once more, and 0.5 mL of reagent solution No. 2 (R2) was added to it. After thoroughly mixing the contents of the tube and letting them sit at room temperature for 20 minutes, 5 mL of NaOH was added, and after another 5 minutes, the absorbance of the mixture was measured at 505 nm.

Determination of aspartate aminotransferase activity (AST):

The analysis kit (Kit) from the French company (Biolabo) was utilized in the enzymatic method. The process depends on the enzyme's capacity to break down the starting materials aspartic acid and alpha-ketoglutarate into amino-aspartic acid and alpha-ketone (pyruvic acid), which can then be converted into a derivative compound by adding the chemical (4-2 dinitrophenylhydrazine) a reddish-brown color was produced. The intensity of the color was then measured, which is directly correlated with the action of the AST enzyme in the blood serum and the enzyme activity was then calculated in U/L units. as previously stated (Tietz 1999, Reitman and Frankel 1957).

As in the following Equation (3.4).

$$L - \text{Aspartate} + \text{Oxaglutarate} \xrightarrow{AST} \text{Oxalacetate} + L - \text{glutamate} \quad (3.4)$$

Procedure:

The reagent solution R1 0.5 mL was poured into a test tube. The tube was then kept in the incubator for five minutes at 37°C. It was then taken out of the incubator and given 0.1 mL of serum. After thorough mixing, the tube was placed back in the incubator for another hour. Then it was removed once more. After thoroughly mixing the contents of the tube and letting them sit the side of room temperature for 20 minutes, 5 mL of sodium hydroxide was added, and the mix was left alone for five minutes. At that point and measured at 505 nm, after measuring the absorbance intensity in the spectrophotometer was calculated as the absorbance of the AST enzyme, from which the enzyme activity was calculated in U/L units.

Determination of alkaline phosphates activity in serum:

The activity of alkaline phosphatase enzyme in serum was estimated using a ready-made kit from the French company (Biolabo) (Kind and King 1954).

Principle:

The chromatic intensity of the activity to the alkaline phosphatase was measured according to the following Equation (3.5).

$$\text{Phenyl phosphate} \frac{\text{Alkaline phosphatase}}{PH10} - \text{Phenol} + \text{Phosphate} \tag{3.5}$$

The liberated phenol was measured in the presence of potassium ferricyanide and 4-amino antipyrine to turn into a red complex, as its absorbance was measured at 510 nm, while the presence of sodium arsenate stopped the enzymatic reaction (Table 3.7) (Tietz 1999).

Table 3.7 Components and concentrations alkaline phosphates activity in serum

Solutions	Components and concentrations
Sub Strate Buffer	Di sodium phenyl phosphate Carbonate-bi carbonate5 mmol/L Buffer PH 10 50 mmol/L Sodium Merthiolate0.1 g/L
Standard	Phenol equal to arsenate 20 king and king
Blocking Reagent	4-amino anti pyrine 60 mmol/L Sodium arsenate 75 g/L
Color Reagent	Potassium ferricyanide 150 mmol/L

Procedure:

Four plastic tubes were used and the following solutions were placed in them (Table 3.8):

Table 3.8 Procedure used for alkaline phosphates

No	Sample tube	Hand tube	Standard tube	Hand solution tube
Sub Strate Buffer	2 mL	2 mL	2 mL	2 mL
It was incubated at 37°C in a water bath for five minutes after which:				
No	**Sample tube**	**Hand tube**	**Standard tube**	**Hand solution tube**
Standard	50 µl	—	—	—
	—	—	50 µL	—
Incubated for 15 minutes at 37°C, then:				
No	**Sample tube**	**Hand tube**	**Standard tube**	**Hand solution tube**
Blocking Reagent	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Mix well and add to it:				
No	**Sample tube**	**Hand tube**	**Standard tube**	**Hand solution tube**
Color Reagent	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Serum	—	50 µL	—	—
Distilled water	—	—	—	50 µL

They were mixed well and left for ten minutes in a dark place after which they were measured by a spectrophotometer at 510 nm.

Calculation:

The absorption of the solution was read in the spectrophotometer, also a concentration of the enzyme in the serum was calculated according to the following Equation (3.6).

Alkaline phosphatase concentration=absorbance of the sample solution-absorbance of the saline solution/absorbance of the standard solution x n. (3.6)

3.2.2.3 Quantity of caffeine concentration

The concentration of caffeine was examined depending on the kit supplied by the American company Bio Vision and depending on the steps attached to the kit. Caffeine (1,3,7-tri methylxanthine) is one of the central nervous system stimulators used to raise blood pressure and reduce stress. The kit can measure minimum limits by an amount 0.3 ng/mL of serum, saliva, or urine samples within a period not exceeding 90 minutes.

1- Standard reference samples.

All reagents, standards and samples were prepared according to the recommendations attached to the examination kit, as 1.5 mL of standard buffer was added to the standard solution tube to dissolve and obtain a concentration (of 27 ng/mL). Tube No. (5) Then 5 tubes containing 1 mL of standard buffer were prepared and 500 microliters were transferred from tube No. (5) sequentially after mixing them into adjacent tubes.

2- Dilution of the serum sample.

Serum samples were diluted with serum solution through put 20 µL of the solution into 180 µL of serum, mixed well and kept for 45 minutes (dilution factor x 40).

3- Fifty µL of standards or samples were added to each hole. Put 50 µL of conjugate working solution and 50 µL of antibody in all pits containing the control or study sample

4- The microliter plate was covered with an airtight bag. The plate was incubated at room temperature 25°C for 45 minutes.

5- All reagents were discarded and each pit was washed by putting 250 µL of 1X wash buffer and incubating for 30 s. The solution was removed well from the pits and the washing process was repeated 3 times.

6- 100 µL of TMB was added to each hole. The plate was then pressed and shaken to ensure complete mixing.

7- The samples should be read at OD 650 nm for the non-caffeinated pit (S0). When its reading is between approximately 0.8 and 1.0. Add 50 µL of the stop solution and tap lightly on the plate to ensure thorough mixing.

8- OD was measured at 450 nm for standards and samples. As the absorbance is on the axis and the focus is on the axis. The standard curve was then drawn by the ELISA Reader as a relationship between the standard concentrations and the absorbance on the linear drawing paper. The absorbance values of the samples were then projected onto the curve to determine the caffeine concentration for each sample (Figure 3.1).

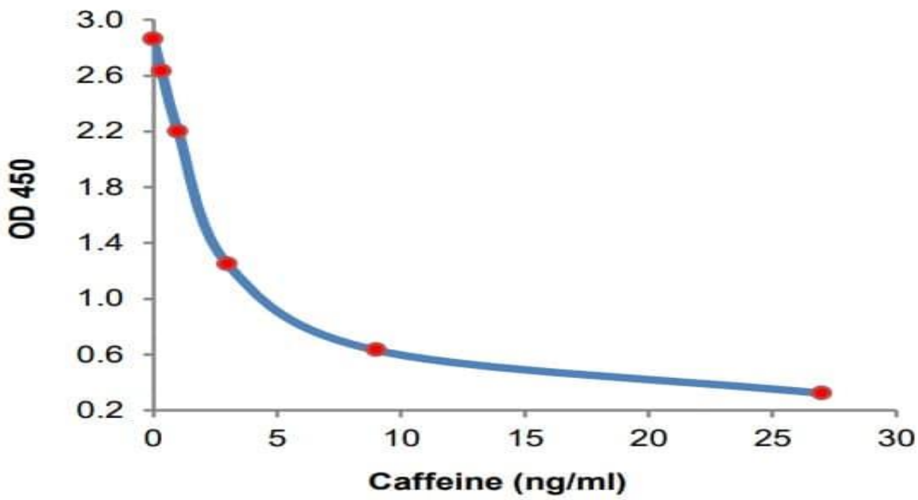

Figure 3.1 Standard curve for Caffeine

3.2.3 Molecular study

3.2.3.1 DNA extraction genomic from blood

The genomic DNA was extracted from the blood of an energy drinker, who numbered 100 samples, as well as the individuals of the control sample, which numbered 20 samples agreeing with the extraction method approved by the processing company (Geneaid) of the United States.

- 200 μL of blood were micropipette-transferred to the 1.5 μL microcentrifuge tubes for each sample participant, followed by the addition of 20 μL of proteinase K solution, which was thoroughly mixed by shaking for 10 seconds. After adding 200 μL of GSB buffer solution to each tube, the mixture was swirled for ten seconds using a vortex device. The tubes were then placed in an incubation bath at 70 °C for ten minutes while the sample was agitated every three minutes. During the time that the sample was allowed to incubate, the elution buffer solution was heated to 60 °C in a water bath so that it would be appropriate for use in the subsequent processes.
- 200 μL of 99% pure absolute ethanol was added to each tube and quickly shaken for 10 s (in case of sedimentation, it must be broken with a pipette).
- Two mL Collection tubes were filled with GS columns, which were then filled with the mixture and centrifuged at 14.000–16.000 rpm for two minutes.
- In place of the 2 mL collection tubes that had previously been removed, GS Column columns were placed in brand new 2 mL collection tubes.
- 400 μL of the W1buffer solution was added to each GS golumn and it was then centrifuged at a speed of 14.000–16.000 rpm.
- GS Column columns were put in new 2 mL collection tubes after the 2 mL collection tubes were removed. Each GS Column was given 400 μL of W1buffer solution, which was at that time centrifuged at 14.000–16.000 rpm for 30–60 seconds. Then, wash buffer, which had 99% absolute ethanol added to it, was added.
- After discarding the precipitate, the centrifugation procedure was carried out once more for three minutes at a speed of 14.000–16.000 x g.

- At room temperature for three minutes, 100 μL of ddH2O and 100 μL of elution buffer (Elution Buffer is put to the center of the columns) were added to 8-GS Columns inserted in 1.5 mL microcentrifuge tubes. centrifuged at 14.000-16.000 x g for 30 seconds.
- GS Column was taken out. 1.5 mL microcentrifuge tubes that had been ultra-frozen, the isolated DNA was kept until the remaining molecular experiments were complete.

3.2.3.2 Determination of concentration and purity for DNA

The purity also the concentration of the extracted DNA was estimated by following the method described by Sambrook *et al.* (1989). By measuring the absorbance of the ultraviolet spectrum at the wavelength 260 nm using a spectrophotometer, where the extracted DNA was diluted for each sample 100 times using water. Distilled, as 20 μL of DNA was taken for each sample and 1980 μL of distilled water was added to each of them, then the following Equation (3.7) was applied.

DNA concentration μg/μL=absorbance reading per 1 mL of sample x dilution inverse (100) x 50/1000 (3.7)

As for the purity of the extracted DNA, it was estimated by dividing the absorbance reading by the side of the wavelength 260 nm by the reading of the absorbance at the wavelength 280 nm and these are Equation (3.8).

Purity ratio of DNA=O.D 260 nm/O.D 280 nm (3.8)

3.2.3.3 Agarose gel electrophoresis

According to stated Sambrook *et al.* (1989). Extracted DNA samples and the result of the PCR technique were discovered by using electrophoresis on an agarose gel:

- To create an agarose gel with a 1.5% concentration and identify the extracted DNA, 100 mL of TBE buffer 1X (Tris base Boric acid EDTA) dissolve 1.5 agarose gel, microwaved for 1-2 minutes and then allowed to cool.
- Install the gel mold (Tray) of the electrophoresis apparatus, place the comb (Wells) used to load the DNA samples and pour the agarose gel gently at a distance of 1 cm from one of the two ends of the mold. Allow resting horizontally for 30 minutes to harden.
- Remove the comb and place the gel and the mold inside the electric relay device's tank, which is occupied with TBE 1X buffer, so that the buffer covers the gel's surface.
- DNA loading in 3 µL for each sample 5 µL of the extracted DNA was combined with dye before being carefully lowered into the pits.
- Load the DNA ladder 100 bp in the case of electrophoresis for the detection of duplicated DNA using the PCR technique to determine the MW of the resulting DNA fragments.
- Next, supply power was connected to the tank, which held the ethidium bromide dye, using wires and positive and negative electrodes. The power supply was then turned on and fixed to 70 volts and two centivolts for 1-2 hours, after which it was turned off and the experiment was finished. electric switch.
- Taken out of the gel from the electrophoresis tank, examined by ultraviolet light using the gel documentation device and photographed using the attached digital camera (Figure 3.2).

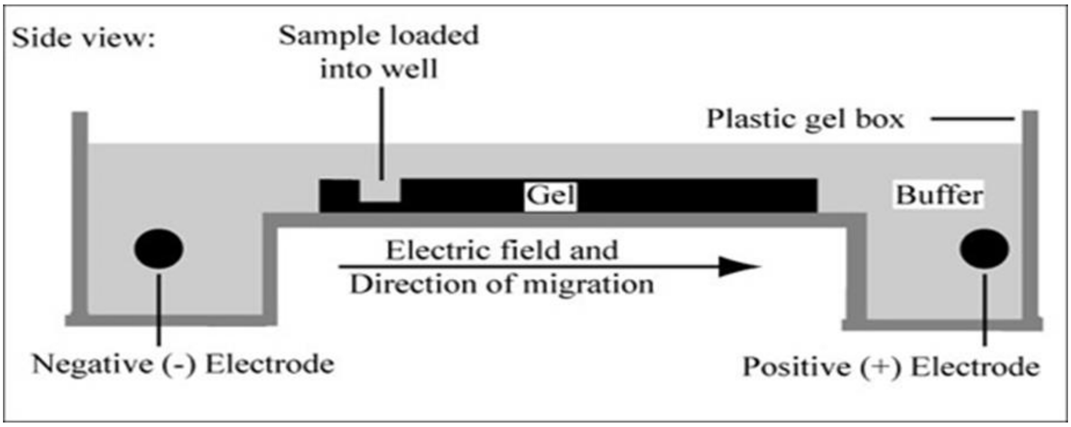

Figure 3.2 Agarose gel electrophoresis tank

3.2.3.4 Estimation of molecular weights

The polymorphs of the fragments produced by the PCR procedure were assessed based on a volume index of the PCR result (DNA Ladder 100bp), which supplied this proof in an agarose gel 11 band with known molecular weights (Sambrook *et al.* 1989) (Figure 3.3).

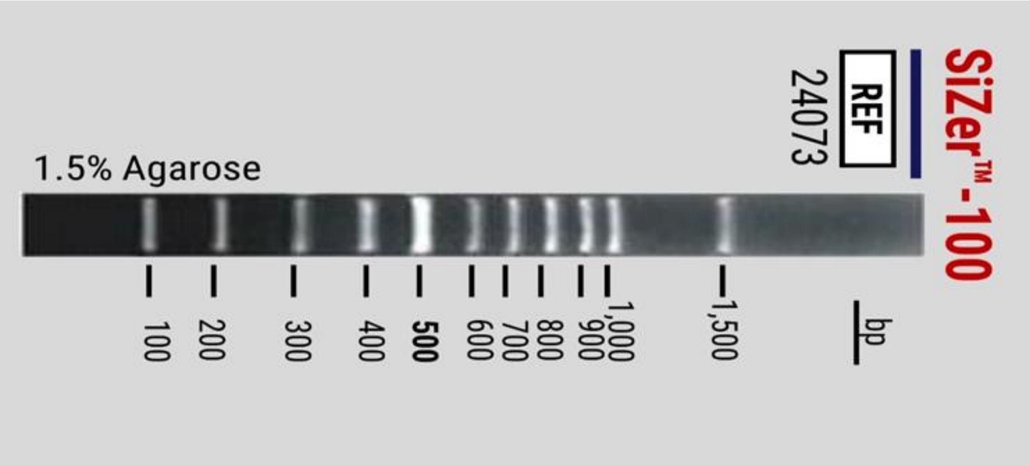

Figure 3.3 DNA markers used in electrophoresis

3.2.3.5 Reaction of PCR

Using the PCR-Premix kit provided by the intron company, the PCR reaction was conducted in a final volume of 25 μL. The elements PCR reaction are shown according to the Table 3.9.

Table 3.9 Describes the workings of the PCR reaction mixture

Apparatuses	Amount
Taq PCR PreMix	5 μL
Forward primer	10 picomols/μL (0.5 μL)
Reverse primer	10 picomols/μL (0.5 μL)
Forward primer	10 picomols/μL (0.5 μL)
Reverse primer	10 picomols/μL (0.5 μL)
DNA	1.5 μL
Distill water	16.5 μL
Final volume	25 μL

PCR reaction components:

1. Specific primers

For each SNP, two pairs of specialized primers were used. These primers were lyophilized and planned by the researcher following the T-ARMS-PCR system, also known as the four-primer amplification refractory mutation system-polymerase chain reaction (Figure 3.4). The American company Bioneer was responsible for producing a result concentration of 10 pcomol/µL, and this concentration was then dissolved in distilled water (Table 3.10, Table 3.11).

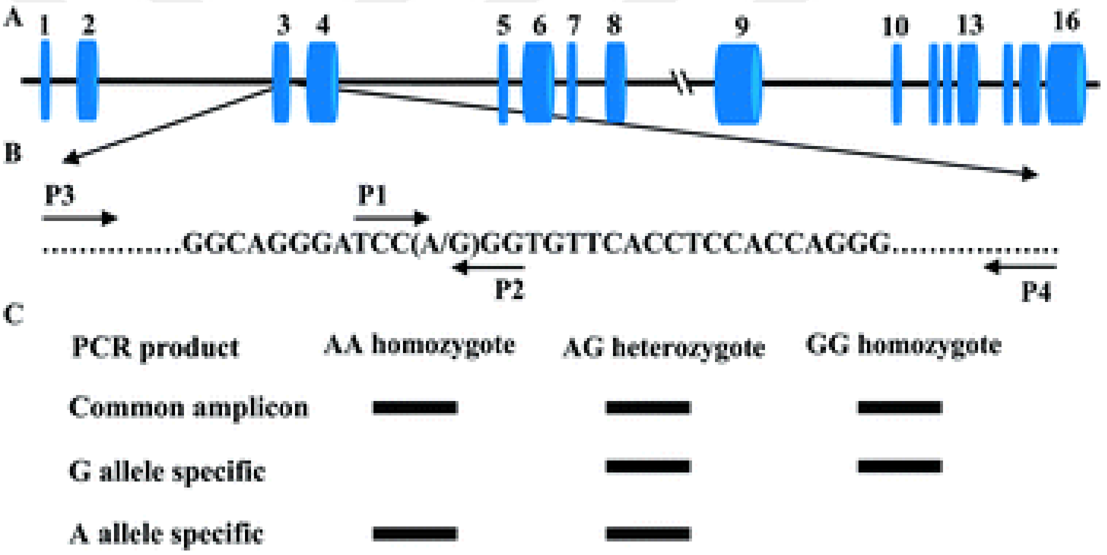

Figure 3.4 Explains how primers work according to T-ARMS (Li *et al.* 2014)

Table 3.10 Primers used in this study to investigate

Primer		Sequence 5'--3'	Tm (°C)	Product size
rs923	IF23	AAAACACTGAATTAGAGAGAAAGGACCAA	60	198
	IR23	CTACTCTCTTCCTCCACTTCATTTTTACC	60	249
	OF23	GAGAGCAGGTTCTCTGCTCAATTC	60	389
	OR23	TGTCTCATTGACCTTCAGATTCCC	60	
rs1544223	IF223	GGAGCAGAGCTGGAAGCACTTAC	60	166
	IR223	AGCTGGCAGAAAGATTGCAGAA	60	238
	OF223	TCAAGATAAGCAACCCTCTTCAGG	60	359
	OR223	TCAACTCGTGCAAGAACTTAGCAG	60	

Table 3.11 The database recycled in the PCR

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	93°C	3 min.	1 cycle
2	Denaturation -2	93°C	35sec	
3	Annealing	60°C*	35sec	35 cycle
4	Extension-1	72°C	35sec	
5	Extension -2	72°C	7 min.	1 cycle

After removing the PCR micro tubes that contained the replicated DNA samples from the device, a volume of 5 microliters was withdrawn from each tube, and the DNA samples were put onto an agarose gel at a concentration of 1.5% for 1-2 hours of electrophoresis.

3.2.3.6 Gene expression

RNA Extraction:

RNA was extracted from the sample using the TRIzol^{TM} reagent according to the manufacturer's procedure, which is as follows:

Sample lysis:

- Approximately 1.5 milliliters from cell culture was precipitated by micro spin centrifugation at 13.000 rpm for 2 minutes, the mixture was discarded and 0.75 mL of TRIzol^{TM} reagent was added to the pellet.
- The lysate was homogenized multiple times by pipetting up and down. The preserved blood, 300 microliters in 900 microliters of Trisol, was removed from the freezer, 300 microliters of chloroform were added and it was separated by centrifuge for 10 minutes at 13.000 cycles and the transparent upper layer containing RNA was taken.

Three phase's separation:

- After adding 0.2 mL of chloroform to the lysate, the tube was securely sealed.
- A combination was first allowed to incubate for 2-3 minutes before being centrifuged at 10,000 revolutions per minute for 5 minutes. This allowed the components to be separated into a lower organic phase, interphase, and a colorless upper aqueous phase.
- The aqueous phase containing RNA was transferred to a new tube.

RNA precipitation:

- A solution of 500 µL of isopropanol was added to the aqueous phase and incubated for 10 minutes before centrifugation at 10.000 rpm. RNA was precipitated, resulting in a white gel-like pellet at the tube's bottom.
- After that, the supernatant was discarded.

RNA washing:

- Each tube received 500 µL of 70% ethanol, which was vortexed briefly before being centrifuged for 5 minutes at 10.000 rpm.
- The pellet was then aspirated with ethanol and air-dried.

RNA solubility:

After rehydrating the pellet in 50 L of nuclease-free water, it was placed in a water bath with the temperature set between 55 and 60 °C for 10-15 minutes.

Estimation of RNA, concentration and purity:

To determine the quality of samples for downstream applications, a Quantus Fluorometer was utilized to measure the quantity of extracted RNA or cDNA. 1 µL of RNA or cDNA was combined with 199 μL of diluted Quant Flour Dye. RNA concentrations were measured after 5 minutes of incubation at room temperature in the dark (Table 3.12).

Table 3.12 Primers used in this study's RT-qPCR along with their sequences

Primer ID	Sequence 5'--3'	primer size	Annealing Temp.	Product size pb
ADOR F	TGCATTTTATGGACGGGAGT	20	57	181
ADOR R	AGGGTTCATCATGGAGTTGG	20		
GAPDH F	CCCACTCCTCCACCTTTGAC	20	58	180
GAPDH R	TTCCTCTTGTGCTCTTGCTG	20		

Quantitative Real-time PCR Assay (RT-qPCR) protocol:

This is the main step in the project, which is divided into two phases: the first is the synthesis of cDNA from RNA using a specific primer for transcripts and a photo script cDNA synthesis kit and the second is the analysis of the results. A new primer was developed using generous prime software and the primer specs were validated using an oligo analyzer program. The operation was carried out in the following steps:

- An aliquot of 8 μL of total RNA was extracted from each sample and placed in a fresh tube.
- Postscript reaction mix containing dNTPs, buffer and other necessary components, ten μl for each sample.
- MuLV Enzyme was then added into the reaction as 2 μL per sample.
- Add aliquot of 2 μL hexamer.
- Incubated for 1 hour by using the thermocycler, as in Table 3.13.

Table 3.13 Thermal cycler steps of conditions cDNA reverse transcription

Condition	Run 1	Run 2	Run 3	Run 4
Temperature	15°C	50°C	60°C	4°C
Time	20 sec	4 min	20 sec	------

It is repeated 12 times and then heated to 95 degrees Celsius to inactivate the enzyme (Relative quantitative PCR). The second stage of this process was completed by selecting cDNA samples of vancomycin subMIC concentration and control at the same run. Each sample has three PCR tubes. Quantity detection based on syber green's fluorescence power. The reaction mix was made up of the following components in the quantities listed in Table 3.14:

Table 3.14 Components of measurable RT-PCR is used in gene expression experiments

Component	Volume per 20 µL Reaction
qPCR Master Mix, (SYBR)	10 µL
Forward Primer	1 µL
Reverse Primer	1 µL
cDNA Template	5 µL
Nuclease-Free Water	to 20 µL

The PCR tubes were spun rapidly (1 minute at 2,000 rpm) to eliminate air bubbles and collect the liquid; the Real-Time PCR software was then configured with the required thermocycling Delta Ct method (Schmittgen and Livak 2008) (Table 3.15).

Table 3.15 RT-PCR cycling program

Loop's steps	Heat	Time	Number of cycles
Initial denaturation	95°C	3min	1
Denaturation	95°C	20 sec	40
Annealing	55°C	20 sec	1
Extension	72°C	20 sec	
Melt Curve	55-95°C		

The Delta Ct (Ct) approach is the simplest then it relates Ct levels between the target gene and the reference gene. The selection of a calibrator sample is required for relative quantification. The calibrator sample might be untreated at the ideal temperature of 37°C, or any sample that will be used to compare the unknown samples. To begin, the Ct among the marker gene and the reference gene is computed for each sample (both unknown and calibrator). As illustrated in the following Equation (3.9).

$$\text{Average Ct=Ct target gene-Ct reference gene} \quad (3.9)$$

The difference between the unknown's Δct and the calibrator's Δct is then computed, yielding the Δct value as in Equation (3.10).

$$\Delta\Delta Ct\text{=(Ct target-Ct reference) sample-(Ct target-Ct reference) Calibrator} \quad (3.10)$$

the normalized target amount in the sample is then equal to $2^{-\Delta\Delta Ct}$ and this value can be used to compare expression levels in samples (Schmittgen and Livak 2008) changes relative in mRNA expression levels were determined by using a comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$).

3.3 Statistical Analysis

GraphPad Prism V7, also Statistical Package for Social Science (SPSS), was used to perform a statistical analysis of the data using the Complete Randomized Design (CRD) model. The T-test was used to compare the arithmetic averages of the transactions at the level of significance (P 0.05) and test. The variables were linearly correlated with the results of the male hormone test and the strength of the correlation was determined using the Pearson momentary correlation coefficient. The significance of the correlation coefficient was then tested (Abu-Salih and Awad 1983). Genotypes, alleles, odds ratio, and confidence intervals using the fisher test and Hardy-Weinberg equilibrium and the program accessible at www.medcalc.org/calc/oddsratio.php. The significance of the differences between the values of the data under study was tested using Duncan's test where possible under different significant levels ($P \leq 0.05$).

4. RESULTS AND DISCUSSION

4.1 Physiological and Biochemical Test

The current study was conducted in Anbar Governorate, Iraq. Samples were collected from energy drink consumers for the period April 2022 to July 2022. 100 samples of energy drink consumers and 20 samples were selected as control samples. Several physiological and biochemical tests were performed (Caffeine, GOT, GPT, ALP, BU, SCR) (Table 4.1).

Table 4.1 The average and range of physiological and hormonal measurements in the blood serum of the study samples

Parameter	Groups		*p-Value*	*r value*
	Patient (No. 100) Mean (±S.D)	**Control (No. 20) Mean (± S.D)**		
Caffeine	223.38 (±113.27) ng/mL	19.33(±5.82) ng/dL	≤ 0.05	
GOT	55.19 (±9.86) IU/L	25.4 (± 6.00) IU/L	≤ 0.05	0.156
GPT	56.94 (± 9.45) IU/L	22.00 (± 10.42) IU/L	≤ 0.05	-0.089
ALP	216.08 (±45.79) IU/L	86.65 (±11.71) IU/L	≤ 0.05	0.374
BU	55.49 (±4.51) mg/dL	28.88 (±6.28) mg/kg	≤ 0.05	0.311
S.CR.	1.77 (± 0.25) mg/dL	0.96(± 0.16) mg/dL	≤ 0.05	0.309

NS= Non-significant, * significant at *p value* ≤ 0.05

4.1.1 Caffeine test

According to the current study's, consumers of energy drinks consumed high significantly more caffeine than control samples (P value < 0.05), where the average concentration of testosterone in Energy drink consumers was 223.38 ng/mL, while the average concentration in the control samples was 19.33 ng/mL (Figure 4.1).

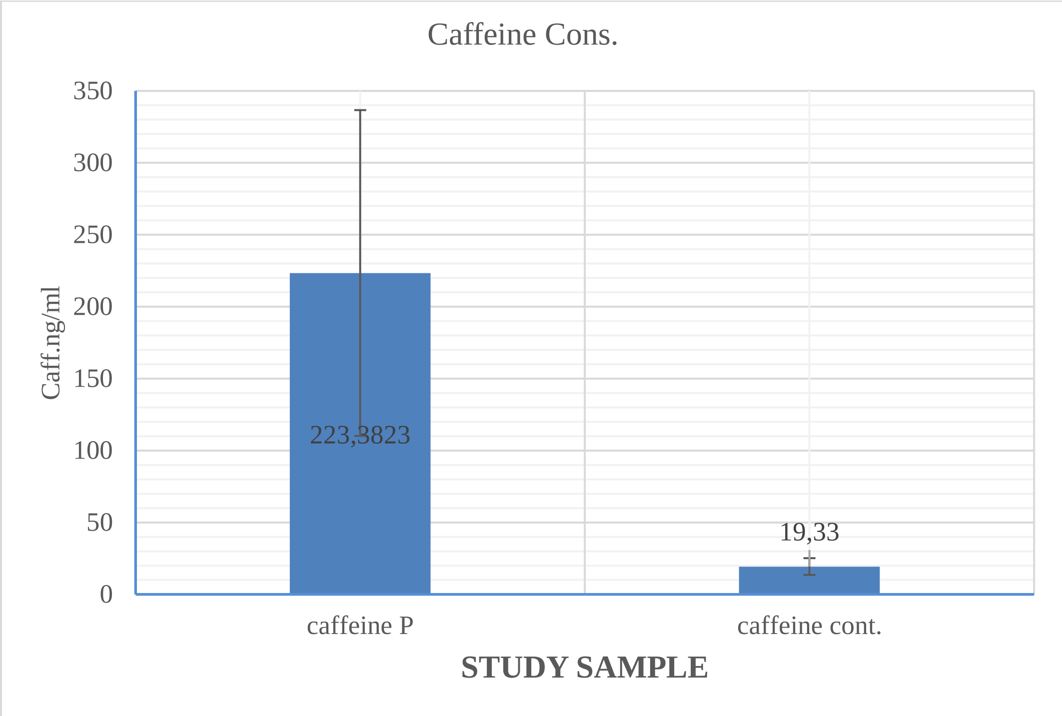

Figure 4.1 Mean of Caffeine concentration

Coffee, tea, cocoa, and other foods containing these substances naturally include caffeine (1,3,7-trimethylxanthine) a plant alkaloid, in the diet. A variety of beverages, particularly energy drinks, can also have it added. A designed caffeinated beverage, such as an energy drink or energy shot, must have a total caffeine content of between 145 and 320 mg/L (Thomson *et al.* 2014). Roldán *et al.* (2018) reported an increase in caffeine levels in the blood in a group of Red Bull users by 4.69 mcg/mL after the 30-minute session. Lassiter *et al.* (2012) also indicated that drinking energy drinks raises the levels of caffeine in the plasma. Another study involving 12 healthy and physically active participants revealed that after consuming energy beverages (containing 1 or 3 mg of caffeine per kg of body weight), caused raised caffeine levels in the blood and then lead to increased blood pressure and heartbeat (Del Coso *et al.* 2012). These results are consistent with the findings of the current study, where a substantial increase in caffeine ranks was observed among energy drinkers compared to the control sample.

4.1.2 Liver function test

T-test was conducted to compare between GOT levels of energy drink consumers and the control group at a level of significance ($P < 0.05$), where the results showed high significant differences between GOT levels of energy drink consumers (55.19) UI/L and control samples (25.4) UI/L (Figure 4.2). The current results of the present study demonstrated the high significant variations in the levels of GPT between consumers of energy drinks and control samples, where the study showed that the concentration of GPT in energy drink consumers is 56.94 IU/L, while the application of GPT in the controller tasters are 22.00 IU/L (Figure 4.2). As for ALP enzyme, the statistical analysis revealed high significant differences between the levels of the ALP enzyme in the energy drink consumers and the control samples at a significant level of ($P<0.05$). The average concentration of the enzyme in the energy drink consumers was 216.08 IU/L, whereas the average concentration of the enzyme in the control samples was 86.65 IU/L (Figure 4.2).

This study is consistent with Harb *et al.* (2011), this study found that a person who had been drinking 4 to 5 cans of an energy drink every day for 3 weeks had extremely elevated levels of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT), where the ALT level was 1203 U/L, and the AST level was 1802 U/L(Harb *et al.* 2016). Also, in another study approved that ALT and AST levels raised in blood for Consumers that have been increased consumption of energy drinks (Vivekanandarajah and Waked 2011). In studies by Huang *et al.* (2014), abnormal liver tests were found with aspartate transaminase (AST) levels of 1,541 U/L, alanine transaminase (ALT) levels of 2,995 U/L, and alkaline phosphatase levels of 231 U/L (Huang et al/ 2014). Energy drinks typically have 80-141 mg of caffeine in an 8-ounce serving, which is equivalent to five ounces of coffee or two 12-ounce cans of caffeinated soda. This higher concentration of caffeine caused GOT and GPT ranks to rise (Luma 2019). In a study conducted in the USA where laboratory tests revealed substantial hepatic dysfunction, Baseline kidney and liver tests had been within the normal range two months before to the investigation but after 3 day from taken a energy drinks liver tests had been measured, which showed a markedly raise in AST and ALT but the ALP levels was normal (Al Yacoub *et al.* 2020).

This study found that taking an energy drink orally caused various grades of liver harm. This is seen in the large rises in serum AST, ALT and ALP ranks caused by energy drinks. Where, Alrasheedi and Abdel-Mageid (2007) found that the consumption of energy drinks leads to the death of liver cells and a reduction in their nuclei, which may result in a loss of liver mass (Alrasheedi and Abdel-Mageid 2007).

In another hand, there are several studies conducted on rats to investigate the relationship and effect between energy drinks and liver function. Mansy *et al.* (2017) found that rats given moderate or high doses of an energy drink exhibited substantial increases in serum AST, ALT and ALP levels at weeks 6 and 12 of the trial, as compared to baseline levels and equivalent values in the control group. The levels of serum AST, ALT, and ALP increased from 66.20, 45.27, and 137.27 U/L, respectively, to 190.62, 107.80, and 421.69 U/L, respectively, when they were treated with 2.2 ml/100 g/day of energy drink for 12 weeks (Mansy *et al.* 2017). Increases in hepatic enzyme levels in the blood assist as undependable indications of liver damage caused by toxic substances. Caffeine energy drinks caused similar elevations in blood AST, ALT and ALP levels in rats (Bukhar *et al.* 2012). It has been shown that rats that consumed energy drinks unaccompanied or in combination with alcohol revealed upper serum total bilirubin, ALT, ALP and AST compared to untreated controls (Ugwuja 2014). According to a study conducted by Luma (2019) on rats after they were given an energy drink (Tiger), the outcomes showed a significant rise in the levels of liver enzymes GOT, GPT and Alkaline phosphate. The aspartate and alanine aminotransferase transferase activities were demonstrated by the clinical chemistry findings (Ebuehi *et al.* 2011). In Contrast, a study that was conducted by Backer and Baeissa (2014) found that all of the groups that consumed energy drinks exhibited a substantial drop in the concentration of aspartate and alanine aminotransferase, with the exception of the Red Bull group. In addition to this, they demonstrated a decline in the alkaline phosphatase levels, with the exception of the bison group (Backer and Baeissa 2014).

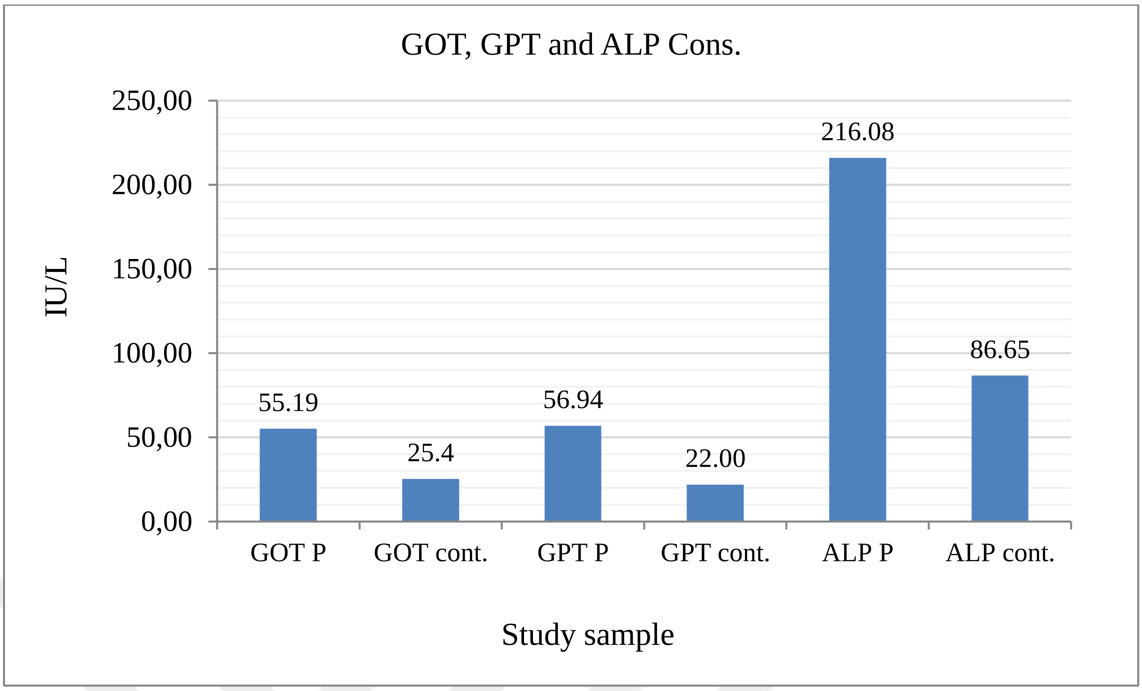

Figure 4.2 Mean of GOT, GPT and ALP concentration

4.1.3 Kidney test

The current study showed higher BU levels (55.49) mg/dL in energy drink consumers compared with a sample of circles (28.88) mg/100mL (Figure 4.3), where demonstrated high significant difference with $P < 0.05$. As for S. Creatinine, the current study's findings showed that S.CR levels had significantly increased. When compared to the control samples, energy drink drinkers had a greater mean concentration of S.CR, with a $P < 0.05$. In comparison to the energy drink drinkers, whose average concentration was 1.77 mg/dL, the control samples' average concentration was 0.96 mg/dL (Figure 4.4).

Serum uric acid and creatinine concentrations were considerably higher among energy drink users in this investigation. Increases in serum urea in addition to creatinine levels be present usually associated with decreased renal function (Mossa and Abbassy 2012). In a study that was carried out by Al Yacoub *et al.* in 2020, laboratory tests revealed substantial renal impairment. Prior to this discovery, two months earlier, both baseline kidney and liver tests had been within the normal range but after 6 day from taken a energy drinks kidney tests had been measured, which showed a markedly raise in renal test, where B. urea (mg/dL) was 85 and S. creatinine (mg/dL) was 4 (Al Yacoub *et al.* 2020). According to the findings of Kelsey *et al.*, acute renal failure is connected with excessive use of energy drinks, with S. creatinine levels measuring 1205 µmol/L. (Kelsey *et al.* 2019). Also, there is another study that revealed that consumption in energy drinks leads to an increase S. creatinine in the blood then lead to a negative affect on renal function, where creatinine levels reached to 5.5 mg/dL (Greene *et al.* 2014). All these studies are consistent with our study that revealed the negative effect of energy drinks on renal function.

These findings are there stable with those of Khayyat *et al.* (2014) who reported that energy drinks caused an increase in blood urea and creatinine levels. These researchers hypothesized that coffee raised urea, uric acid and creatinine levels by inhibiting adenosine A2A receptors, resulting in interstitial infection, increased proteinuria and negative alterations in kidney function and structure. Energy drinks have an influence on renal function in mice, according to Mansy *et al.* (2017). When compared to baseline levels of parameters and similar values in the control group, mice exposed to large doses of energy drinks exhibited substantial increases in blood creatinine and urea levels at week 12, Where it was found that when rats were treated with an energy drink at a rate of 2.2 ml/100 g/day for a period of 12 weeks, there was an increase in the levels of serum creatinine and blood ure from 0.33 and 7.57 mg/dL to 0.73 and 23.48, respectively. However, some other studies based on energy drink consumption reported results that contradict these findings. Consumption of energy drinks has been linked to reduced levels of the creatinine marker, according to some research (Ebuehi *et al.* 2011). On the other hand, the findings of other researchers did not point to any significant link between consumption of caffeine and levels of serum urea or creatinine (Akande and Banjoko 2011). These discrepancies in the effect of energy drink consumption can be attributed to mismatches in the ingredients of these energy drinks as well as the proportion of each ingredient. Eltahir *et al.* (2020). Study when evaluating blood urea and creatine levels revealed not any significant changes in blood urea levels in the test group compared to the healthy control group. On the other hand, a non-significant increase in serum creatinine was observed in the vigor drink group when compared to the control group.

Energy drinks have caffeine, which causes diuresis leading to increased urinary output and natriuresis instead of hydration (Higgins *et al.* 2010).

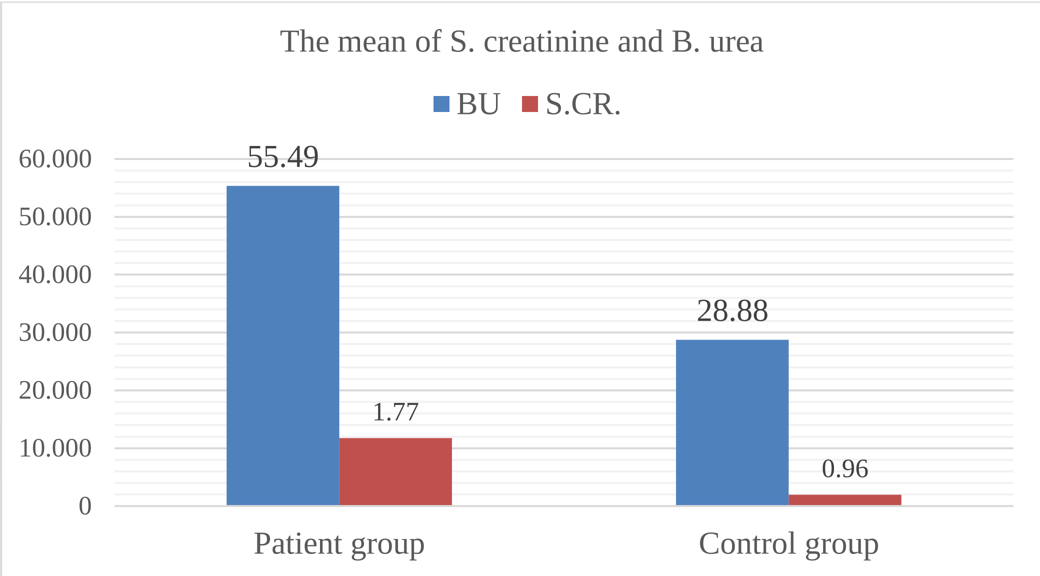

Figure 4.3 Mean of blood urea and serum creatinineconcentration

4.2 Correlation Coefficient of Caffeine with Physiological Variables

The correlation coefficient was measured between caffeine levels in the studied samples and some physiological variables (Table 4.2).

Table 4.2 Correlation coefficient was measured between caffeine levels in the studied samples and some physiological variables

	Caffeine	*GOT*	*GPT*	*ALP*	*BUN*	*S.CR.*
Caffeine	1					
GOT	0.156	1				
GPT	-0.089	0.563	1			
ALP	0.374	0.201	-0.416	1		
BUN	0.311	-0.572	-0.479	0.207	1	
S.CR.	0.309	0.029	-0.341	0.566	0.442	1

4.2.1 Correlation coefficient between caffeine and GOT

The correlation of caffeine concentration with GOT concentration was studied as correlation coefficients. The study showed no correlation between caffeine concentration and GOT (Figure 4.5). As the correlation strength is (r=0.156).

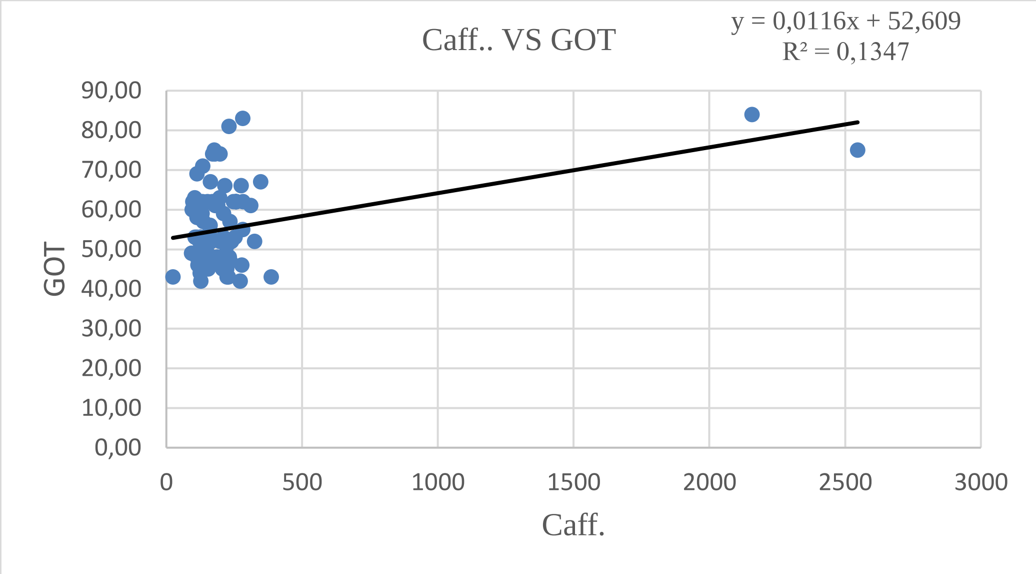

Figure 4.5 Correlation coefficient between caffeine and GOT

4.2.2 Correlation coefficient between caffeine and GPT

The correlation of caffeine concentration with GPT concentration was studied as correlation coefficients. The study showed no correlation between caffeine concentration and GPT (Figure 4.6). As the correlation strength is (r=-0.089).

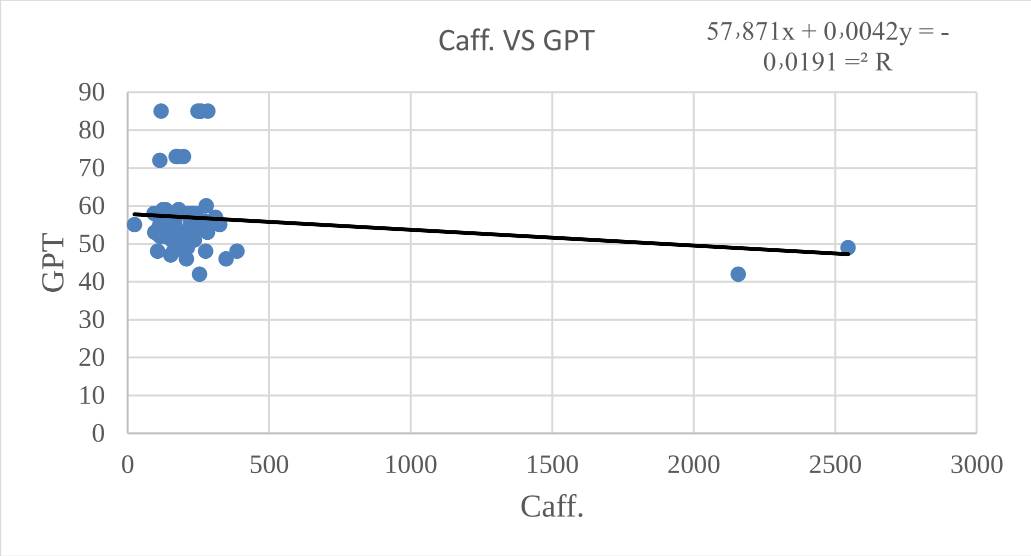

Figure 4.6 Correlation coefficient between caffeine and GPT

4.2.3 Correlation coefficient between caffeine and ALP

The correlation of caffeine concentration with ALP concentration was studied as correlation coefficients. The study showed a positive correlation between caffeine concentration and ALP (Figure 4.7). As the correlation strength is (r=0.374).

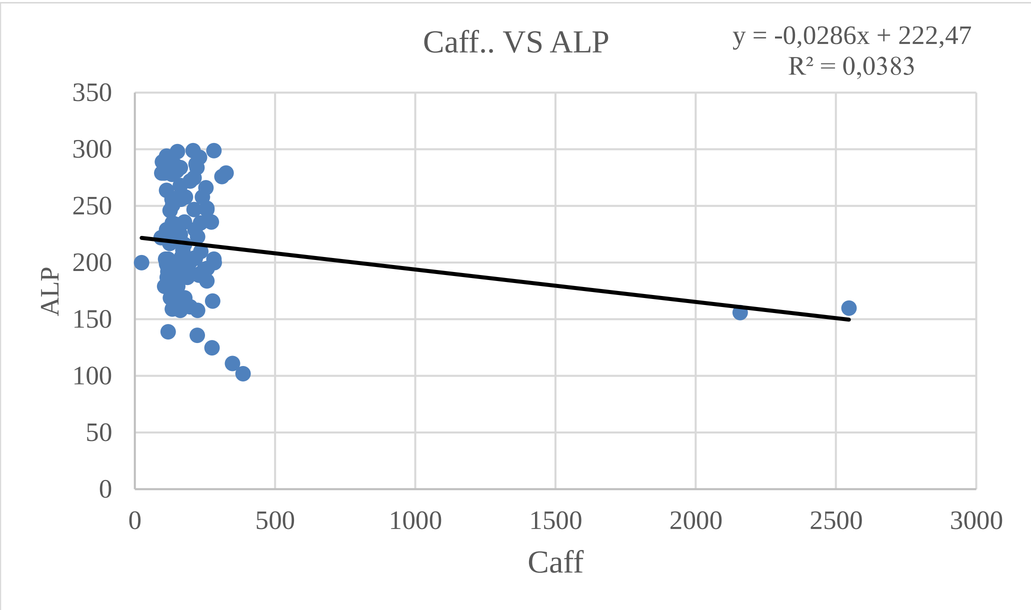

Figure 4.7 Correlation coefficient between caffeine and ALP

The potential advantages of caffeine for health are debatable. Despite the widespread belief that coffee use is unhealthy, a recent large population-based study found a slight decrease in all-cause mortality associated with increased coffee consumption, primarily due to a lower risk of cardiovascular death (Lopez-Garcia *et al.* 2008). Similar to this, higher caffeine intake, particularly from coffee, takes remained associated with a lower incidence of chronic liver disease. The National Health and Nutrition Examination Survey [NHANES] I and III, two recent population-based studies, found that higher caffeine drinking (>2 cups per day) was related to a lower risk of elevated alanine aminotransferase (ALT) levels and a lower risk of chronic liver disease (Ruhl and Everhart 2005a, Ruhl and Everhart 2005b). According to research using NHANES III data, daily coffee drinkers were 44% less likely than non-drinkers to experience elevated ALT levels. Furthermore, a different large cohort study of 330 patients with cirrhosis caused by alcohol and non-alcohol found a strong negative correlation between coffee consumption (>4 cups per 24h) and higher blood enzymes, chiefly in those who drank heavily (Klatsky *et al.* 2006). Prior studies have given away that drinking coffee was related to lower levels of the blood enzymes gamma-glutamyl transferase (GGT) and ALT (Modi *et al.* 2010).

4.2.4 Correlation coefficient between caffeine and blood urea

The correlation of caffeine concentration with blood urea concentration was studied as correlation coefficients. The study showed a positive correlation between caffeine concentration and blood urea (Figure 4.8). As the correlation strength is (r=0.311).

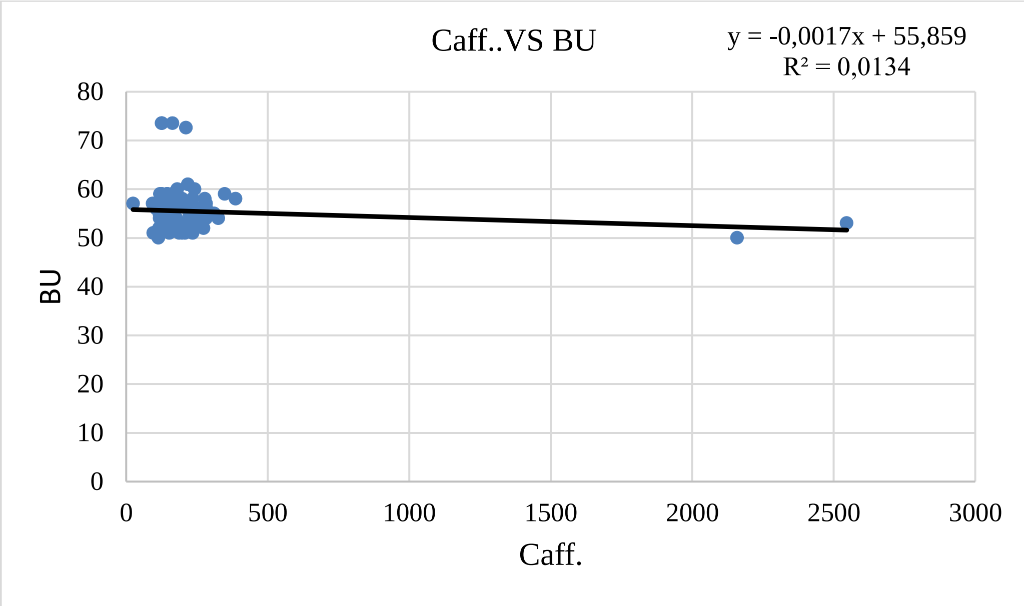

Figure 4.8 Correlation coefficient between caffeine and blood urea

4.2.5 Correlation coefficient between caffeine and creatinine

The correlation of caffeine concentration with creatinine concentration was studied as correlation coefficients. The study showed a positive correlation between caffeine concentration and creatinine (Figure 4.9). As the correlation strength is (r=0.309).

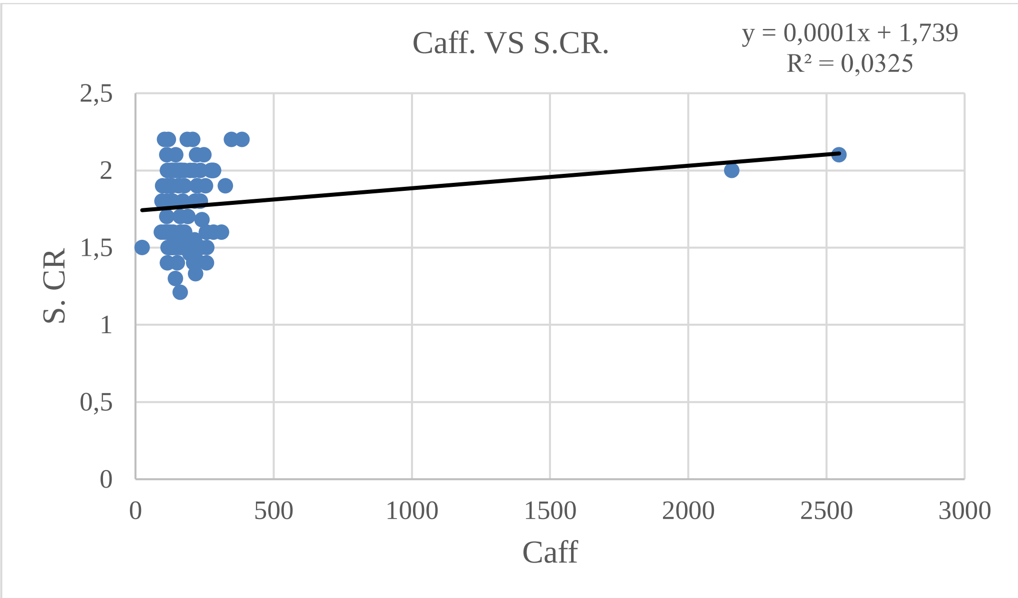

Figure 4.9 Correlation coefficient between caffeine and creatinine

4.3 Molecular Studies

The current study involved the extraction of DNA from blood samples of 100 energy drink customers using the extraction kit offered by the Korean business Gene aid, which was explained in the chapter on materials and methods of work. While the control samples were 20 samples. Utilizing the spectrophotometer method, the quantity and pureness of the extracted DNA were also determined. Both parameters were acceptable and compatible with the tetra primer ARMS-PCR reaction. where the purity was between 1.6 and 1.9 and the concentration ranged from 35 to 58 ng/L.

To check for the incidence of DNA by resulting in its movement in (0.8%) agarose gel and using ethidium bromide dye, all extracted samples were electrophoresed in a gel electrophoresis device. This procedure ensured the success of the extraction process (Figure 4.10).

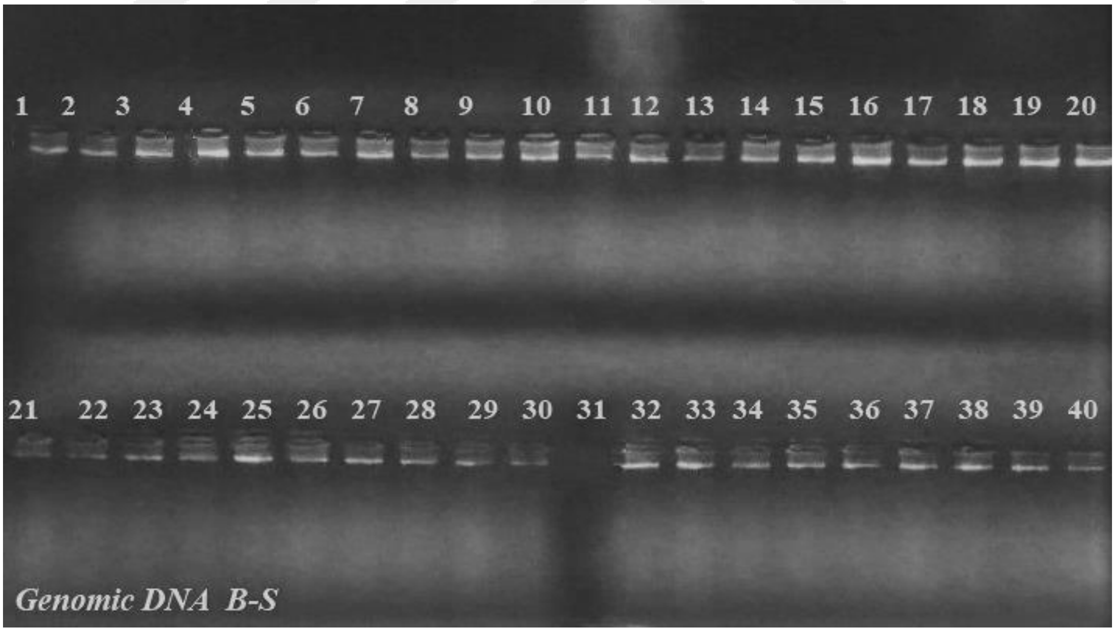

Figure 4.4 Whole genome on agarose gel electrophoresis at a concentration of 0.8% and the time was 80 min at 70 volts

4.3.1 Genotyping of rs923

The results of the polymerase chain reaction in the current study showed a discrepancy in the number and sizes of bands, where the AA genotype gave two bands of size 198 and 389 bp, while the GG genotype gave two bands of size. 249 and 389 bp, while the AG genotype yielded three bands with sizes 198, 249 and 389 bp (Figure 4.11) (Figure 4.12).

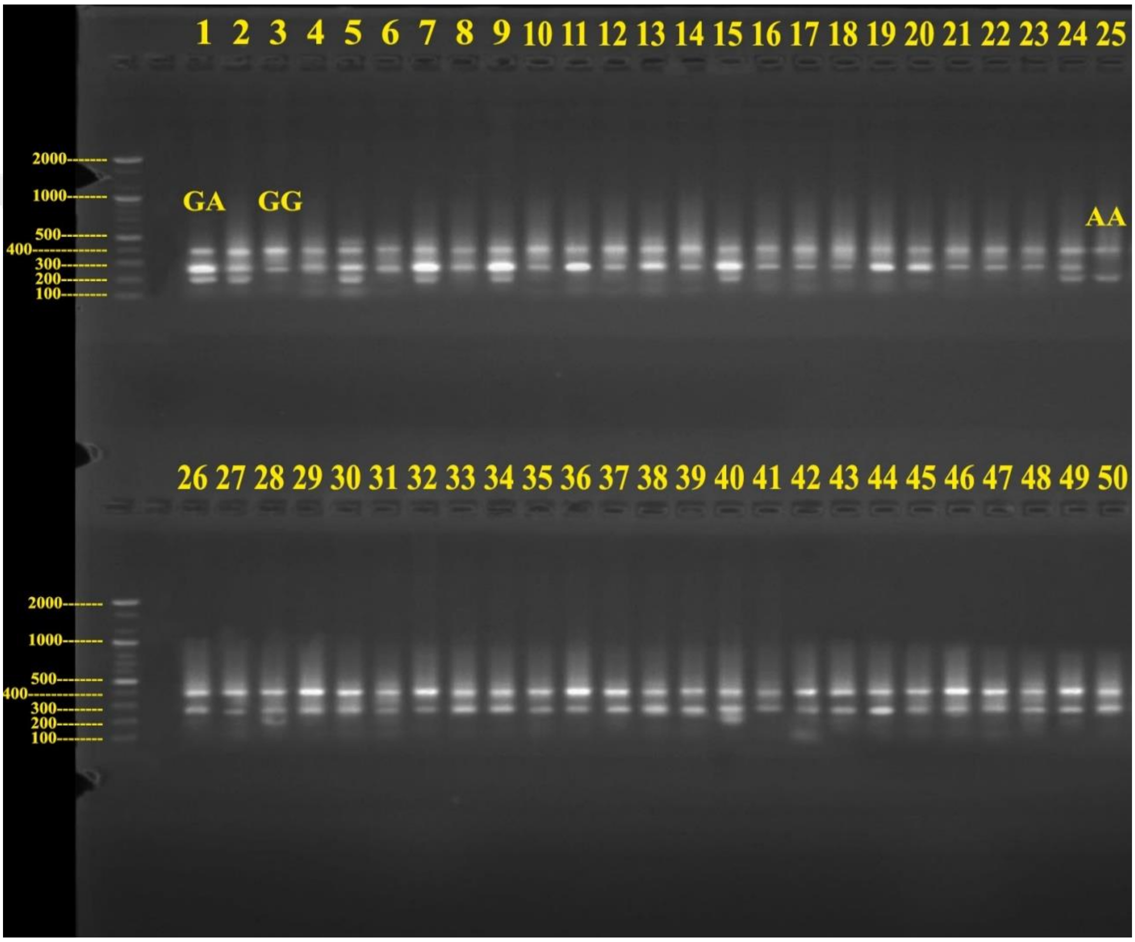

Figure 4.5 Genotype and Allele frequency of rs923 SNP in Patients groups. (1.5% agarose, 70 V for 1 h) (GG=249+389, GA=198+249+389 and AA=198+389)

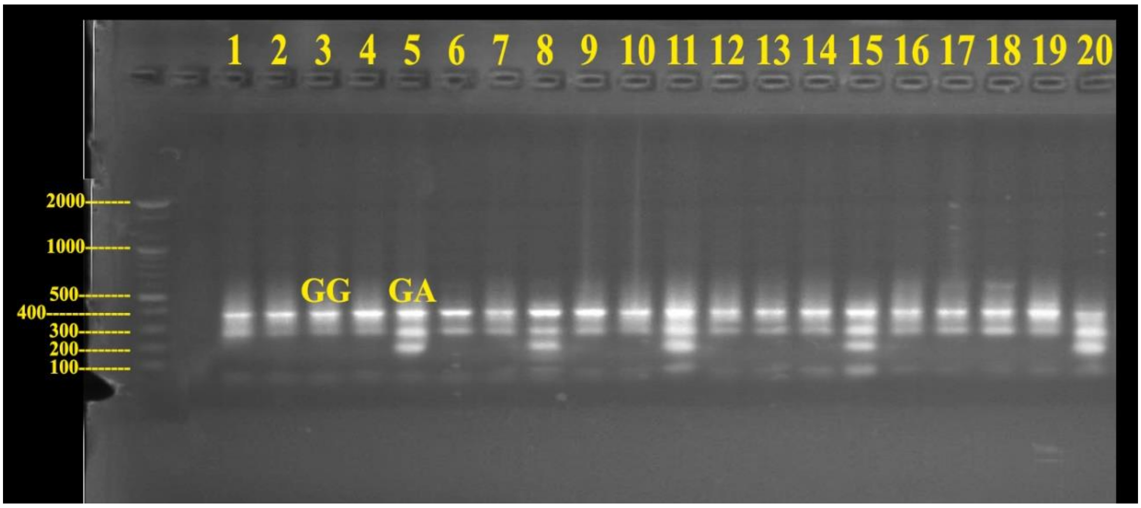

Figure 4.6 Genotype and Allele frequency of rs923 SNP in Control groups. (1.5% agarose, 70 V for 1 h) (GG=249+389, GA=198+249+389 and AA=198+389)

The prevalence of genotypes among Energy drink consumers was 80 (80%) for the GG genotype, 18 (18%) for the GA genotype and 2 (2%) for the AA genotype. As for the prevalence of genotypes in the control samples, it was distributed by 15 (75%) for the GG genotype, 5 (25%) for the GA genotype and the AA genotype did not appear in any of the control samples 0 (0%) (Table 4.3).

Table 4.3 Genotype and Allele frequency of rs923 SNP in patients and control groups

rs923 Genotype	Patient (No.= 100)				Control (No.=20)			
	Observed		Expected		Observed		Expected	
	%	No.	%	No.	%	No.	%	No.
GG	80	80	79.21	79.2	75	15	76.56	15.31
GA	18	18	19.58	19.6	25	5	21.88	4.38
AA	2	2	1.21	1.2	-	0	1.56	0.31
HWE Analysis	*p*-value=0.5683 Significant				*p*-value=0.5229 non-Significant			

The statistical analysis of the rs923 genotypes is displayed in Table 4.3. The prevalence of the GG genotype was highest in both energy drink drinkers and control samples 80% and 75%, respectively, where a statistical analysis revealed no differences between energy drink drinkers and control samples. Additionally, the prevalence of the GA genotype was comparable in the control samples and the energy drink users 18% and 25%, respectively, whereas the AA genotype was least noticeable in both groups.

The rs923 SNP is located on chromosome 1 at position 111499761 and has a G>A change. The association of rs923 with some diseases has been studied. Zheng *et al.* (2020) investigated the relationship of rs923 with Richter syndrome. Campbell (2014) studied the role of rs923 in autistic patients. The prevalence of the allele for rs923 was studied. In most studies, the results showed that the frequency of the alternative allele A was higher compared to the reference allele G. Where the results were recorded on (NCBI) The National Center for Biotechnology Information website showed that the highest frequency of the reference allele G was in Africans=G 0.2566, while the lowest frequency was G=0.1586 among Latin Americans. These results contradicted our results, which showed that the frequency of the reference allele was higher compared to the frequency of the alternative allele.

4.3.2 Genotyping of rs1544223

The results of the polymerase chain reaction in the current study showed a discrepancy in the number and sizes of bands, where the CC genotype gave two bands of size 166 and 359 bp, while the TT genotype gave two bands of size 238 and 359 bp, while the CT genotype yielded three bands with sizes 166, 238 and 359 bp (Figure 4.13) (Figure 4.14).

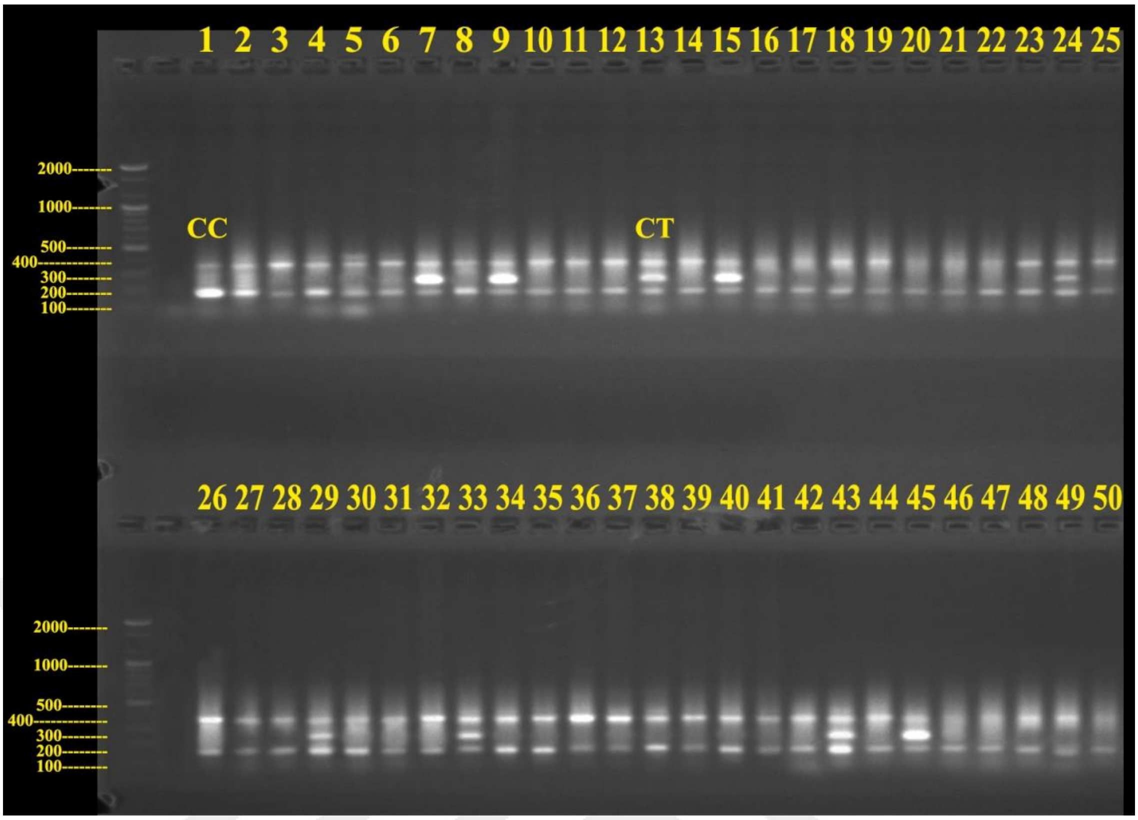

Figure 4.7 Genotype and Allele frequency of rs1544223 SNP in Patients groups. (1.5% agarose, 70 V for 1 h) (CC=166+359, CT=166+238+359 and TT=238+359)

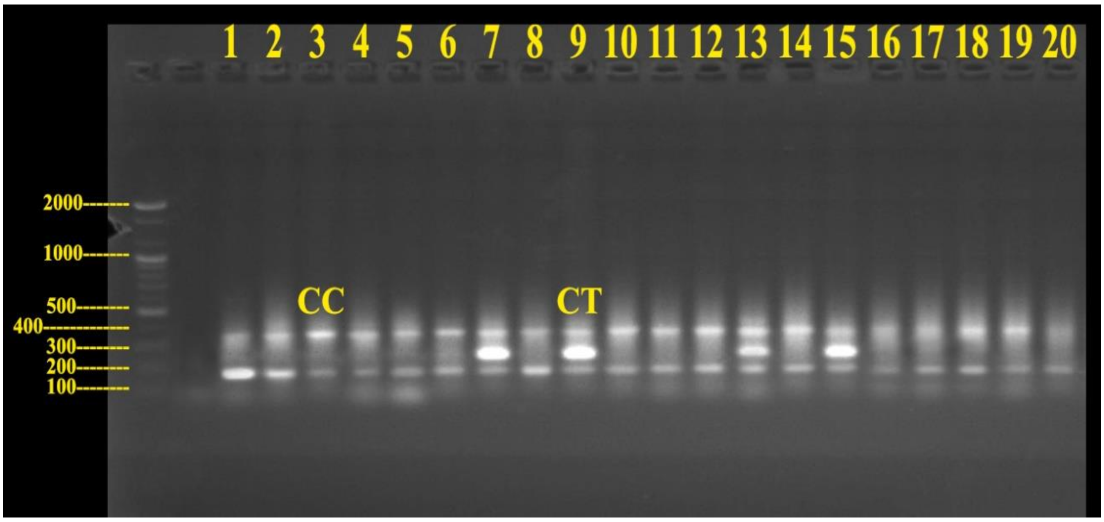

Figure 4.8 Genotype and Allele frequency of rs1544223 SNP in Control groups. (1.5% agarose, 70 V for 1 h) (CC=166+359, CT=166+238+359 and TT=238+259)

The prevalence of genotypes among energy drink consumers was 82 (82%) for the CC genotype, 18 (18%) for the CT genotype and 0 (0%) for the TT genotype. As for the prevalence of genotypes in the control samples, it was distributed by 16 (80%) for the CC genotype and 4 (20%) for the CT genotype and the CC genotype did not appear in any of the control samples (Table 4.4).

Table 4.4 Genotype and Allele frequency of rs1544223 SNP in patients and control groups

rs1544223 Genotype	Patient (No.= 100)				Control (No.=20)			
	Observed		Expected		Observed		Expected	
	%	No.	%	No.	%	No.	%	No.
CC	82	82	82.81	82.8	80	16	81	16.2
CT	18	18	16.38	16.4	20	4	18	3.6
TT	-	0	0.81	0.8	-	0	1	0.2
HWE Analysis	*p*-value=0.4843 Significant				*p*-value=0.6193 non-Significant			

The statistical analysis of the rs1544223 genotypes is presented in Table 4.4. The prevalence of the CC genotype was highest in both energy drink drinkers and control samples 82% and 80%, respectively, where a statistical analysis revealed that there were no significant differences between energy drink drinkers and control samples. Additionally, the prevalence of the CT genotype was similar in both the control samples and the energy drink users 18% and 20%, respectively, whereas the TT genotype was the least noticeable in both groups.

The effect of the ADORA3 gene polymorphism as an influencing factor on consumers of energy drinks has never been investigated before. Both the study and control samples contained the rs1544223 SNP. The study revealed that CC genotypes were the most prevalent. The C allele was found to be more common than the T allele, according to the current study.

Numerous diseases and physiological conditions have been studied to determine how the rs1544223 SNP affects them. According to He *et al.* (2018), the rs1544223 SNP reduces transcriptional activity and affects the risk of chronic heart failure in Chinese. examining the level of ADORA3 mRNA expression also demonstrated decreased ADORA3 gene expression concerning AA/GA (He *et al.* 2018).

4.3.3 Gene expression of ADORA3

In this study, a quantitative RT-PCR assay analyzed the mRNA expression of the ADORA3 gene by comparing isolates between control isolates and four genotypes (two genotypes per SNP).

Concerning the rs923 SNP, the results indicated an increase in ADORA3 gene expression in GG genotype carriers, while there was no change in ADORA3 gene expression in GA genotype carriers. As for the rs1544223 SNP, the quantitative RT-PCR test displayed an increase in gene expression in both the CC and CT genotypes. The reason study gene expression is that there are no significant differences in the genotypes between the samples of energy drink users and the control. For this reason, wanted to know the effect of each genotype on the expression of the ADORA3 gene and whether is there a difference between the effect of drinking alcohol on the different genotypes (Table 4.5, Figure 4.15, Figure 4.16, Figure 4.17).

Table 4.5 Fold change of gene expression of ADORA3 gene depending on ΔΔCt method for different genotypes in drinker sample

	Sample	ADORA3 Admitted	CT HK	CT Control Average	CT HK Average	ΔCTE	ΔCTC	ΔΔCt	Expression Fold Change2^-ΔΔCt
	Control	0	0	22.18	16.78				1
rs923	1GG	23.09	20.27	22.18	16.78	2.82	5.40	-2.58	5.979396995
	2GG	22.90	21.13	22.18	16.78	1.77	5.40	-3.63	12.38051995
	3GG	23.69	22.28	22.18	16.78	1.41	5.40	-3.99	15.88947993
	4GG	21.41	21.19	22.18	16.78	0.22	5.40	-5.18	36.25228433
	1GA	22.49	23.13	18.63	19.27	-0.64	-0.64	0.00	1
	2GA	24.05	25.09	18.63	19.27	-1.04	-0.64	-0.40	1.319507911
	3GA	23.40	24.12	18.63	19.27	-0.72	-0.64	-0.08	1.057018041
	4GA	22.99	23.39	18.63	19.27	-0.40	-0.64	0.24	0.846745312
rs1544223	1CC	26.17	24.10	23.29	17.25	2.07	6.04	-3.97	15.67072476
	2CC	25.01	23.56	23.29	17.25	1.45	6.04	-4.59	24.08394796
	3CC	27.18	24.90	23.29	17.25	2.28	6.04	-3.76	13.547925
	4CC	25.49	24.17	23.29	17.25	1.32	6.04	-4.72	26.35491255
	1CT	25.96	24.97	24.05	17.83	0.99	6.22	-5.23	37.53071838
	2CT	27.03	25.11	24.05	17.83	1.92	6.22	-4.30	19.69831061
	3CT	25.11	23.91	24.05	17.83	1.20	6.22	-5.02	32.44670335
	4CT	26.78	24.50	24.05	17.83	2.28	6.22	-3.94	15.34822591

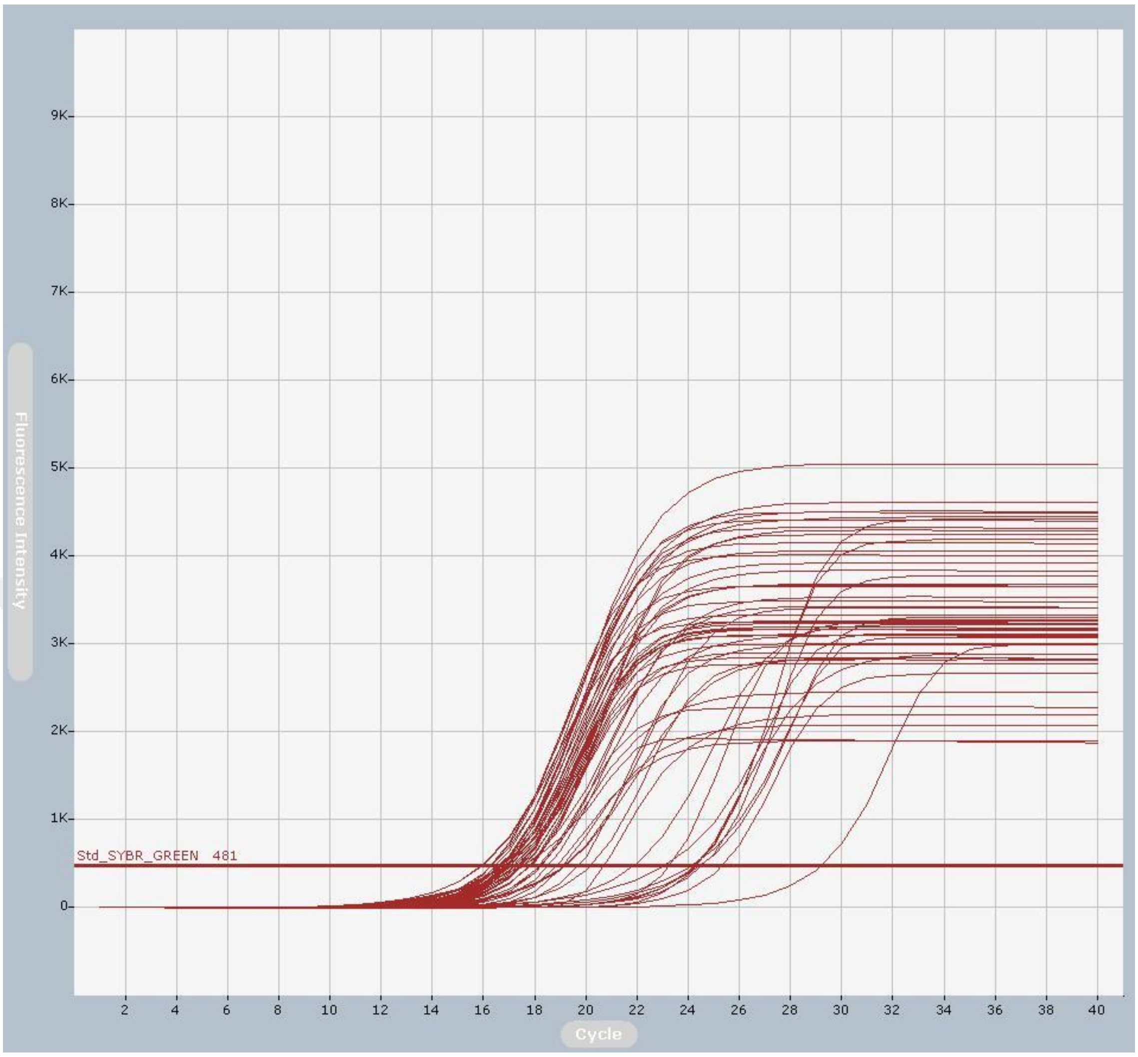

Figure 4.9 Amplification curve plot in qPCR and CT value for studied samples with the housekeeping gene

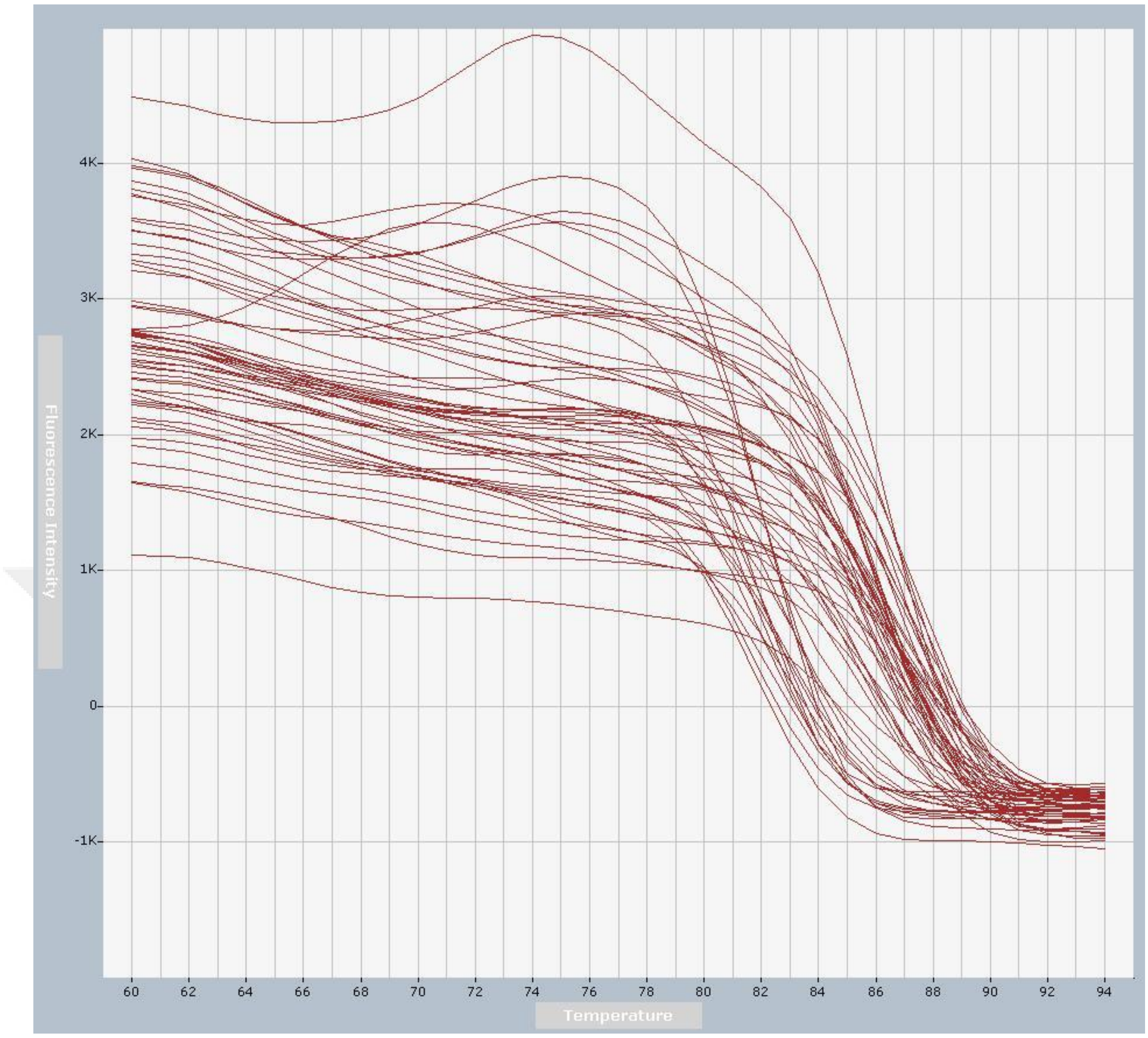

Figure 4.10 Melting curve analysis by qPCR for amplified product

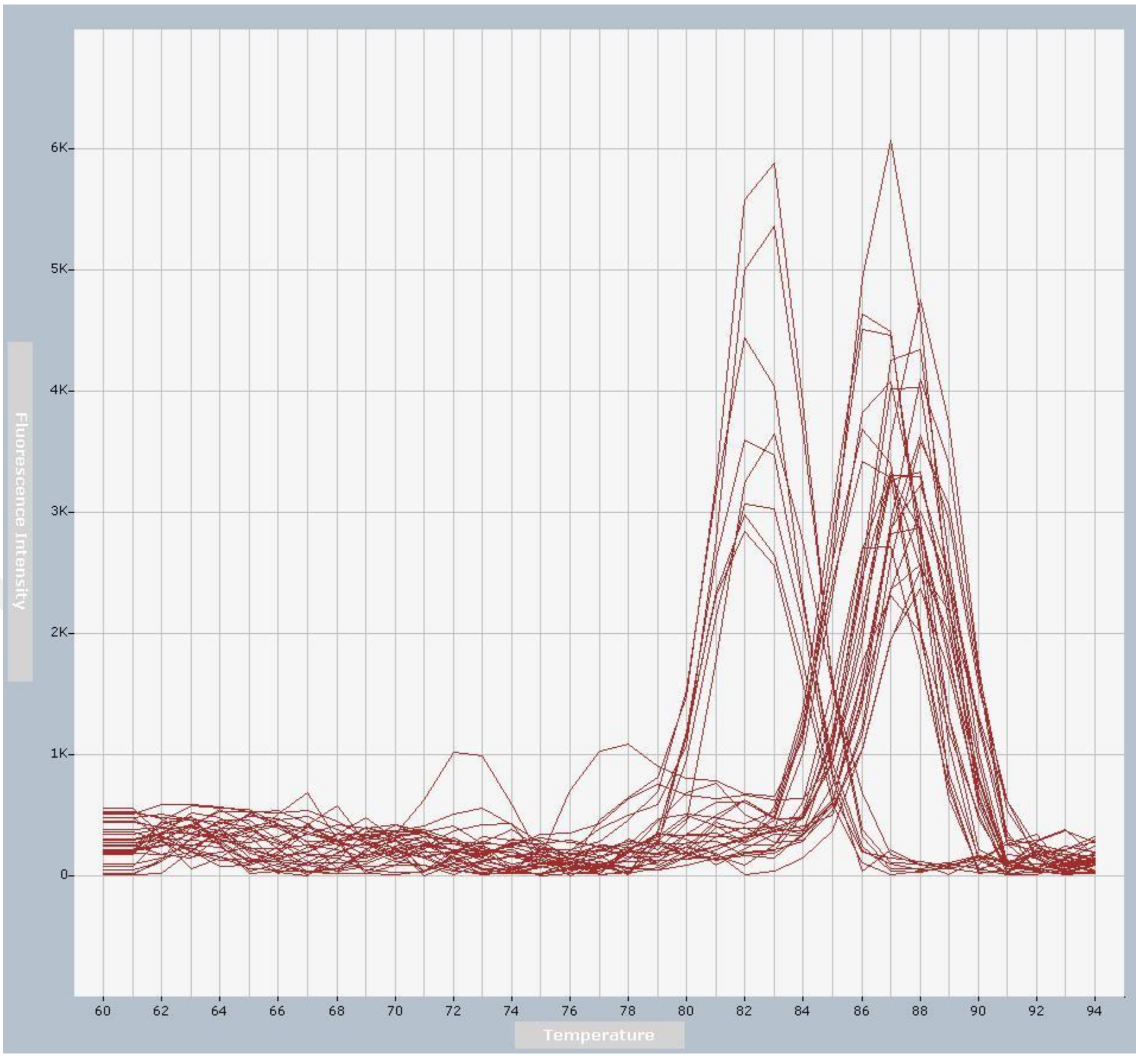

Figure 4.11 Primer specificity for both ADOR3 and housekeeping gene

The GA genotypes were not affected by the consumption of energy drinks, as their average gene expression was close to normal, while the rest of the genotypes were affected differently by the effect of the drinks on the expression of the ADORA3 gene. This means that the GA in the rs923 SNP has a protective role in the effects of beverages.

The most precise and sensitive approach for determining gene expression is the qRT-PCR technique. It has thus emerged as the industry benchmark for gene expression study. Comparing the amount of a certain gene's expression across various samples is the premise behind this method. A Ct value (cycle threshold) was used to quantify amplification, with higher Ct values denoting low gene expression and lower Ct values denoting high gene expression (Derveaux *et al.* 2010).

The transcriptional activity of the promoter regions containing various alleles varied in the current study and there were statistical differences in the levels of ADORA3 mRNA among the four genotypes. All of this suggests that rs1544223 and rs923 may affect promoter activity, which in turn may affect the level of gene expression. The A DORA3 gene's 5' untranslated regions (5' UTR) contained Rs1544223. In eukaryotic cells, mRNA translation initiation is primarily regulated by a highly up-regulated 5' UTR (Hughes 2006, Pickering and Willis 2005). Additionally, the mRNA's half-life can be impacted by the 5' UTR, which indirectly controls mRNA stability (Anthonisen *et al.* 2001). We made a hypothesis based on these biological results that different genotypes of rs1544223 and rs923 could result in unique 5' UTR sequences disrupt the ability of this region to start mRNA translation and have an impact on mRNA stability. This could be how rs1544223 results in different expression levels (He *et al.* 2018).

The involvement of rs1544223 in ADORA3 expression may influence this receptor's cardio protection. In rat models, minor overexpression of ADORA3 improves cardio protection with no deleterious consequences, whereas in mice, six-fold overexpression causes dilated cardiomyopathy (Black *et al.* 2002, Peculis *et al.* 2011). Jacobson and Gao (2006) also observe that ADORA3 is expressed at a very low level in the heart. As a result, either this receptor is very effectively connected to signaling pathways that protect the heart, or its cardioprotective effects are also produced by ADORA3 activation in cells other than heart tissue. All of this suggests that the cardioprotective effect of this receptor is highly reliant on its expression level.

In this study, individuals with different genotypes showed higher gene expression several times compared to control samples. This may be attributed to the fact that consuming energy drinks increases the gene expression of the ADORA3 gene and this is what the current study showed, as the levels of gene expression were much higher in three genotypes. compared with control samples. This expression is higher than what was shown by other studies, as one study showed that the gene expression of the studied genotypes was twice as high (He *et al.* 2018), while the gene expression in our current study was tens of times higher. The mechanism by which energy drinks increase expression is poorly understood and energy drinks extensive studies**.**

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusion

- The relationship between energy drink consumption and elevated liver enzymes.
- The study showed an association between the consumption of energy drinks and high levels of urea and creatinine.
- The study indicated that there is no connection between the consumption of energy drinks and the genotypes of both rs1544223 and rs923 SNP.
- The study noted that there was an increase in the levels of gene expression among carriers of the genotypes GG, CC and CT among the drinkers of energy drinks compared to the control samples.
- The study noted stability in gene expression levels in GA genotype carriers compared with comparison samples.

5.2 Recommendation

- Studying the influence of energy drinks on heart function.
- Investigate the consequences of consuming energy drinks on liver and kidney tissues.
- Examine the impact of energy drink use on the gene regulation of ADOR-A33.

- We recommend taking samples from other sports venues who consume large amounts of energy drinks containing high concentrations of caffeine.

- We recommend increasing the number of studied samples to discover the largest number of variables.

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