SELINUS UNIVERSITY
OF SCIENCES AND LITERATURE

# THE RELATIONSHIP BETWEEN ANGIOTENSIN RECEPTOR (AGTR.1) AND HIGH BLOOD PRESSURE

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A DISSERTATION

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# Abstract

There are reported associations between a polymorphism of the angiotensin II type 1 receptor (AGTR.1/A1166C) gene and hypertension in some populations.

To study the possibility of an association between single nucleotide polymorphism (SNP) A1166C in AGTR.1 and high blood pressure in the world, we collected information from 11 practical studies from around the world that were conducted on this subject, including methods, results, background, and conclusions.

The AGTR.1 polymorphism was assessed in these studies using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based method.

Results: The results indicated that AGTR.1 A/C1166 polymorphism has a significant association with hypertension in Canada, San Luis, Korean, Czech and Spain. In Germany, Japan and Italy was AGTR.1 A/C1166 polymorphism not associated with hypertension, while AGTR.1 A/C1166 polymorphism has a possible association with hypertension in Iran and Lebanon.

**Keywords:** A1166C SNP, angiotensin receptor, gene polymorphism, hypertension, PCR-RFLP.

# Acknowledgements


"The more that you read, the more things you will know, the more that you learn, the more place you will go"

Dr. Seuss

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# 1. Introduction

## 1.1. Importance of the research:

High blood pressure is a major health problem because of its wide prevalence and because it predisposes to cardiovascular disease and kidney disease [1]. High blood pressure affects about a quarter of the world's population [2] and has been identified as a leading cause of death. However, high blood pressure results from the interaction of various genetic and environmental factors, where genetic causes are responsible for 30-40% of all cases of high blood pressure [3].

Science is still far from understanding the exact genetic background of high blood pressure disease, as there is a disagreement about the role of the A1166C polymorphism in the AGTR1 gene as an indicator of susceptibility to high blood pressure. Several studies in different countries have shown that people who have the A1166C polymorphism in the AGTR1 gene may be more susceptible for high blood pressure, while other studies did not show any role for this polymorphism in increasing susceptibility to high blood pressure.

In the researches, they try to study the society and contribute to resolving the global debate about the value of this polymorphism as an indicator of high blood pressure. They study the presence of the A1166C polymorphism in the AGTR1 gene in hypertensive patients in the society and compare it with a group of healthy subjects.

## 1.2. Research Objectives:

1. Determining the genotype distribution of the polymorphism of the angiotensin receptor gene AGTR1 A1166C in a sample of the society.
2. Investigating the genotype of the studied SNP in hypertensive patients in the studied society and determining its distribution ratio compared to healthy subjects to show its association with hypertension.

### 1.3. Thesis Outlines

The thesis is structured as follows:

**Chapter 2** presents the Literature Review of information collected in the research.

**Chapter 3** introduces Information about the blood pressure and the factors influencing it, as well as the types of blood pressure measurements, in addition to defining high blood pressure, classifying its levels, determining the factors and causes predisposing it, and also dealing with the classification of high blood pressure according to organic damage, as well as special patterns of high blood pressure.

**Chapter 4** describes the Renin-angiotensin system, its components and its relationship with pathological hypertension, in addition to the forms of angiotensin and the effects of angiotensin II on various organs of the body. This chapter also describes the angiotensin II receptors of the first and second types and the mechanism of regulation of these receptors. It also deals with the definition of genetic polymorphism, the types and mechanisms of genetic mutations, in addition to the genes responsible for high blood pressure and their types.

**Chapter 5** presents the experimental part including the definition of the materials and devices used in the research and their different types. It also talks about laboratory research methods and the stages of DNA extraction and focuses on the PCR and electrophoresis mechanism.

**Chapter 6** explains the scientific studies in different countries that were conducted to determine the possible relationship between high blood pressure and the gene A1166C responsible for the angiotensin 1 receptor (AGTR.1). It also explains the results of these studies.

**Chapter 7** discusses the results of the studies.

**Chapter 8** summarizes our contributions and gives some perspective points for future directions.

# 2. Literature Review

The relationship between the A1166C polymorphism in the AGTR1 gene and hypertension is still not entirely clear, as this nucleotide is located in the 3rd region outside of the 3′-UTR [130].

It has been suggested that this polymorphism may interfere with the regulation of AGTR1 expression [131].

Evidence also indicates that there are gender differences in the effectiveness of the RAS device, which is responsible for the differences in the value of blood pressure between men and women [132-1135].

It was suggested that there is an interaction between sex and the A1166C polymorphism that affects hypertension, as it was found that the AC and CC genotypes are associated with high blood pressure in Canadian women compared to the AA genotype of the AGTR1 gene [133].

Studies of the association between high blood pressure and this polymorphism began in 1994 when the scientist Bonnardeaux and his colleagues indicated that there was a large prevalence of the C allele among people with high blood pressure compared to healthy subjects in France [98] and since then many studies have been conducted around the world to clarify the association between polymorphism AGTR1 and high blood pressure.

Numerous studies have considered the role of polymorphisms in the angiotensin receptor gene AGTR1 as risk factors for high blood pressure. Studies conducted to determine the relationship between the polymorphism A1166C in the AGTR1 gene and high blood pressure showed conflicting results, as studies in Lebanon [137], France [110], Argentina [138] and Canada [139] showed that there is an important relationship between the A1166C polymorphism and the risk of developing high blood pressure, while other studies did not show any relationship between them as in the studies of Japan [140] and Iran [141].

According to previous studies, it was predicted that the presence of the A1166C polymorphism is associated with susceptibility to arterial hypertension.

Indeed, Zhu and his colleagues published in 2006 [142] in his study that included 150 people from the Chinese community that the A1166C polymorphism in the AGTR1 gene is associated with high blood pressure and atherosclerosis, and in another study conducted by Rehman and his colleagues in 2007 in Malaysia [143] it was found that the A1166C polymorphism it is not associated with high blood pressure

A 2007 study by Miyama and colleagues showed that the A1166C polymorphism in the AGTR1 gene does not predispose to essential hypertension [144].

# 3. Data - High blood pressure

## 3.1. Introduction:

The human genome contains thousands of genes whose inactivation leads to an abnormal phenotype that appears in some cases as a disease or disorder. This disease or disorder may be simple or complex, depending on the nature and number of genotypes that control this phenotype in addition to the interaction with environmental factors.

High blood pressure (Hypertension) is one of the most common diseases in the world, and it was considered a major risk factor for heart, cerebrovascular and renal diseases.

It leads also to an increase in the death rate if the patient is not treated and monitored closely. Moreover, it causes an economic burden on both the patient and the country.

In the past two decades, research focused largely on understanding the relative factors, including the genetic elements, and their interaction with many other factors, in order to find appropriate treatment methods for high blood pressure. The regulating of blood pressure is crucial to maintaining adequate blood flow in the body. The blood pressure is the measurement of force applied to artery wall.

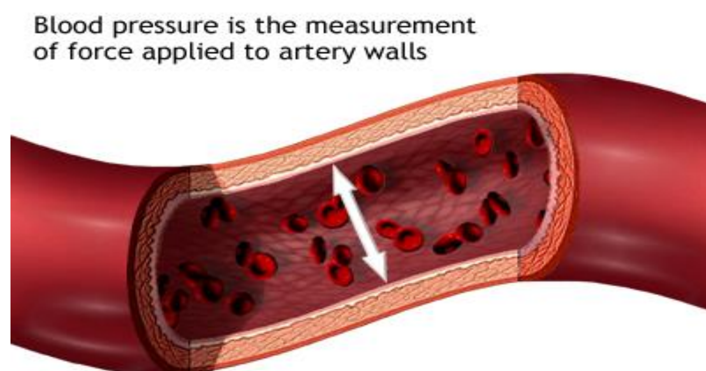



**Fig. 3.1**: Blood pressure

### 3.2. Blood pressure

Blood pressure is the force that circulating blood exerts on the walls of the arteries, the major blood vessels of the body. Blood pressure is vital to all organs because it is the force of the blood that presses against the walls of the blood vessels when the heart pumps. The artery (a blood vessel) carries blood from the heart to the various organs.

Blood vessels can be compared to tubes. A large vessel can be compared to a water hose, and a small vessel can be compared to the narrower tip of the water hose. When the tip of the water hose is pinched or tightened, a higher pressure is pressed against the walls of the hose.

The level of blood pressure essentially depends physically on two factors: the ejection volume of the heart per unit time (cardiac output) and the level of peripheral resistance.

This includes the flow resistance, which is caused by the fine vascular branches in the area of the terminal flow path and is influenced by the widening or narrowing of the vessels by the autonomic nervous system. The higher the cardiac output and/or the peripheral resistance, the higher the blood pressure.

**Table 3.1:** Factors leading to an increase in peripheral vascular resistance and cardiac output and result in Hypertension

**Hypertension because of:**

| Increased peripheral vascular resistance | Increased cardiac output |
|---|---|
| • Stress<br>• Activation of the sympathetic pathway<br>• atherosclerosis<br>• Renal artery disease<br>• Excessive angiotensin II<br>• Pheochromocytoma<br>• Excessive catecholamine<br>• Hypothyroidism | • Increased blood volume<br>• Narrowing of the renal artery<br>• Excessive aldosterone in blood<br>• Excessive secretion of antidiuretic hormone ADH<br>• Aortic stenosis<br>• Pregnancy<br>• Stress |

| | |
|---|---|
| • Diabetes<br>• Cerebral ischemia | • Activation of the sympathetic pathway<br>• Pheochromocytoma<br>• Excessive secretion of catecholamine in the blood |

**3.3. Types of blood pressure measurement:**

There are two types of blood pressure measurements, systolic and diastolic. That means Blood pressure is reported in two numbers, such as 120/70 mmHg (mmHg or mm of mercury is the medically preferred unit of measurement) [4].

- The first number, called systolic blood pressure, represents the pressure in the arteries when the heart muscle contracts (when the heart beat is heard).
- The second number, called diastolic blood pressure, represents the pressure in the arteries when the heart muscles relax between the heart beats.

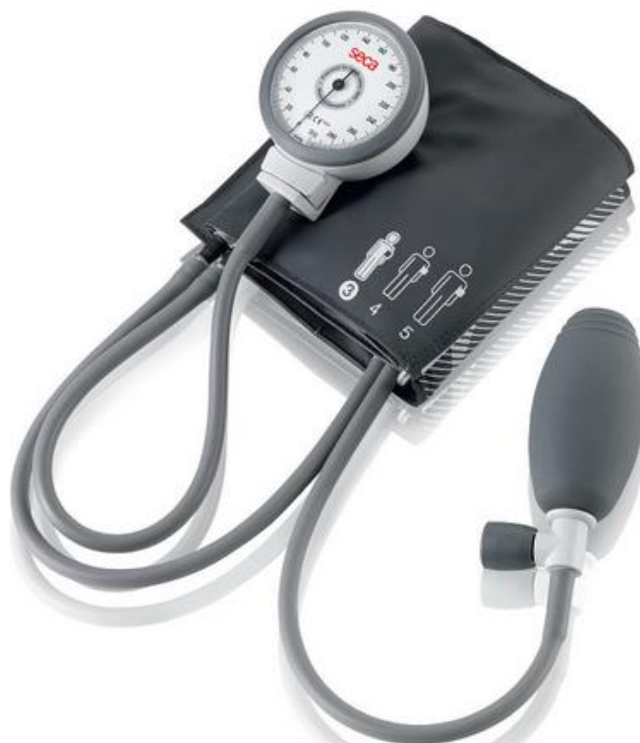



**Fig. 3.2**: Blood pressure measurement

### 3.4. Hypertension [5]:

- Hypertension – or high blood pressure – is a serious medical condition that is characterized by a persistent increase in blood pressure and its consequences.
- Hypertension is diagnosed if, when it is measured on two different days, the systolic blood pressure readings on both days is ≥140 mmHg and/or the diastolic blood pressure readings on both days is ≥90 mmHg.
- Hypertension is a leading cause of premature death worldwide.
- One of the global non-communicable disease targets is to reduce the prevalence of hypertension by 33% between 2010 and 2030.
- Globally, an estimated 1.28 billion adults aged 30 to 79 years have hypertension, with most (two-thirds) living in low- and middle-income countries.
- An estimated 46% of adults with hypertension do not know they have the condition.
- Less than half of adults (42%) with hypertension are diagnosed and treated.
- About 1 in 5 adults (21%) with hypertension have it under control.

### 3.5. Classification of blood pressure levels:

- Optimal blood pressure is less than 120 mmHg systolic and less than 80 mmHg.
- Normal blood pressure in most adults is less than 140 mmHg systolic and less than 90 mmHg diastolic.
- People with blood pressure ranging from 120 – 139 mmHg systolic and 80 – 90 mm Hg diastolic, may be predisposed to have hypertension and may be advised to modify their lifestyle and diet, but not all patients need to be treated with medications.
- Adults with hypertension may have:
    - <u>stage I (light)</u> hypertension (a blood pressure ranging from 140-159 mmHg systolic and 90-99 mmHg diastolic).
    - <u>stage II (average)</u> hypertension (a systolic blood pressure ranging from 160-179 mmHg and a diastolic pressure ranging from 100-109 mmHg). Patients with cardiac risk.

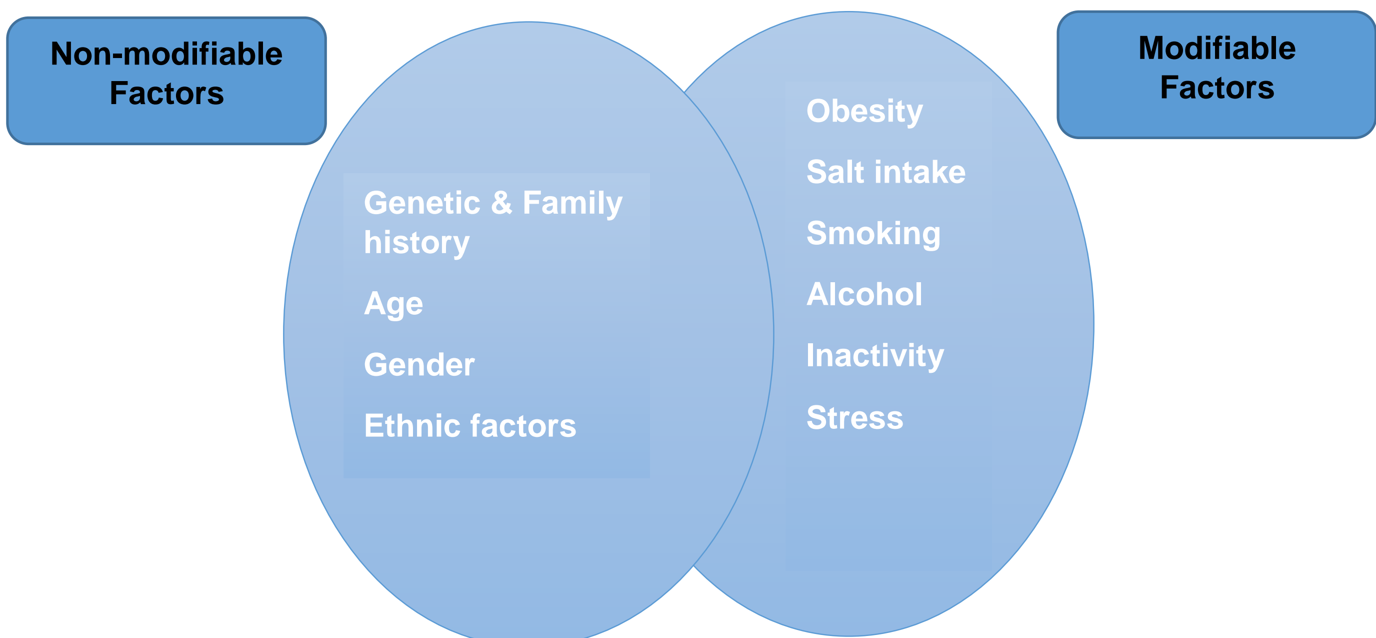
- stage III (extreme) hypertension (systolic blood pressure equal or higher than 180 mmHg, and a diastolic blood equal or higher than 110 mmHg). Patients with high cardiac/ cerebral risk.

Patients with cardiac risk factors may need to be treated for a blood pressure above 120/70.

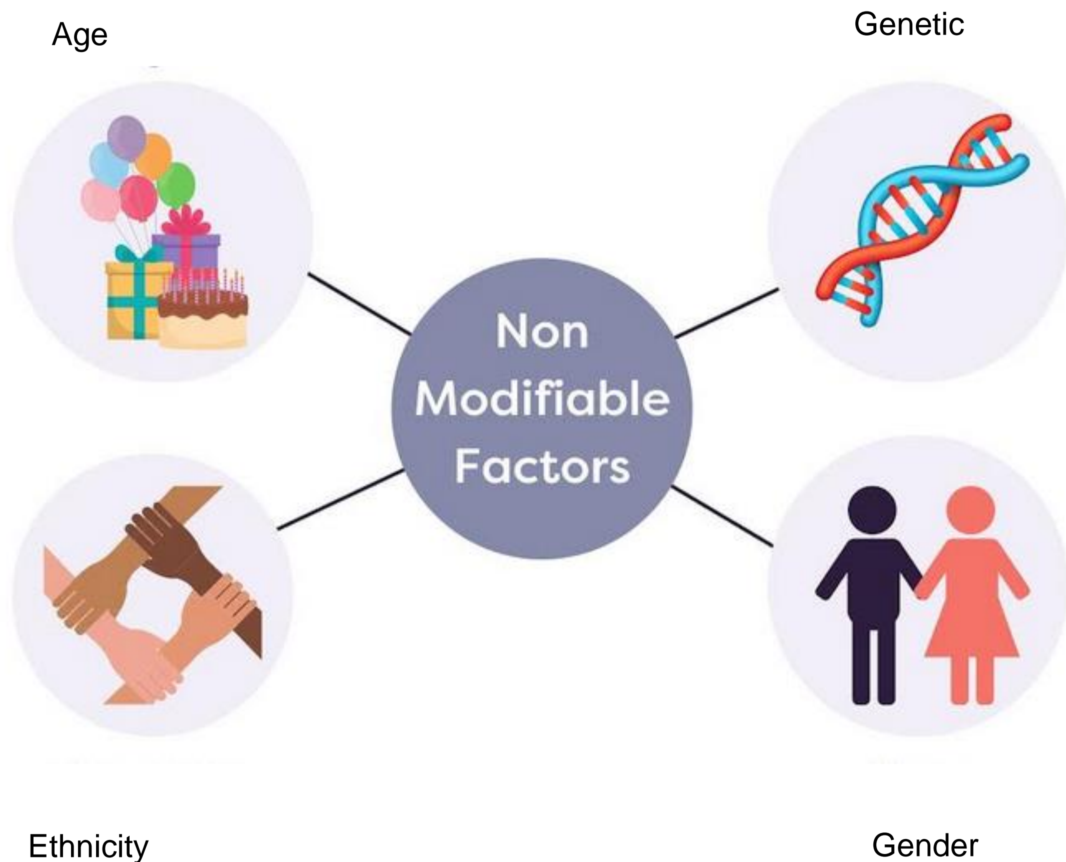
**Table 3.2:** Classification of blood pressure levels [6].

| Classification | Systolic blood pressure (mmHg) | Diastolic blood pressure (mmHg) |
|---|---|---|
| **optimal** | <120 | <80 |
| **normal** | 120-139 | 80-90 |
| **Hypertension (light)** | 140-159 | 90-99 |
| **Hypertension (average)** | 160-179 | 100-109 |
| **Hypertension (extreme)** | >180 | >110 |

**3.6. Predisposing factors of Hypertension [7]:**



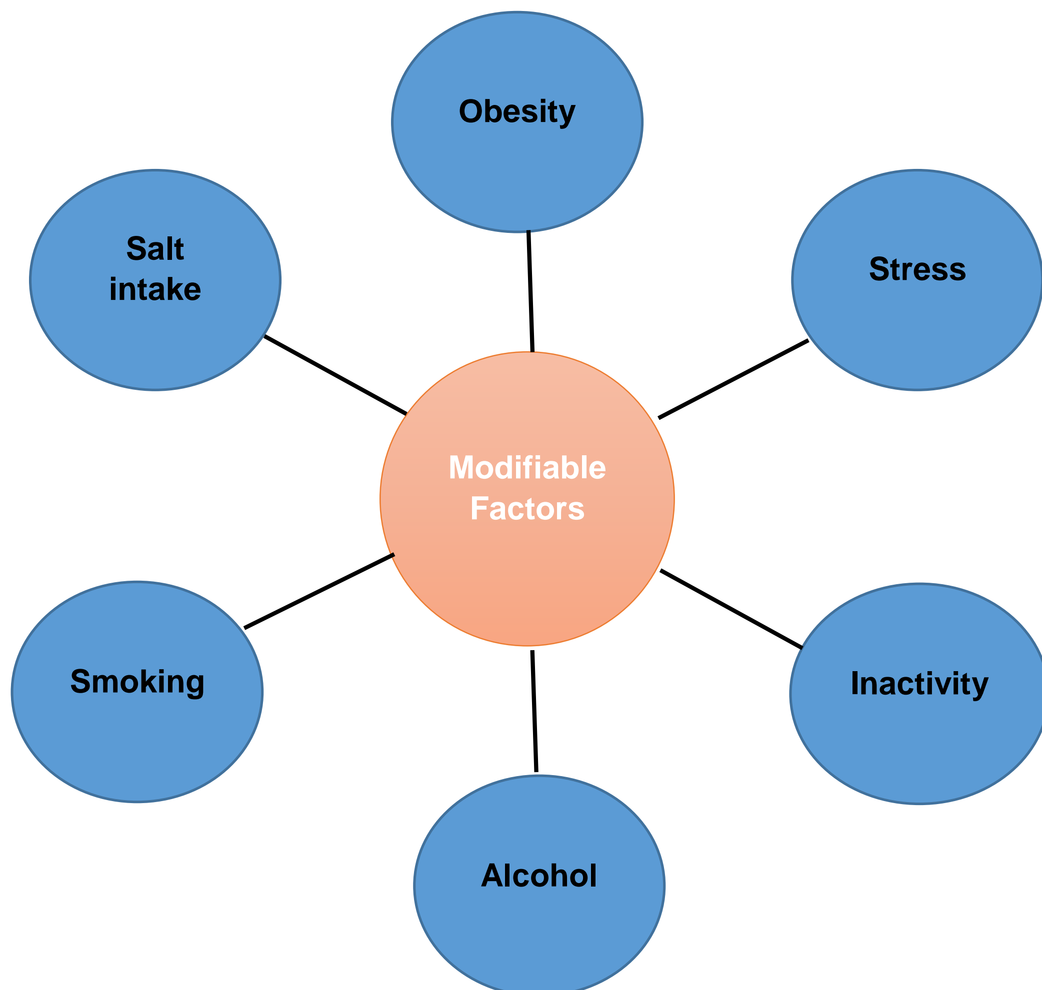
### 3.6.1. Non-modifiable Factors:




- Genetic and Family history is a useful tool for identifying health risks and preventing disease, and genes likely play some role in hypertension. Hypertension appears to run in families, so it is important to know more about family history. The genetic predisposition that makes certain families more prone to hypertension appears to be related to increased intracellular sodium levels and decreased potassium-sodium ratios.
- Age is an unmodifiable risk factor for hypertension. There is a relative relationship between age and hypertension. In the life of the individual, there is a continuous increase in systolic and diastolic blood pressure. It is at birth 70/45 mm Hg and gradually rises to 100/60 mmHg in adolescence and up to 125/70 mmHg between the ages of twenty and forty. It may then rise to 140/90 mmHg at the age of sixty. However, epidemiologic studies have shown a poorer prognosis in clients whose hypertension began at a young age.

- Gender: There is a striking age-related sexual dimorphism in the prevalence of hypertension. Men experience hypertension at higher rates and at an earlier age than do women until after 60 years. Women have lower systolic blood pressure (SBP) than men in early adulthood, whereas the opposite is true after the sixth decade of life [8].
- Ethnic factors: The difference in prevalence of essential hypertension depends on ethnicity, being higher in black Americans (32.4%) compared to white Americans (23.3%). The reason has been attributed to heredity, greater salt intake, and greater environmental stress [9].

### 3.6.2. Modifiable factors:



- Obesity or additional weight: Obesity, in particular that located in the upper body with increased amounts of intra-abdominal fat, is an important cause of hypertension; the combination may be related to hyperinsulinemia secondary to insulin resistance.

  The excess fat cells in the body caused by obesity need also large amounts of oxygen and nutrients for their survival, so they force the heart to pump larger amounts of blood and thus increase the blood pressure. The more weigh means the more blood you need to supply to tissues. Overweight (Body-Mass-Index: BMI > 25) [21].

- Salt intake: There is a strong relationship between the amount of salt consumed and raised levels of blood pressure.

In countries with low-salt diets, the increase in blood pressure with age seen in most Western countries does not occur [10]. Lower sodium intake lowers blood pressure, with a greater effect in people with hypertension [11].

- Smoking: There is now no doubt that cigarette smoking is a strong potent cardiovascular risk factor [12]. Smoking cessation is probably likely to be the most effective lifestyle intervention for the prevention of a variety of cardiovascular diseases, including stroke and Myocardial infarction [13]. Smokers with hypertension are at higher risk of developing severe forms of hypertension, including malignant and renovascular hypertension. Smokers have twice the overall mortality compared with nonsmokers [14].

- Drinking too much alcohol: Having more than two drinks a day for men and more than one drink a day for women increases the risk of hypertension. Excessive alcohol consumption can drive blood pressure to unhealthy levels. Consuming more than three drinks at a time temporarily raises blood pressure. Repeated binge drinking can lead to a long-term increase in blood pressure [15].

- Inactivity: Lack of regular exercise can increase the possibility of hypertension. Exercise is a key component of lifestyle therapy for the primary prevention and treatment of hypertension. There are beneficial effects of exercise on hypertension with reductions in both systolic and diastolic blood pressure with as much as 5-7 mmHg reductions in those with hypertension [16]. The reduction in blood pressure with physical activity is thought to be due to attenuation in peripheral vascular resistance, which may be due to neurohormonal and structural responses with reductions in sympathetic nerve activity and an increase in arterial lumen diameters, respectively [17].

- Stress: Most patients with hypertension attribute great importance to psychological stress in regulating blood pressure and the need to take antihypertensive medications. Although acute psychological stress is associated with a temporary increase in blood pressure, epidemiological studies do not consistently show that chronic psychological stress affects blood pressure in the long term [18,19,20].

### 3.7. Classification of high blood pressure according to causes: [22].

High blood pressure is classified according to causes into two types

- Primary or essential hypertension; this type occurs with no known cause. This type develops slowly over many years and as we age. It is by far the most common type of hypertension and it represents 90- 95% of the diagnosed cases.

- Secondary hypertension; This type may appear suddenly or can cause higher blood pressure than the primary hypertension. It occurs as a result of pre-existing disorders such as:
    - Narrowing of the arteries that supply the kidneys.
    - Chronic kidney disease or failure.

- Abnormalities in the endocrine system, such as overactive adrenal glands, problems with the thyroid gland, etc.
- Congenital (condition present at birth) defects in the large blood vessels
- Certain medications, such as birth control pills, cold medications (decongestant pills for sinus), anti-inflammatory pain killer drugs, cortisone, etc.
- Illegal drugs, such as cocaine.
- Pregnancy in some women.

Secondary hypertension cases represent 5-10% of the total cases of high blood pressure. In this type the cause is diagnosed with various laboratory and clinical tests.

### 3.8. Classification of high blood pressure according to the severity of organ damage [23]:

Hypertension is classified according to the following degrees of severity:

- **Stage I:** Hypertension without demonstrable organ damage to heart, kidneys, brain, ocular fundus.
- **Stage II:** Hypertension with demonstrable damage to heart, kidneys, brain, ocular fundus.
- **Stage III:** Hypertension with damage to heart, kidneys, brain, ocular fundus and marked functional impairment of one of these organs.
- **Stage IV:** Hypertension with rapidly progressive functional impairment of any of the above organs.

### 3.9. Special patterns of Hypertension:

- High blood pressure due to leukoplakia: high blood pressure let's just go to the doctor's office.
- High systolic blood pressure: systolic lower pressure is elevated, accompanied by a normal lower diastolic pressure.

- Malignant hypertension: severe hypertension is associated with bulimia nervosa and optic neuralgia.
- Nubian hypertension: an abnormality (fluctuation) in blood pressure values.
- Borderline hypertension: a low blood pressure that ranges from the upper normal limits.
- Acute high blood pressure: it is caused by hardening of the arteries and their lack of elasticity in the tissues.
- Pulmonary hypertension: low pressure is raised in the pulmonary circulation.
- High blood pressure in the iliac vessels: due to stenosis of the iliac artery.
- Pregnancy hypertension: occurs during the pregnancy.

# 4. Data - Renin-angiotensin

### 4.1. Introduction:

Many biological activities in the human body need the renin-angiotensin system (RAS). An important example is the pathophysiological mechanisms of hypertension.

Understanding this action helps us to develop new therapeutic methods to inhibit the effects of this system. In this chapter, we explain the relationship between the renin-angiotensin system and hypertension.

At first, we present briefly about the history, biochemistry, cell biology, and overall effects of the RAS component.

### 4.2. Renin-angiotensin system:

#### 4.2.1. The history of the RAS component:

The RAS is a hormone system that plays an important role in circulatory system, blood pressure, and regulation of electrolytes and fluids.

In 1898, Tiergersted and Bergman established through unpurified salt extracts that the kidney contains a pressure-increasing hormone called renin. In 1934, Golblatt demonstrated that constriction of the renal arteries in dogs caused persistent hypertension [24]. The clarification was the reduction in vascular area and in consequence, the increase in force leads to the increase in blood pressure. In 1940, Golblatt explained that renin is actually a protein that acts on a substrate in plasma [24].

In the following 20 years, the designation of this substrate was controversial, since the two major research groups dealing with the subject at that time (one from Argentina and one from the United States) presented different designated ones. The group from Argentina called the substrate hipertensin and the group from USA called it angiotonin, until these names were changed to angiotensin, the actual press agent, after 20 years.

The precursor of this peptide was called angiotensinogen [25]. Therefore, time had an idea of this simplified system, Fig. 4.1.

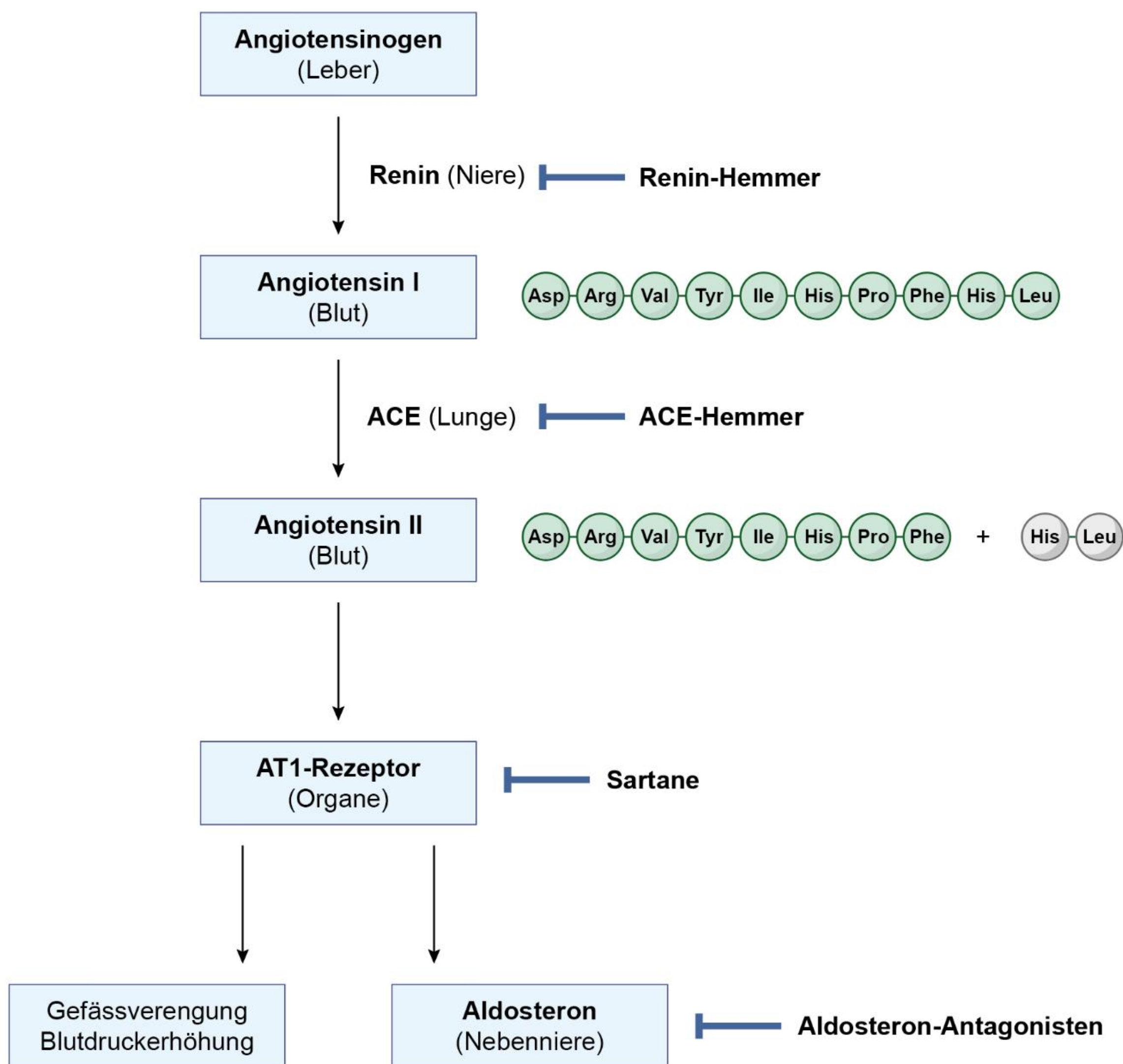



**Fig. 4.1:** Stages of angiotensin formation

### 4.2.2. Forms of angiotensin:

The two forms of angiotensin identified in the 1950s are called angiotensin I and II. **Angiotensin I** is a chain of 10 peptides, which is why it is also called a decapeptide. **Angiotensin II** is formed by cleavage of two peptides from angiotensin I to form an octapeptide. This cleavage involves an enzyme located on the luminal surface of the endothelial cells of the vasculature called angiotensin converting enzyme (ACE).

In the big picture, the peptide angiotensin II is more active, meaning that angiotensin II has the main vasoconstrictor effect.

Thus, in the mid-1950s, the overall picture of this system was completed both by the presentation of the angiotensin II and by the observation that the RAS system simultaneously regulates the secretion of aldosterone. Based on these findings, the 1970s and 1980s saw further development of these polypeptides, which interfere with the components of the RAS, i.e., directly inhibit the release of renin or ACE, as well as angiotensin receptor antagonists. To date, these findings have made it possible to improve quality of life, as these drugs are mainly used to treat hypertension, and more recent studies show their effects in inflammatory diseases. To understand this (subtle) relationship between RAS and inflammatory processes, it is necessary to know the components of this system.

### 4.3. Renin angiotensin system components:

#### 4.3.1. Renin:

- Renin is produced, stored and secreted in so-called juxtaglomerular cells, cells that circulate in the renal artery and are present in the afferent arterioles, the infiltrating glomeruli, to promote renal blood flow in this region.
- The release of renin by juxtaglomerular cells (CJG) is controlled by three main pathways: two acts locally in the kidney and the third acts indirectly via the CNS, where norepinephrine is released from renal noradrenergic nerves.
  1. The macula densa is a mechanism that controls the release of renin. It is a complex mechanism that relies on receptors, cyclic adenosine monophosphate (cAMP), and also prostaglandins. In general, the macula densa is located adjacent to the CJG and is composed of columnar epithelial cells. When the NaCl flux in the macula densa changes, the cells emit chemical signals that cause the CJG to inhibit or stimulate renin as the amount of NaCl increases or decreases. These signals are mediated across the macula by both adenosine and prostaglandin, the former acting to increase NaCl and the latter to decrease it. Regardless of which protein acts (adenosine or prostaglandin), the answer to these questions is the binding of these G-protein-coupled receptors, which trigger a cellular messenger (cAMP)-dependent signal. Thus, while adenosine acts at the A1 adenosine receptor, it inhibits renin release, whereas prostaglandin stimulates it.

2. The second mechanism by which renin release is controlled is the intrarenal baroreceptor pathway. It is controlled by raising and lowering blood pressure in the preglomerular vessels and thus by a mechanical phenomenon. Through this mechanical modulation, CJG inhibits or stimulates the release of renal prostaglandin, which acts in part through the intrarenal baroreceptor.
3. Finally, the third mechanism is invoked via the beta-adrenergic receptors. In this case, regulation occurs via the CNS. After the release and action of this neurotransmitter, norepinephrine is released from the postganglionic sympathetic nerves by binding to the beta-adrenergic receptors and stimulating the sympathetic nervous system and consequently the secretion of renin by the CJG.

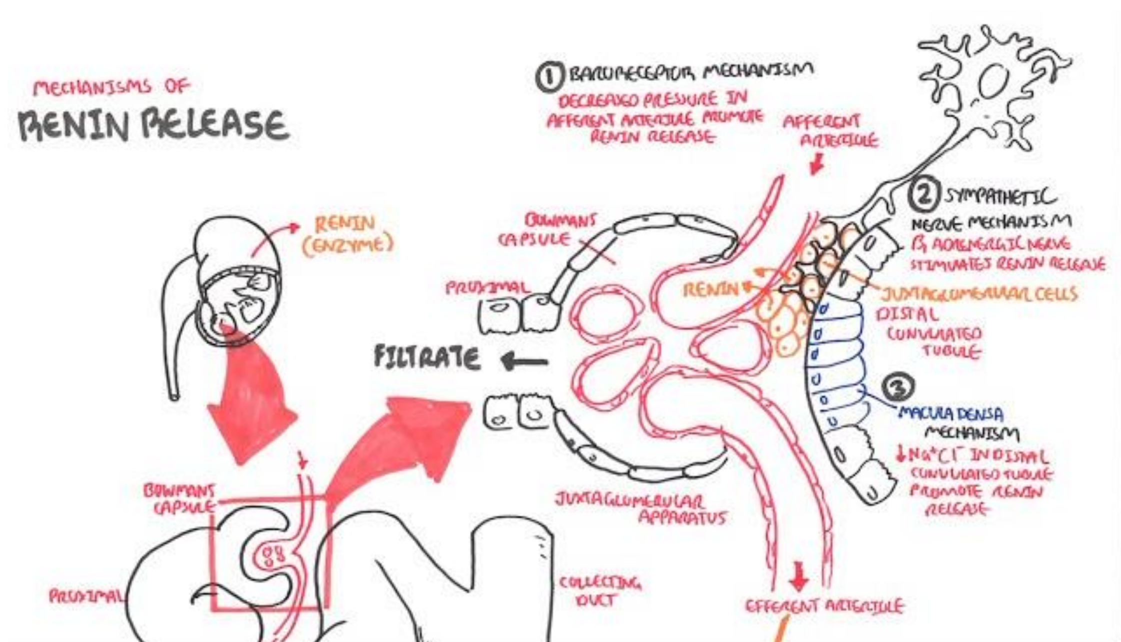



**Fig. 4.2:** Mechanisms of renin release

A physiological network is involved in these three mechanisms of regulation of renin secretion, which will be discussed below:

1. increased release of renin leads to increased release of angiotensin II. This in turn binds to AT1 receptors in the CJG. This binding leads to inhibition of renin secretion in a mechanism known as the short feedback loop.

2. In addition to angiotensin II, binding to these AT1 receptors also leads to an increase in blood pressure. Increased pressure leads to a decrease in renin secretion through the action of the high-pressure baroreceptors, increased pressure in the preglomerular vessels, and decreased pressure natriuresis (decrease in NaCl reabsorption). This mechanism of reduction in renin secretion via increased blood pressure resulting from the action of angiotensin II is known as the negative feedback loop.

- Renin is the major protease capable of determining the rate of angiotensin II production.
- The active form of renin contains 340 amino acids. It is synthesized as a pre-pro enzyme containing 406 amino acid residues, soon after which this precursor is processed to produce a pro-renin that is more mature but lacks activity. Shortly thereafter, the pro-renin is activated by an as yet undescribed enzyme that cleaves 43 amino acids of the amino-terminal tail to produce active renin.
- Renin is secreted by a process known as exocytosis. The major substrate of this aspartyl protease is an alpha-2 globulin stock, i.e., angiotensinogen, which is secreted by hepatocytes.
- The renin that cleaves the peptide bonds amino-terminal tail of angiotensinogen (leucyl-leucine in mice and rats) and (leucine-valine in humans), resulting in angiotensin I, is an active renin. Therefore, the synthesis of this protease occurs in several steps.

### 4.3.2. Angiotensinogen:

- Angiotensinogen is synthesized in liver tissue, although it may have made its transcription in adipose tissue, CNS and kidney.
- It is a globular protein, has a (MW= 55,000 to 60,000) and is the main substrate of renin.
- There is a close relationship between the amount of angiotensinogen circulating in plasma and elevated blood pressure, so that the use of estrogen-containing oral contraceptives leads to an increase in serum angiotensinogen and thus to

an increase in blood pressure. Here, the close relationship with the inflammatory process also becomes clearer. It has been shown that prostaglandin release from renin is directly interfered with, i.e., prostaglandins increase the secretion of this hormone by binding to adenosine receptors.

- There is a very close relationship between the synthesis and secretion of angiotensinogen by stimuli such as inflammation, insulin, estrogens, glucocorticoids, thyroid hormones, and angiotensin II, i.e., all of these stimuli increase the synthesis and secretion of the dodecahydrate peptide.

#### 4.3.3. Angiotensin Converting Enzyme (ACE):

- ACE is a glycoprotein ectoenzyme that not only converts angiotensin I to angiotensin II but can also deactivate bradykinin because ACE is very nonspecific and can cleave dipeptide units with many amino acids as substrate. For this reason, the ACE inhibitors such as captopril and lisinopril, for example, are able to increase bradykinin and decrease angiotensin II.
- The in vivo rapid conversion of angiotensin I to II occurs through the action of ACE, which is present on the luminal surface of endothelial cells throughout the vasculature.
- In addition to these actions of ACE, some studies show the existence of a carboxypeptidase-related enzyme called ACE2, which is capable of cleaving angiotensin I into (angiotensin 1-9) and angiotensin II (angiotensin 1-7) [26]. The classical ACE inhibitors do not inhibit this enzyme. Its physiological significance has not yet been clarified.

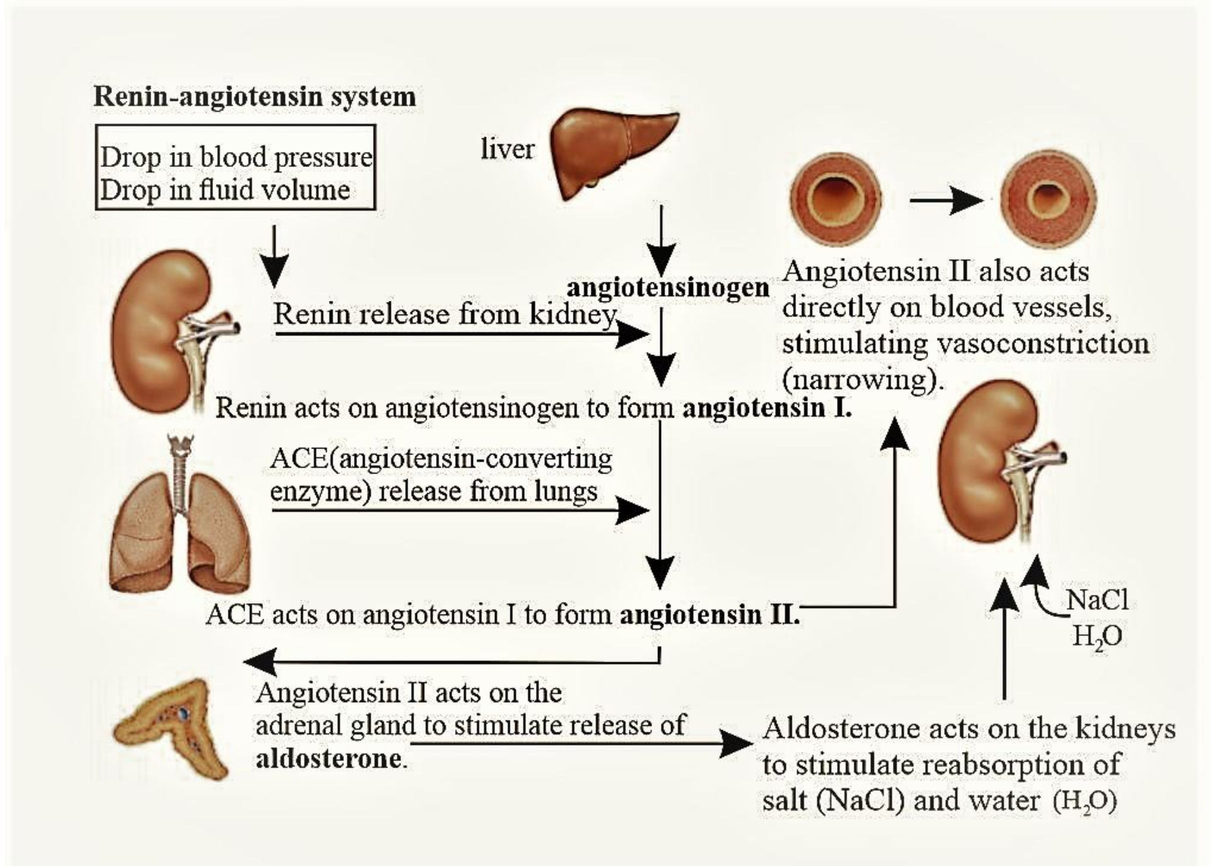



**Fig. 4.3:** Role of Angiotensin Converting Enzyme (ACE)

### 4.4. The renin-angiotensin system and its relationship with pathophysiology hypertension:

- Hypertension is one of the most common cardiovascular diseases and its prevalence increases with age.
- The hypertension is defined as a permanent increase in blood pressure to > 140/90 mmHg, and the most individuals with this pressure rank belong to the risk group of cardiovascular disease.
- The RAS plays a key role in regulating blood pressure, both in the short and long term. This occurs because the increase, even in modest concentrations of angiotensin II, leads to an acute increase in blood pressure.
- In the presence of intravenously administered angiotensin II, blood pressure increases in a few seconds and decreases to the normal rate after a few minutes. This mechanism, known as the immediate pressure response, is due to a rapid

increase in total peripheral resistance. This increase in resistance is a response that maintains arterial blood pressure in an acute hypotensive response.

- On the other hand, there is a slow pressure response that also occurs through the action of angiotensin II. This pressure response remains stable over a long period of time. This slow increase in pressure is most likely due to decreased renal excretion, resulting in an increase in fluid and salt retention that steadily increases with increasing pressure. In conjunction with these renal effects, angiotensin II also induces synthesis of endothelin-1 and superoxide anions in this response, which may contribute to this type of slow pressure response.
- Angiotensin II is about 40 times more potent than norepinephrine, and the effective concentration (EC50) for acute blood pressure elevation by angiotensin II is about 0.3 nmol/L.
- The direct action of angiotensin II is on cardiac contractility and heart rate but indirectly in the rapid blood pressure rise leads to activation of the baroreceptor reflex, and in a negative feedback loop, this occurs with the reduction of sympathetic tone and increase vagal tone.
- Other classic effects of angiotensin II include various other effects that do not merely increase blood pressure. They range from increased expression of proto-oncogenes to the inflammatory process.
- In the last decade, studies have shown that RAS blockers have anti-atherosclerosis effects not only by regulating blood pressure, but also anti-inflammatory and antioxidant effects [27]. According to studies, angiotensin II also acts on the expression of adhesion molecules such as intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), P-selectin molecules expressed in the inflammatory process, and promotes the expression of chemokines, growth factors, and cytokines. Angiotensin promotes the activation of certain stellate cells that actively promote collagen deposition and fibrosis. Thus, regardless of the mechanisms involved, ACE inhibitors have broad clinical utility as antihypertensive agents but also great potential for the therapy of other vascular diseases, as observed in experimental models.

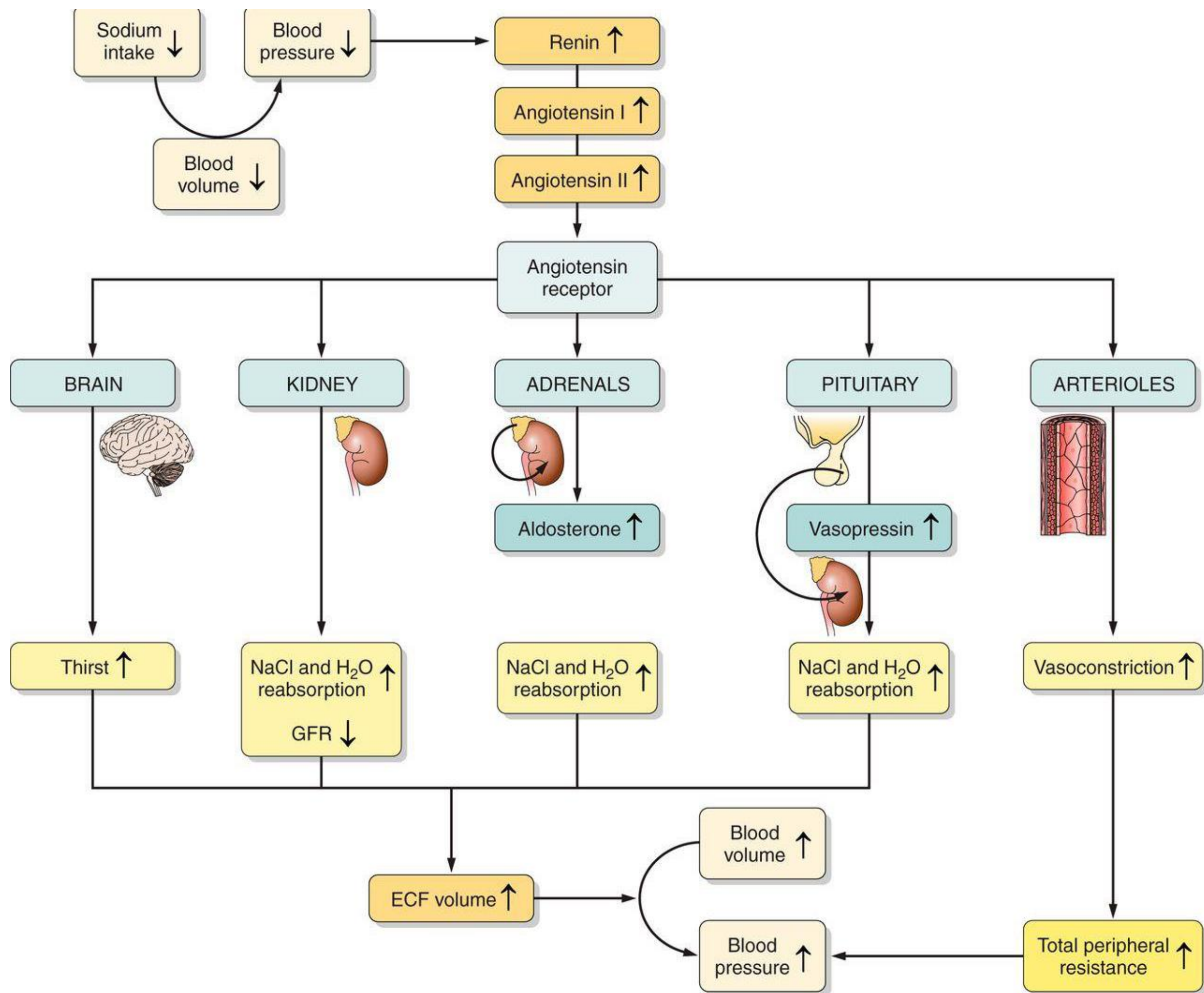



**Fig. 4.4:** Actions of Angiotensin in human Body

### 4.5. Actions of Angiotensin II

Angiotensin II plays its role through its connection with specific angiotensin receptors located on the cell surface. This connection leads to effective effects in the circulation and various organs (Fig. 4.4).

#### 4.5.1. In the blood vessels

- Angiotensin II leads to contraction of the smooth muscles of the vessels, and as a result, hypertension occurs in the arterioles.
- Angiotensin II stimulate the production of Beta- Transforming growth factor and leads to hypertrophy of the smooth muscles of the vessels and increase in the

peripheral resistance of Blood vessels [28] and increase of the blood pressure [29].

### 4.5.2. In the adrenal gland

The connection of angiotensin II with its receptors leads to increase in the secretion of aldosterone and because of this leads to increases in the retention of water and salt, and increase in plasma volume [30], and consequently to an increase in blood pressure and develop of edema (Fig. 4.5).

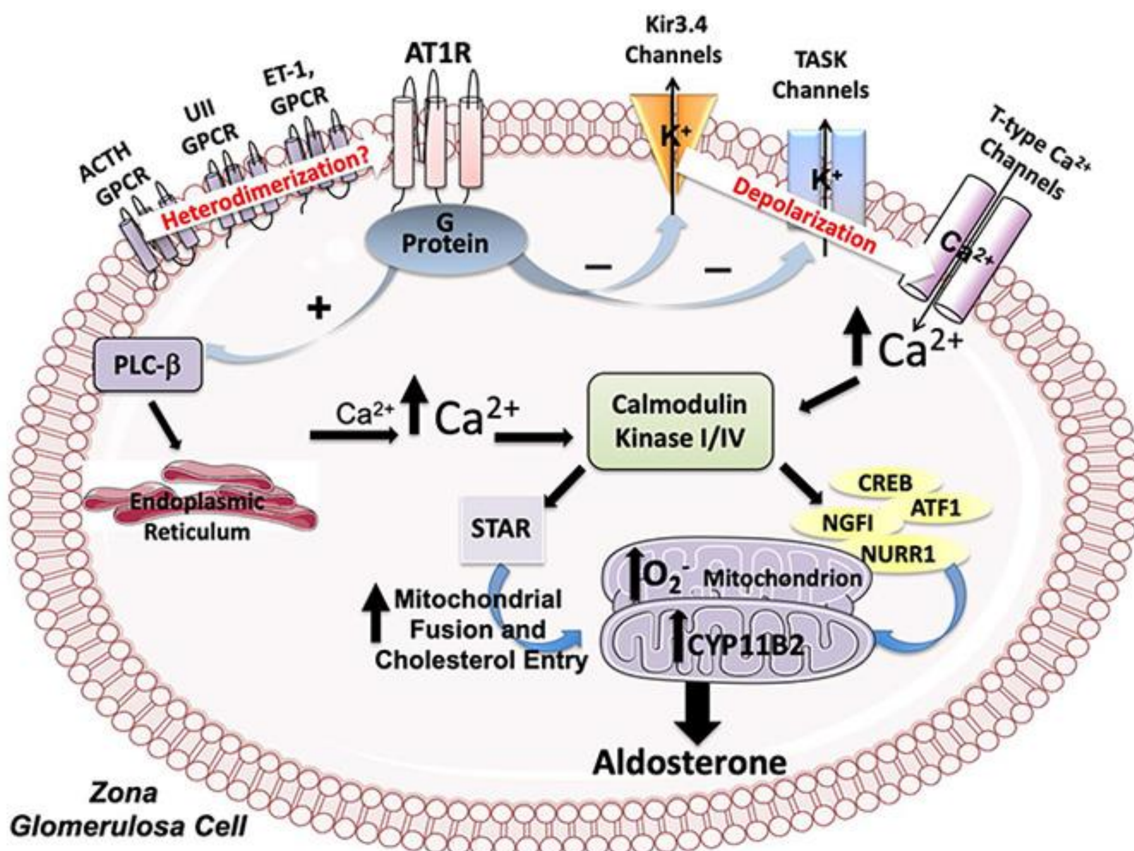



**Fig. 4.5:** Molecular pathways modulated by Ang II in adrenal zona glomerulosa cells.

### 4.5.3. In the kidney

- The renal blood vessels are very sensitive to Angiotensin II-induced vasoconstriction. The increase in vascular resistance especially in the efferent arteriole leads to a decrease in renal blood flow (RBF) and consequently to a decrease in glomerular filtration rate (GFR) [31].

- Angiotensin II causes directly an increase in resorption of Sodium and to decrease in excretion of uric acid in urine.
- Angiotensin II cause the shrinkage of mesangial cells in Kidney and leads to decrease in renal blood flow (RBF)
- Angiotensin inhibit the synthesis of renin in the kidney through the feedback mechanism. (Fig. 4.6).

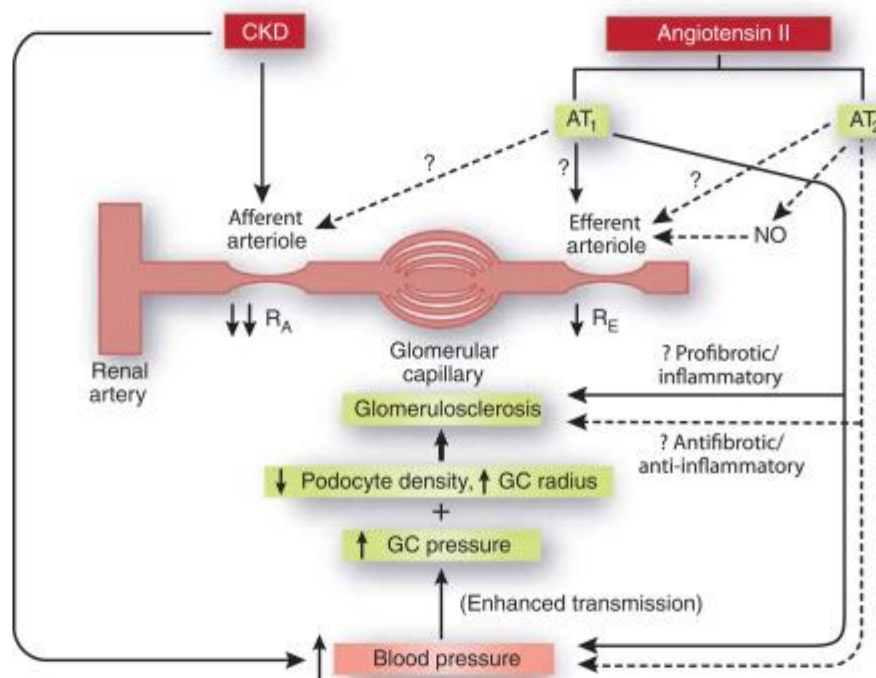



**Fig. 4.6:** Actions of Angiotensin in the Kidney

### 4.5.4. In the nervous system:

- Angiotensin affects several areas of the brain [32] and it stimulate especially the vascular regulation center and lead to increase in blood pressure.
- Angiotensin II causes an increase in secretion of vasopressin in the pituitary gland and leads to retention of sodium in blood and consequently to increase in blood volume and increase of blood pressure.
- Angiotensin II stimulates the sympathetic transportation [33], which leads to increase in cardiac output and blood pressure. (Fig. 4.7)

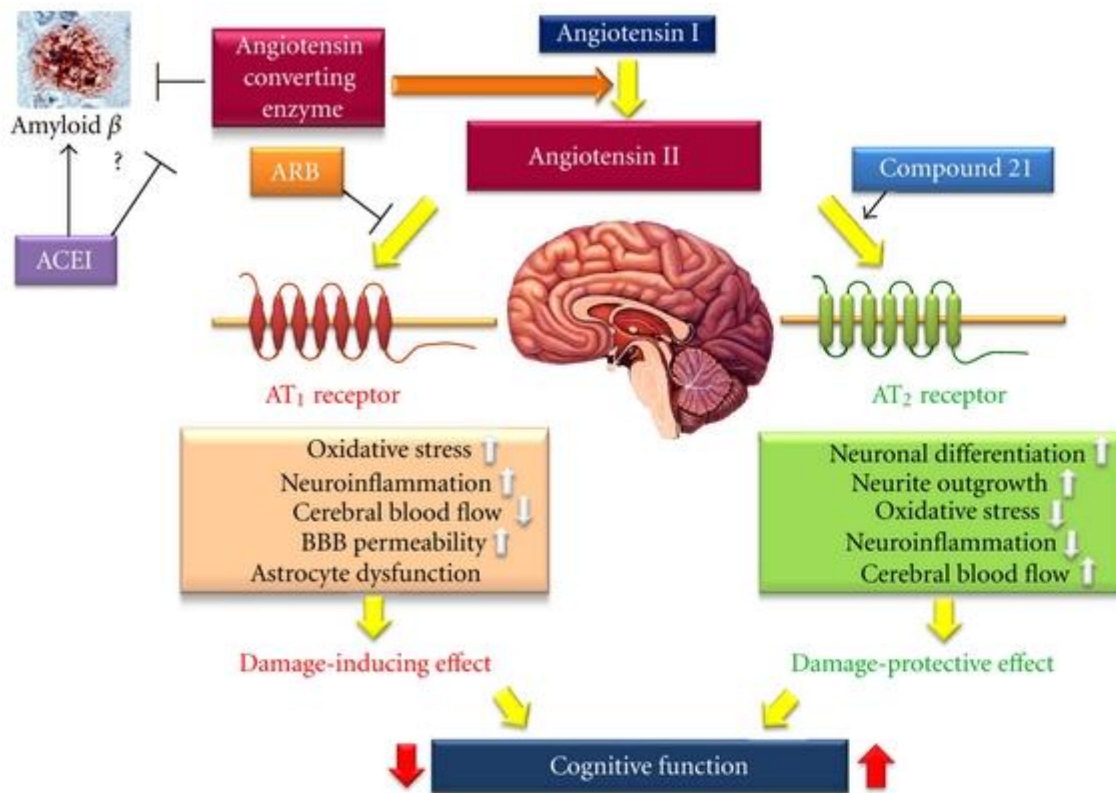



**Fig. 4.7:** Actions of Angiotensin in the nervous system

### 4.5.5. In the heart:

Angiotensin may cause heart failure. There are two mechanisms:

- Angiotensin II causes increase in aldosterone secretion and leads to salt and water retention and increase in plasma volume and leads consequently to increase in cardiac preload and workload.
- Angiotensin II causes peripheral vasoconstriction [34] and leads to increase in cardiac afterload and leads consequently to decrease in cardiac output [35]. (Fig. 4.8)

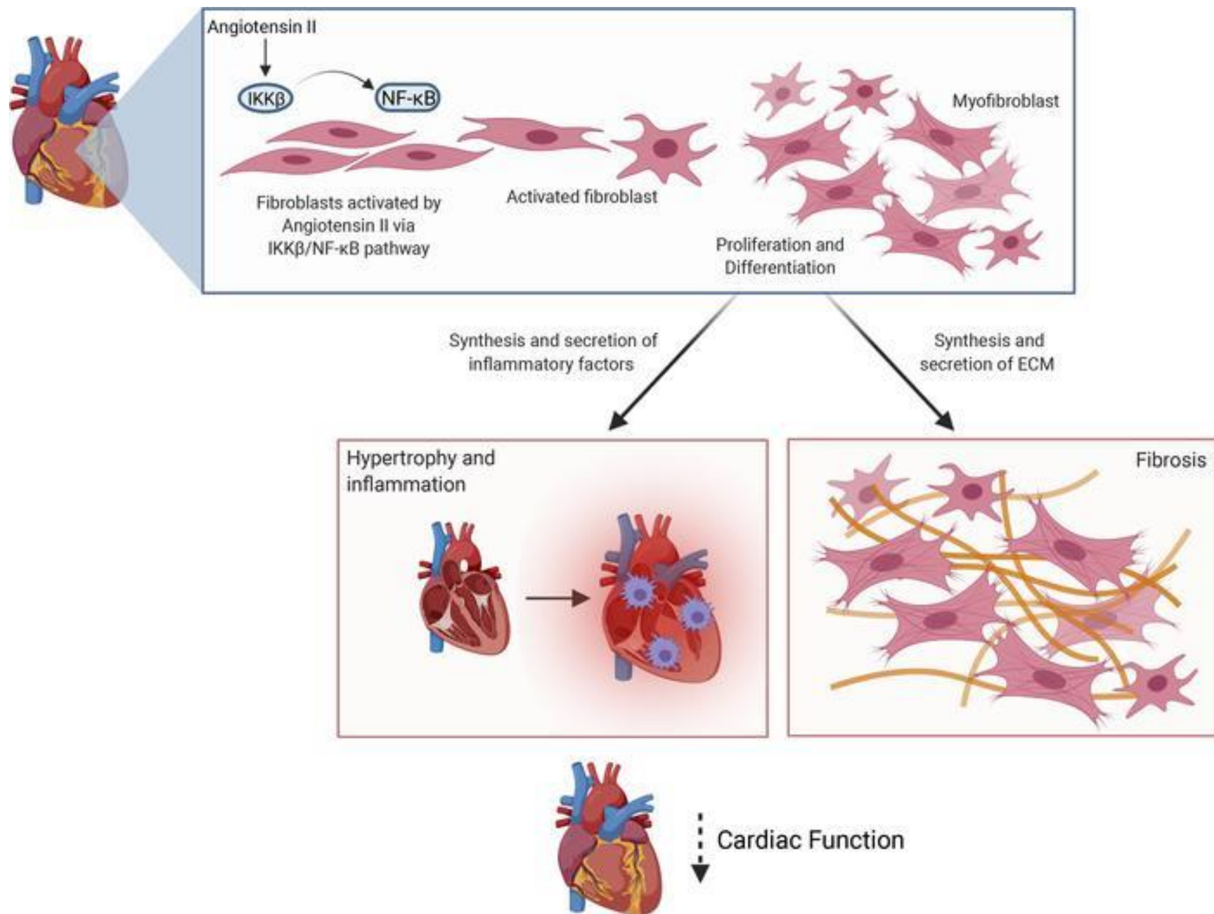



**Fig. 4.8:** Actions of Angiotensin in the heart

### 4.6. The Angiotensin II Receptors:

There are two types of angiotensin II receptors: type 1 and type 2, and both belong to protein G-related receptors [36].

These two types of angiotensin II receptors AGTR1 and ADTR2 have different distribution in the tissues as well as different mechanisms of signal transport [37].

#### 4.6.1. Angiotensin II receptors Type 1:

- **Distribution:** These receptors are present in almost all organs, for example the liver, adrenal glands, brain, lungs, and heart.
- **Structure:** The angiotensin II receptor is composed of 359 amino acids; its molecular weight is 40 kilo daltons. It belongs to the protein G-associated

receptor group. The extracellular region contains three regions that bind to a polysaccharide molecule and are known as a glycan. The occurrence of mutations in these regions does not affect the binding of angiotensin to the receptor. Four cysteine molecules form disulfide bonds in the extracellular region (Cys101, Cys18, Cys274 and Cys180) and are essential for angiotensin II binding [38]

Like other muscarinic and adrenergic receptors, the cytoplasmic tail of the receptor AGTR 1 contain several molecules of the amino acids serine and threonine, which are phosphorylated by a type of kinase that is specific for receptors that binds to protein G (G-protein receptor kinases GRKs) and any modification in these functional regions would be responsible for a change in receptor function in cardiovascular diseases CVD.

In the receptor, there are two chemical bridges that are necessary for anchoring the hormone angiotensin to the receptor [39,40,41,42,43]:

1. The first bridge between the arginine molecule Arg2 of the angiotensin side chain and the aspartic acid Asp281 present in the receptor.
2. The second bridge is between the carboxylate side α -COOH of angiotensin and the lysine molecule Lys199 in the receptor.

These bridges do not play any role in activating the receptor AGTR1, but there are two other interactions necessary to activate this receptor:

1. The first interaction between the phenylalanine molecule Phe8 in angiotensin II and the histidine molecule His256 in the receptor AGTR 1 [44].
2. The second interaction between the tyrosine molecule Tyr4 in angiotensin II and the asparagine molecule Asn111 in the receptor AGTR 1 [45-47].

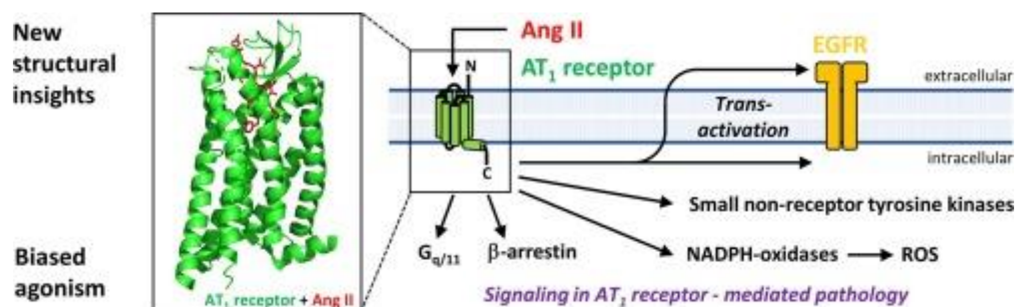



**Fig. 4.9:** The structure of angiotensin II receptors Type 1

- **Importance:** It is not possible to distinguish between the two types of the receptor (AGTR 1A and AGTR 1B) functionally, but studies within the organism showed that the type AGTR 1A is more important than the type AGTR 1B in regulating blood pressure.
- **Genetics:** The AGTR genes are located on chromosome3. Two types of this receptor (AGTR 1A and AGTR 1B) have been discovered in rats, and they are 95% similar in amino acids.
- **The relationship between polymorphisms in genes and hypertension:**

  Genetic changes in the RAS system are associated with cardiovascular diseases. Numerous evidences indicate that genes play an important role in individual differences in response to angiotensin II.

  Genetic mapping also showed the presence of single-nucleotide polymorphisms in the AGTR1 gene, which are associated with an increase in the development of risk factors for cardiovascular disease. The genetic form (A1166C) of AGTR1 gene is believed to be associated with high blood pressure [48], arterial sclerosis [49], and myocardial infarction [50].

  A study conducted on hypertensive patients with a high-salt diet showed an association between the A1166C genotype and increased sensitivity to angiotensin II [51].

  Studies also showed the role of polymorphism in the AGTR1 gene with hyperlipidemia. In patients with familial hypercholesterolemia, many genetic conditions with multiple genes cause a deficiency in the number of LDL receptors. It has been shown that the A1166C genotype in these patients increases the risk of cardiovascular diseases [52].

  However, the importance of the role of polymorphism of the AGTR1 gene in high blood pressure remains controversial [53, 54].
- **Oligomerization:** Angiotensin II receptors Type 1 not only regulate cellular functions, but also form oligomerization with many other receptors such as bradykinin receptors, adrenergic receptors, and dopamine receptors [55-57].

  It has recently been shown that the AGTR2 receptors can bind directly with the AGTR1 receptors and interfere with the functions of the AGTR1 receptors.

And the inhibition of AGTR1 receptors signal transduction by AGTR2 is done by activating the AGTR2 receptors [55].

The researchers (Hansen and colleagues) showed that the AGTR1-like dimer is necessary and unaffected by the presence of blockers or potentiators and is formed prior to the expression of this receptor on the cell surface.

The bradykinin receptor enhances AGTR1 signaling. In gestational hypertension, the presence of the AGTR1/B2 dimer enhances the astringent effects of angiotensin II.

There are also effects resulting from the direct interaction between beta-adrenergic receptors and AGTR1

Valsartan, an angiotensin receptor blocker, inhibits signal transduction at both AGTR1 and beta-adrenergic receptors in rats [58].

Also, beta-blockers can interfere with angiotensin II signaling in heart failure. Because of this, beta-blockers have become a major treatment in patients with chronic heart failure [58, 59].

- **Regulating of the action of angiotensin II receptors Type 1:** The receptor acts as a control point to regulate the effects of angiotensin II on the target tissue. Therefore, it is necessary to understand the mechanism that controls the number of receptors on the cell surface.

  An increase in angiotensin II levels leads to a decrease in the number of its receptors [42-44].

In addition, other factors can increase or decrease the expression of this receptor in vascular smooth muscle cells. The following table shows substances that increase or decrease the number of angiotensin II receptors AGTR 1 in the cardiovascular system:

| Substances that decrease the number of receptors AGTR1 | Substances that increase the number of receptors AGTR1 |
|---|---|
| ▪ Angiotensin II [67]<br>▪ Interferon gamma [68]<br>▪ Estrogen [65]<br>▪ Vitamin A [69]<br>▪ Statin [62]<br>▪ Human growth facto [70, 71]<br>▪ Platelet-derived growth factor [72]<br>▪ Thyroid hormone [73]<br>▪ Nitrogen oxide [74] | ▪ LDL [60]<br>▪ Insulin [64]<br>▪ Progesterone [65]<br>▪ Erythropoietin [66] |

**Table 4.1:** Regulation of angiotensin II receptors AGTR 1 in the cardiovascular system

Understanding the receptor regulation mechanism gives us an understanding of the association of high blood pressure with other diseases such as hyperlipidemia and increased insulin in the blood.

Studies have shown that LDL increases the number of angiotensin receptors by increasing the stability of post-translational mRNA, thus increasing angiotensin II-induced vasoconstriction in hyperlipidemia men [72, 73].

In addition, insulin can increase the production of angiotensin receptors through increasing the stability of post-translational mRNA and thus, the mechanism of the relationship between increased insulin in the blood and high blood pressure becomes clear [63].

In contrast, statins, which are inhibitors of the enzyme that reduce 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA), can reduce the number of angiotensin II receptors Type 1 and thus reduce angiotensin II signaling [62].

#### 4.6.2. Angiotensin II receptors Type 2:

- **Structure:** Like the AGTR1 receptor, AGTR2 receptor consists of seven transmembrane regions. The AGTR2 receptor has a molecular weight of 41 kDa. The AGTR2 receptor is only 34% similar to the AGTR1 receptor in structure [75]. It consists of 363 amino acids that are mainly expressed in fetal tissues such as the fetal aorta, gastrointestinal mesenchyme, connective tissue, skeletal system, brain and adrenal medulla.
- **Importance:** Although most of the vascular effects of angiotensin are mediated by the AGTR1 receptor, studies have shown that the AGTR2 receptor also plays other important roles in vascular smooth muscle cells.

  Expression of this receptor decreases after birth, which indicates its importance. in embryonic development [76].
- **Oligomerization:** Studies indicate that AGTR2 receptors can block AGTR1 receptors by inhibiting signal transduction by activating tyrosine molecules or serine-threonine molecules by phosphatases.

  It was also found that the formation of dimers of the two types of receptors leads to interruption in the AGTR1 signal [77, 78].

### 4.7. Methods of signal transduction associated with protein G:

Vasoconstriction is one of the important effects of angiotensin II. This effect is achieved through the G protein-coupled signal transduction methods shown in Fig. 4.10.

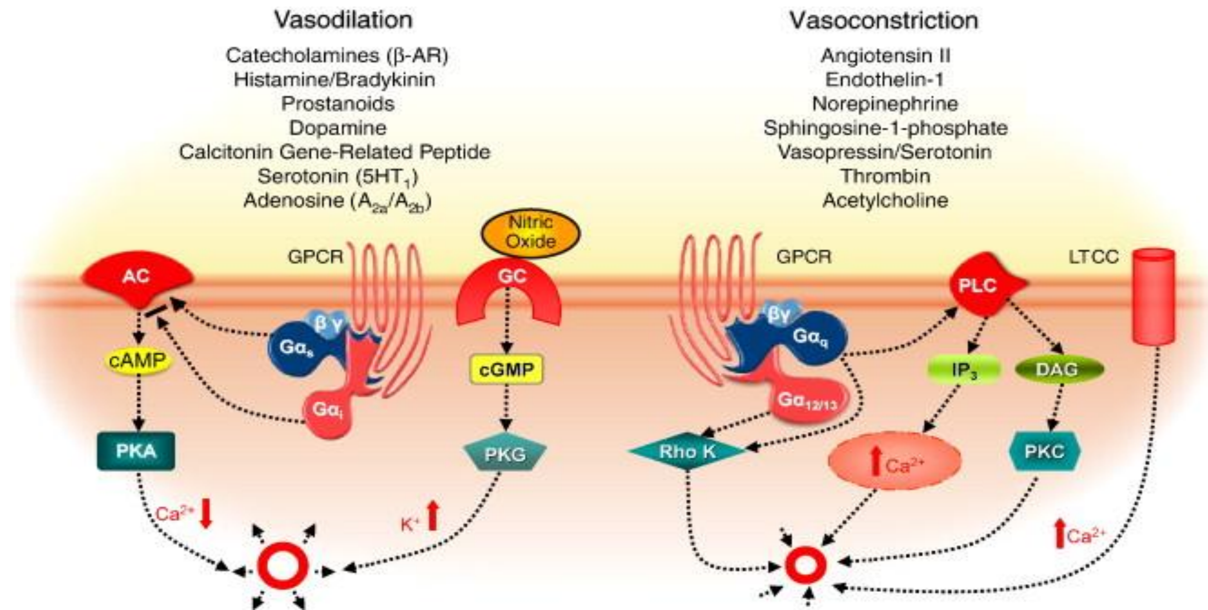



**Fig 4.10:** G protein-coupled signal transduction methods and Vasodilatation/Vasoconstriction

Studies have indicated that when the angiotensin receptor is activated, it binds to complexes G-beta-y, G-alpha-12/13, G-alpha-q11, which leads to the activation of the enzyme phospholipase C (PLC), phospholipase A2 (PLA2) and phospholipase D (PLD) [79].

PLC activation gives inositol triphosphate (IP3) and diacylglycerol (DAG) within seconds. IP3 binds to its receptors and this leads to the opening of channels responsible for the influx of intracytoplasmic calcium.

Calcium ions bind with calmodulin and lead to the activation of the enzyme myosin light chain kinase (MLCK), which in turn phosphorylates light myosin chains and increases the interaction of actin with myosin, thus increasing the contractility of vascular smooth muscle cells [80].

In order to counteract this effect, cells have the enzyme myosin light chain phosphatase (MLCP) which reverses the role of MLCK.

MLCP is inhibited by the enzyme Rho kinase, which leads to prolonged contraction of vascular smooth muscle [81, 82]. DAG can activate PLC which is involved in increasing the pH in the medium and is essential for cell contraction [83].

On the other hand, activation of the AGTR1 receptor leads to the activation of PLD, which hydrolyzes phosphatidylcholine (PC) and converts it to choline and phosphatidic acid (PA), and the latter is rapidly converted to DAG, which leads to prolonged activation of the PLC and prolonged contraction of the muscles.

Angiotensin has shown the ability to phosphorylate and activate PLA2, which leads to the production of arachidonic acid (AA) and its derivatives such as:

- Prostaglandins such as PGE2, PGI2, which are vasodilators and counteract the action of PGH2 and thromboxane A2, which induce vasoconstriction.
- Leukotrienes involved in vasoconstriction, hypertension, and inflammatory diseases.
- Hydroxyeicosatetraenoic acids (HETEs) Which is a precursor that raises blood pressure and causes smooth muscle contraction by the action of angiotensin II by facilitating the entry of calcium ions into the cell [84].

  This mechanism is reversed by epoxyeicosatrienoic acid (EETs) and dihydroxyeicosatetraenoic (DiHETEs), which are antihypertensives.

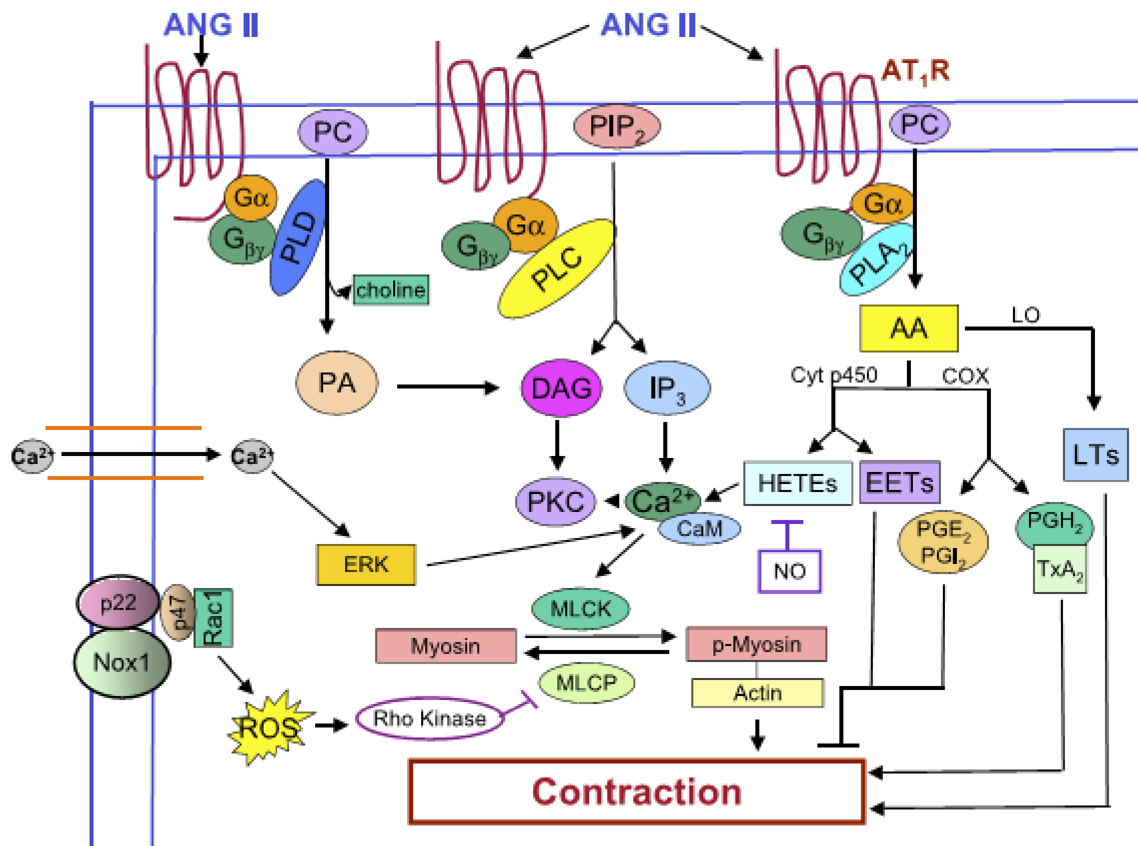



**Fig. 4.11:** The role of angiotensin II in the contraction of vascular smooth muscle cells

## 4.8. Genetic Polymorphism:

### 4.8.1. The definition of genetic polymorphism:

Genetic polymorphism, in genomics terms, refers to the presence of two or more different forms of a given DNA sequence that can occur between different individuals or populations. The most common type of polymorphism involves variation in a single nucleotide (also known as a single nucleotide polymorphism, or SNP). Other polymorphisms can be much larger, involving longer stretches of DNA. The incidence is greater than 1% in society.

(Poly) means many, and (morph) means form, Thus, (polymorphism) means genetic variations in the genetic total of a society.

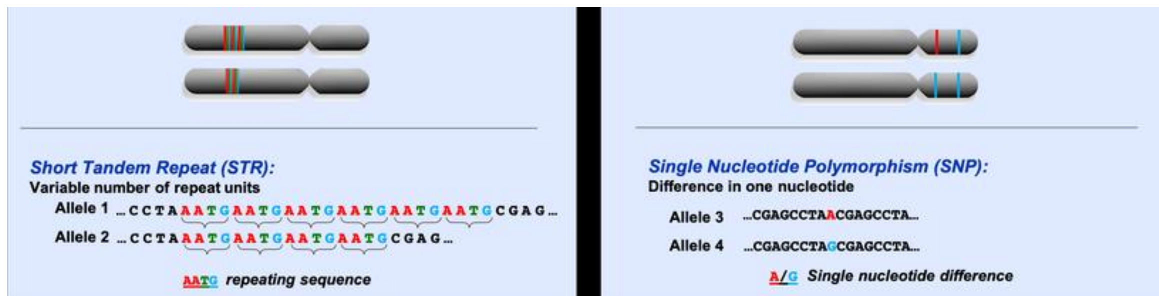



**Fig. 4.12:** Single nucleotide polymorphism and the short tandem repeat polymorphism

DNA sequences differ between individuals in small and large groups, and most of these variations in DNA sequences are stable and usually occur either in the form of single nucleotide polymorphism (SNPs) or in the form of short tandem repeats morphism by insertion and deleting of nucleotides.

The single nucleotide polymorphism (SNPs) is stable and consist of two alleles, one of which represents the natural pattern (wild-type) and the other allele represents the mutant pattern. Polymorphism occurs in this type when only one nucleotide (A, T, C or G) is changed in the genome sequence.

The incidence of single nucleotide polymorphism SNPs in each gene is 4 - 8 SNPs, and they occur either in exons (which are the coding regions) or in introns (which are the non-coding regions).

When comparing the genomes between people, we find that 24,000-40,000 sites in which a base substitution occurs, which leads to a change in the resulting amino acid, but this substitution does not affect the function of the resulting protein [87].

#### 4.8.2. The definition of mutation:

The term mutation is applied to the differences that occur in the DNA chain that lead to the emergence of diseases and the percentage of their presence in society is less than 1% [85].

#### 4.8.3. Mutation mechanism:

Mutations may occur by chance or as a result of the induction of external factors such as viruses and radiation [88].

- Transcription errors: Mutations can occur when there is an error and are called transcription errors when they occur when DNA is replicated. A lot of these errors that cause disturbances in DNA occur as a result of external factors such as sunlight, cigarette smoking, and radiation, and these mutations can be transmitted to children.
- Germ line errors: Mutations can occur during the division of cells responsible for the production of sperm and eggs, so mutant gametes are formed, and the disease appears in children only, without any problem in parents.

| | **Mutation** | **Polymorphism** |
|---|---|---|
| **Allele frequency** | <1% | >1% |
| **association with diseases** | yes | no |
| **Amino acid change** | yes | perhaps |
| **evolution occurs** | yes | perhaps |
| **Presence in intact samples** | no | yes |
| **Effects in vitro** | yes | perhaps |
| **Effects in vivo** | yes | perhaps |

**Table 4.2:** Comparison of polymorphism and mutation

### 4.8.4. Types of DNA alterations resulting from Single Base Substitutions:

DNA changes resulting from a single base substitution are divided into two types:

- Polymorphisms
- point mutations

Polymorphisms in human DNA are generally classified into two groups:

- Single-nucleotide polymorphisms
- polynucleotide polymorphisms

Point mutations (Fig. 4.13) can be classified into

- silent,
- nonsense,
- and missense mutations

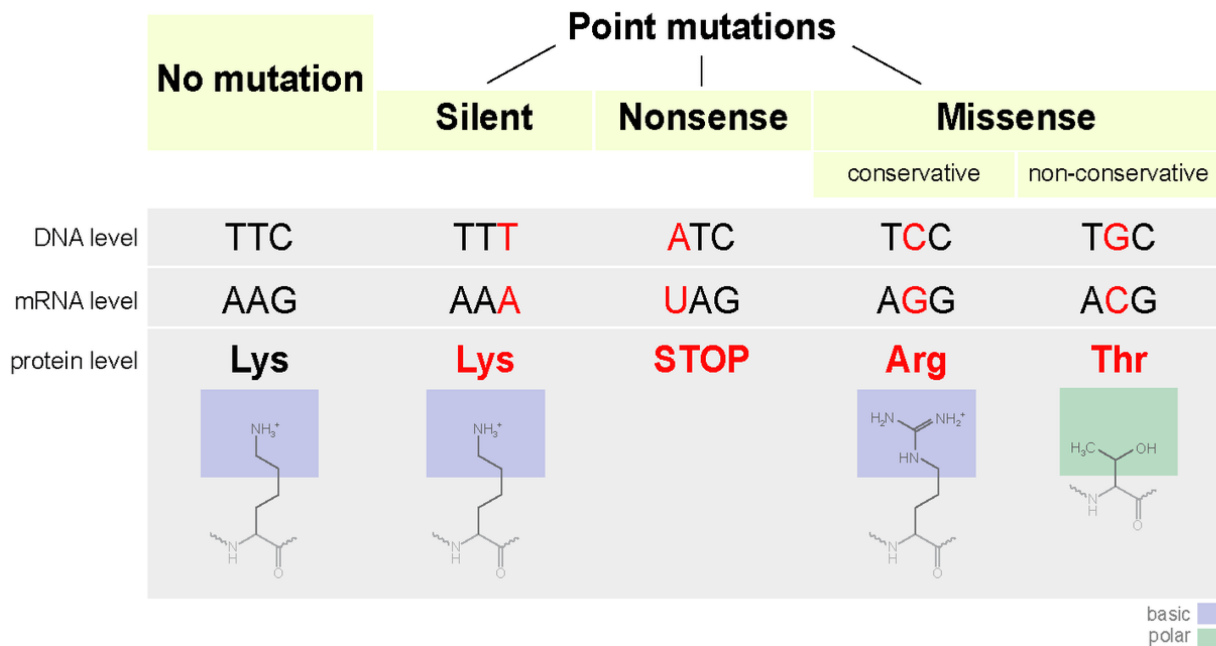



**Fig. 4.13:** Types of point mutations in the DNA sequence

### 4.8.5. Single-nucleotide polymorphisms:

Single nucleotide polymorphisms are considered the most prevalent genetic variations among humans. The results from the Human Genome Project showed that the DNA of any two people is identical by more than 99.99%, so that 0.1% represents all genetic differences between humans.

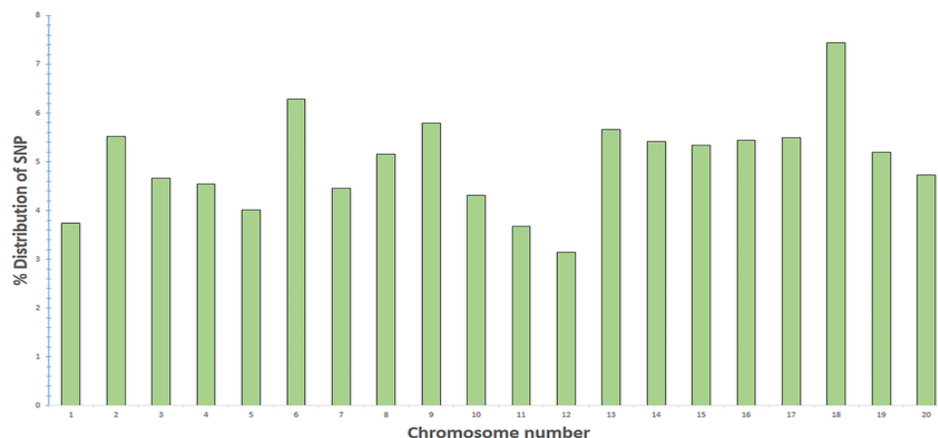



**Fig. 4.14:** Percentage of Distribution of SNP in chromosomes

In other words, one person may have an A-T pair in a specific region of the DNA, and another person may have a G-C pair in the same location on the DNA, as shown in Fig. 4.15.

This type of polymorphisms does not usually show any effect on the health or appearance of the individual. The number of SNPs in humans is not known, but it is likely that the number is between 10-30 million, or one SNP in every 100-300 nucleotide pairs.

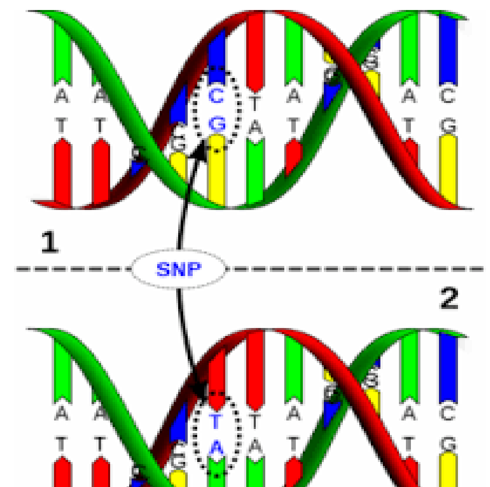


**Fig. 4.15:** Single nucleotide polymorphism

There are 4 million common SNPs in which the frequency of two alleles per SNP is more than 20%.

Most of the SNPs do not affect the health or development of the individual, but it has been proven that some of these genetic variations are very important when studying the health of individuals.

Polymorphisms usually affect the structure, function, or organization of a gene, and in some cases can determine individuals who are predisposed to developing a disease [89]. Some SNP alleles represent actual functional variations that participate in the risk of disease [90]. Individuals who have a specific allele are more likely to develop the disease than individuals who do not carry this allele

To find the genetic regions that participate in the disease, the frequency of a number of alleles is compared between infected and non-infected individuals. If the frequency of the SNP alleles in a specific region in those with the disease is more than the frequency of the alleles in those without the disease, then we can say that this SNP and its alleles are associated with the disease.

### 4.8.6. Silent Mutations:

Silent mutations are mutations that do not cause a change in the resulting final protein, and this mutation can only be detected by sequencing the gene, and most of the amino acids involved in the structure of proteins are symbolized by different codons, for example when changing the third base in the CAG codon to adenine, that will give CAA, but the structure of the protein remains the same Because the mutated codon encodes the same amino acid, this type of mutation is considered silent and has no effect.

### 4.8.7. Missense Mutations:

Missense Mutation is a point mutation in which a single nucleotide is changed so that the resulting codon codes for a different amino acid.

Fig. 4.16 illustrates this type of mutation in sickle cell disease, as the seventeenth nucleotide change in the hemoglobin beta chain gene from A to T causes a change in the codon from GAG to GTG, and as a result a change occurs in the sixth amino acid in the chain from glutamic acid to valine and this seemingly simple change in the beta globin gene leads to a change in the structure of hemoglobin and thus affects the physiology and health of the individual.

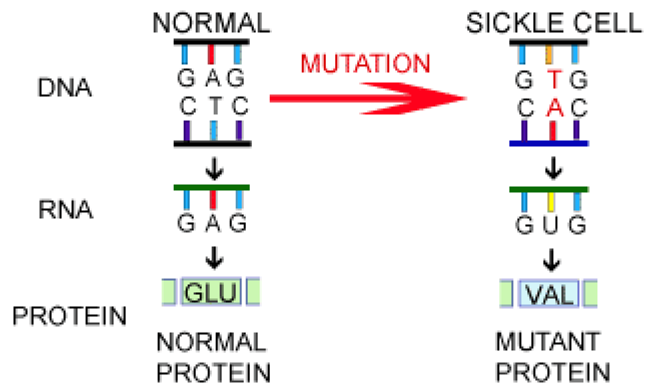



**Fig. 4.16:** Missense mutation in sickle cell disease

### 4.8.8. Nonsense Mutations:

In this type of mutation, the new base causes a change in the codon that encodes the amino acid and turns it into one of the stop codons (TAA, TAG, TGA).

This type of mutation occurs in 15-30% of all genetic diseases, such as haemophilia, retinitis pigmentosa, muscular dystrophy, and cystic fibrosis, as shown in Fig. 4.17.

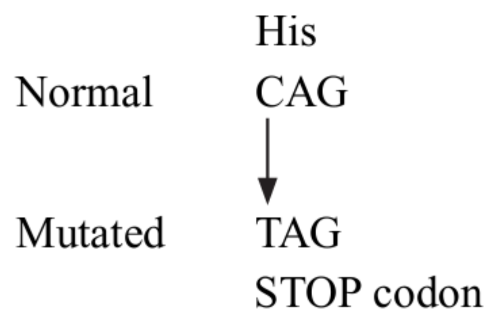



**Fig. 4.17:** An example of Nonsense mutations occurring in patients with cystic fibrosis

### 4.8.9. Frame-shift Mutations

These mutations occur as a result of adding or deleting a number of nucleotides from the DNA sequence so that this number is not divisible by 3 as shown in Fig. 4.18.

This mutation makes the DNA sequence, starting from the region of deletion or addition, responsible for encoding amino acids that are completely different from the original ones.

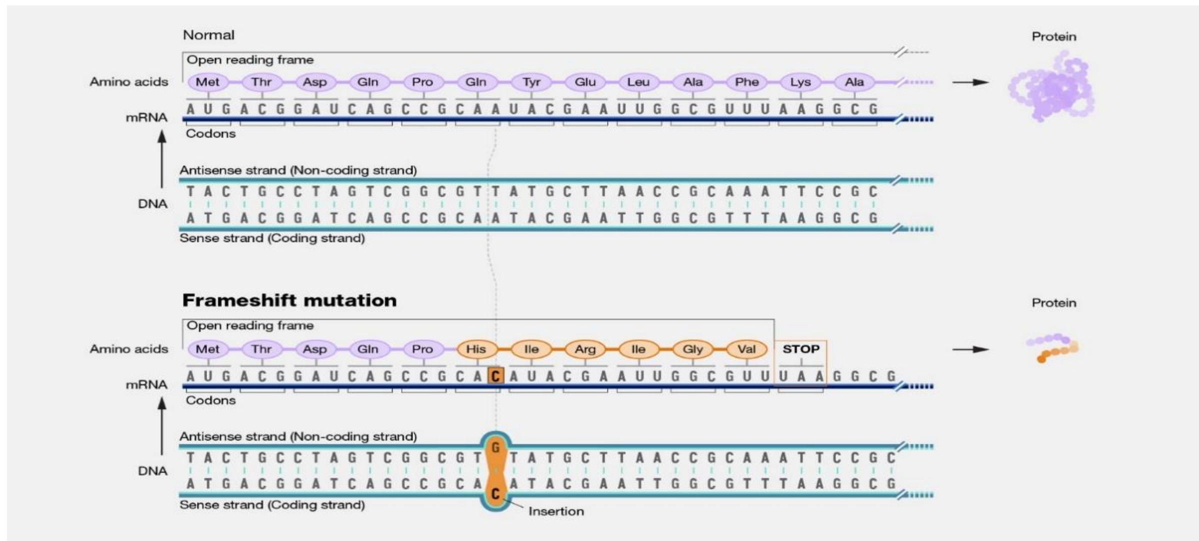



**Fig. 4.18:** Frame-shift mutation

### 4.9. Candidate Genes in Hypertension:

Essential hypertension is a multifactorial, multigenetic disorder that results from genetic and environmental factors [91].

This disease does not have a specific cause, but it results from a defect in the mechanisms of regulating blood pressure, such as a defect in vascular stimulating factors or in local or neurohormonal factors present in the blood circulation.

Genetic variations in these factors play a role in the inheritance of essential hypertension, which is a major risk factor for heart disease, stroke, peripheral vascular disease, and kidney disease [92].

The rates of essential high blood pressure increase with age and with the presence of other cardiovascular risk factors such as dyslipidemia, glucose intolerance, increased insulin in the blood, abdominal obesity, and increased urea in the blood. Studies have shown, for example, that uric acid in experimental animals increases vasoconstriction through Activation of the renin-angiotensin system and by reducing the circulating nitrogen oxide in the blood [144].

Other environmental factors can enhance the incidence of this disease, such as a diet based on high sodium intake, alcohol consumption, or increased nervous tension.

The family story also has a fundamental role, as it was noted that the emergence of essential hypertension in individuals is greater when there is a strong family history

Studies have identified a number of genes that predispose to high blood pressure, these studies have also identified the interaction of these genes with each other on the one hand and with the environment on the other hand as shown on Fig. 4.19.

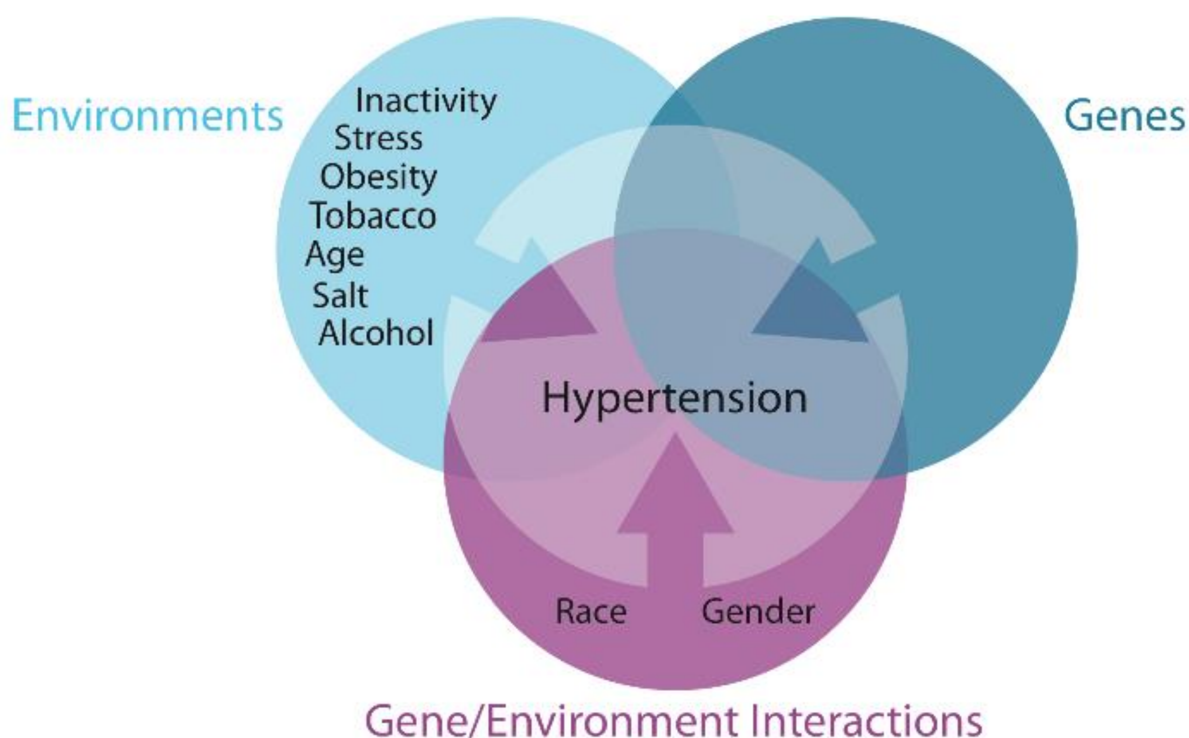



**Fig. 4.19:** Effect of genetic factors and environment on high blood pressure

Among the genes that predispose to high blood pressure are the encoding renin-angiotensin-aldosterone (RAAS), the catecholamine and adrenergic system, G-protein signal transduction genes, sodium channel systems, hormone receptor genes such as glucagon receptors and insulin-like growth factor [93,94]. Apolipoproteins and cytokines

The renin-angiotensin-aldosterone (RAAS) system is the most invasive device for the risk of developing high blood pressure

### 4.9.1. Genes that encoding renin-angiotensin-aldosterone system (RAAS):

The (RAAS), is responsible for controlling blood pressure and sodium balance in the body. It also plays a major role in regulating kidney function.

Genetic variations of the components of this system showed an association with susceptibility to high blood pressure, such as the genes of renin, angiotensin, angiotensin II receptor type 1, AGTR1, angiotensin-converting enzyme ACE [95] and aldosterone (CYP11B2).

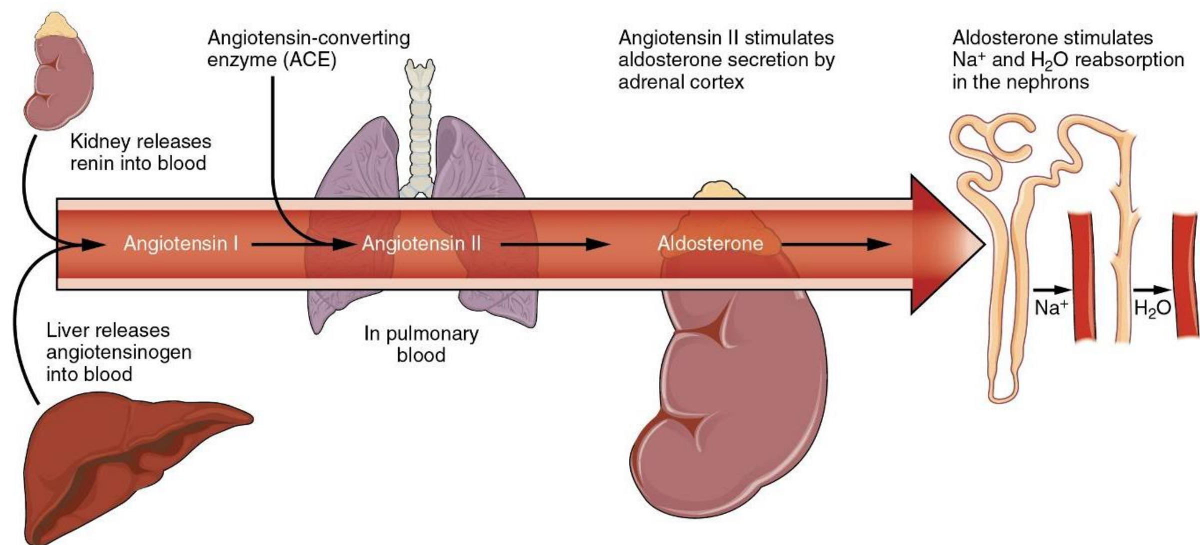
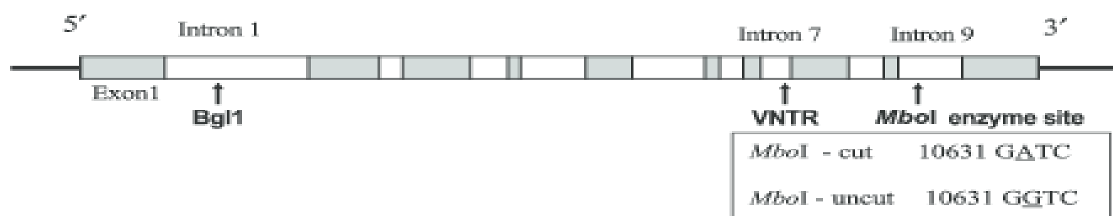



**Fig. 4.20:** Effect of renin-angiotensin-aldosterone system on Hypertension

### 4.9.2. Renin gene:

Renin plays the role of a coenzyme in the production of angiotensin. The renin gene is symbolized by the symbol (REN), and it is located at site 1q32, as shown in Fig. 4.21.



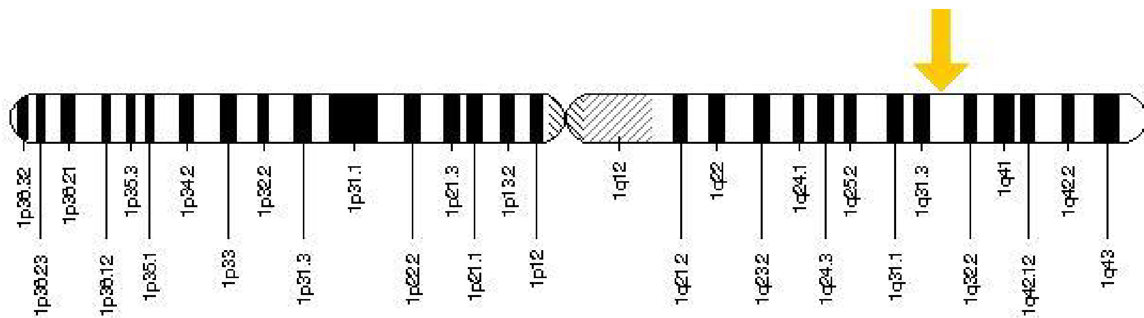


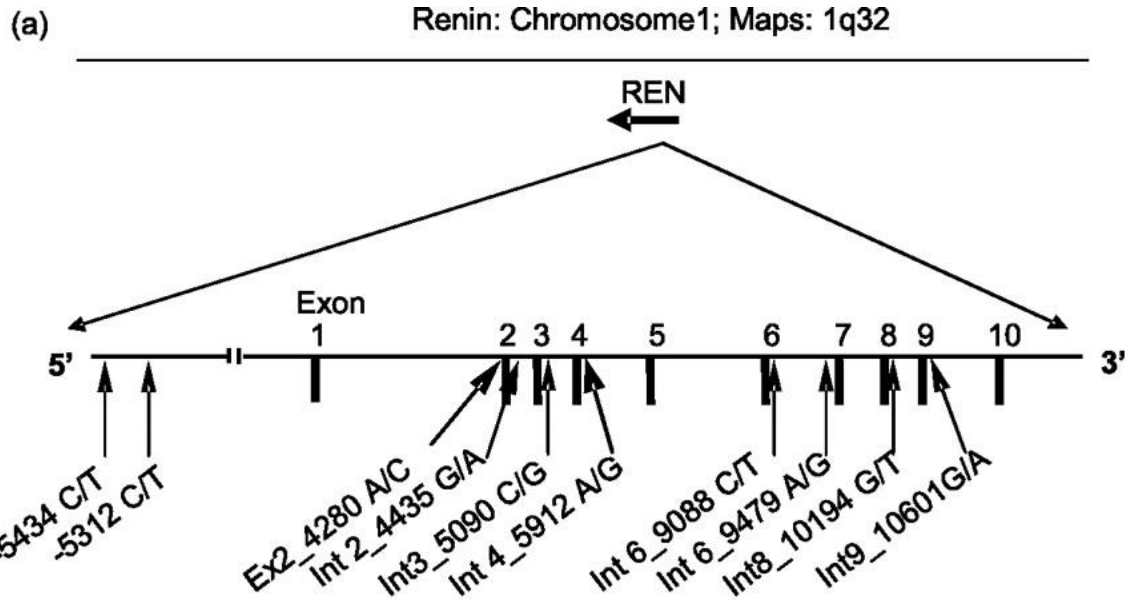
**Fig. 4.21:** A schematic diagram of the first chromosome showing the location of the renin gene

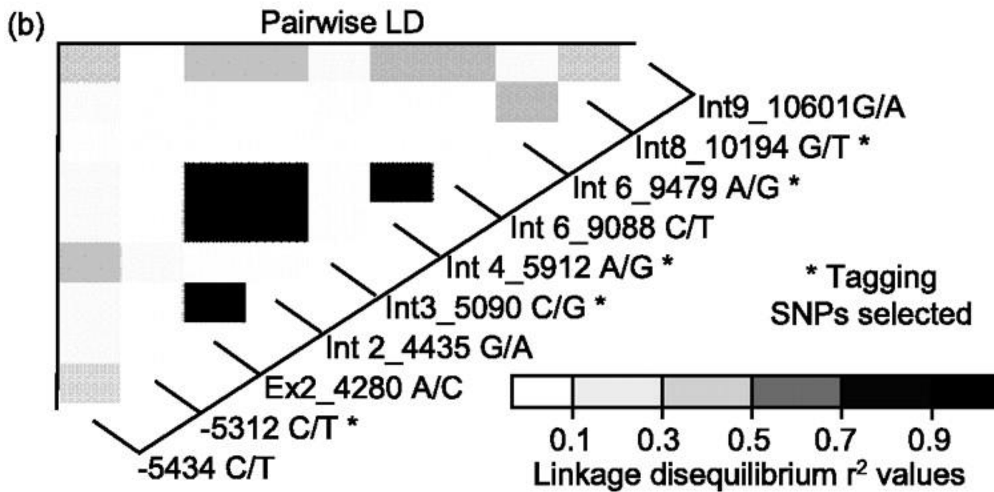
Several studies have shown that the renin gene plays a role in increasing the risk of developing high blood pressure, and it was found that the mutation of the type of addition / deletion in the renin gene is associated with primary hypertension [97]. Many single-nucleotide polymorphisms in the renin gene are associated with the risk of developing high blood pressure.

Fig. 4.22 shows some polymorphisms of the resonance gene. In single-SNP analyses, the site most strongly associated with hypertension was found to be the locus rs6693954 located in intron 9 [86]. It was also found that the 5312T allele in the RNA gene is associated with high diastolic blood pressure [99].

In addition, it was found that the Bg1I type in intron 1 and the single nucleotide polymorphism G1051A in exon 9 are associated with high blood pressure in white Americans and Arabs in the United Arab Emirates [96, 86], and an association between C4021T and C3212T with high blood pressure was also determined In African Americans [100].

Also, a single-nucleotide polymorphism in intron 4 (A54620025C) was associated with hypertension in Spanish women [101]. All these studies give evidence that the resonance gene is one of the genes that predisposes to high blood pressure




(c)

| Haplotype | Frequencies | |
|---|---|---|
| | Employee Population | Hypertensive Population |
| 1. CGAAG | 0.41 | 0.35 |
| 2. CCAAG | 0.21 | 0.23 |
| 3. TCAAG | 0.18 | 0.19 |
| 4. CCGAG | 0.10 | 0.12 |
| 5. CCAAT | 0.05 | 0.05 |
| 6. CCAGT | 0.05 | 0.06 |
| Other rare | <0.01 | <0.01 |

**Fig. 4.22:** Polymorphisms in the renin gene

### 4.9.3. Angiotensinogen gene:

Angiotensinogen gene is located at position 1q42-43 and contains 5 exons as shown in Fig. 4.23 and has a length of 13,000 nucleotide pairs [102].

Angiotensinogen is cleaved by renin to give a decapeptide angiotensin called angiotensin I, which is a precursor of angiotensin II. There are many evidences confirming the genetic effect of the Angiotensinogen gene site on blood pressure through numerous studies on diverse ethnic groups.

A number of SNPs have been identified, shown in Fig. 4.24, such as: M235T, where the amino acid threonine replaces the amino acid methionine. Another example is T174M, where the amino acid methionine replaces the amino acid threonine. One more example is G217A. Several studies have shown that alleles 235T and T174 increase the risk of developing high blood pressure [103-106].

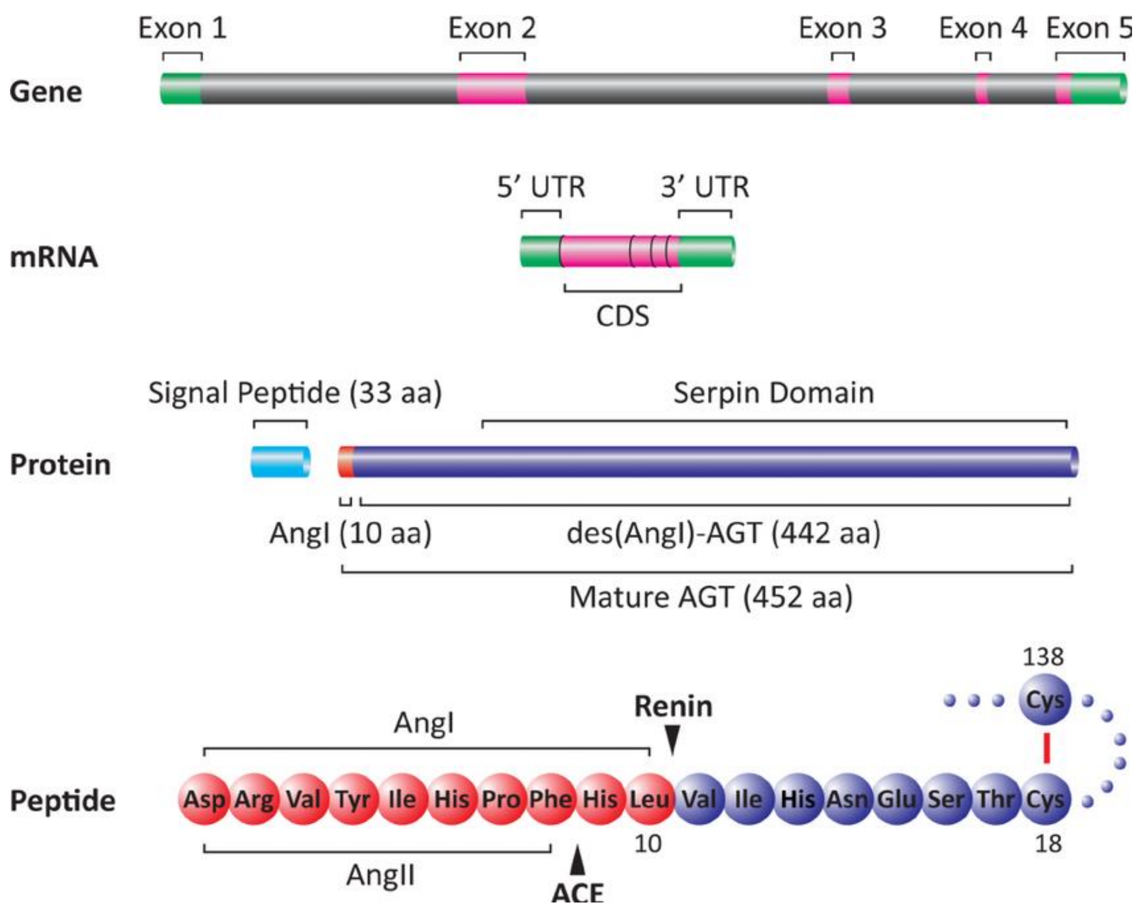



**Fig. 4.23:** Angiotensinogen gene

It has also been shown that there is a positive association with high blood pressure due to several mutations in the inducer site, including the presence of adenine A in the place of guanine G before 6 nitrogenous bases of the translation start site (G-6A) [107].

This mutation has been identified in the original Taiwanese. The prevalence of -6A and M235T variants of the angiotensinogen gene is high and significantly associated with hypertension [107].

The M268T variant is one of the studied variants among the polymorphisms that occur on the Angiotensinogen gene, and it has been studied in many groups, and it is a gene that predisposes to high blood pressure [108].

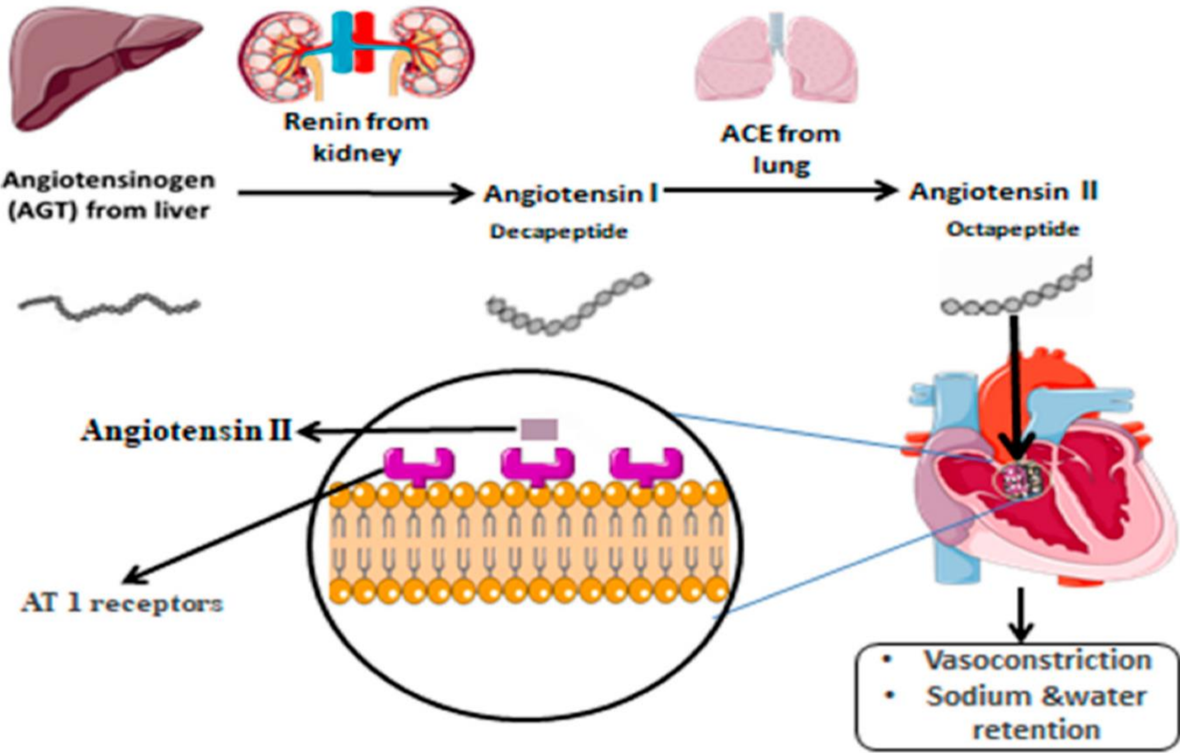



**Fig. 4.24:** Genetic Polymorphism of Angiotensinogen

### 4.9.4. Angiotensin-converting-enzyme (ACE) gene:

This gene is located on chromosome 17 at position 17q23, as shown in Fig. 4.25. In addition to this enzyme's role in the production of angiotensin II, it destroys bradykinin, which is a vasodilator and a sodium-reducing substrate.

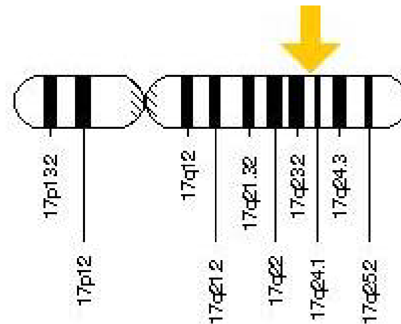



**Fig. 4.25:** Illustration of chromosome 17 showing where the ACE gene is located

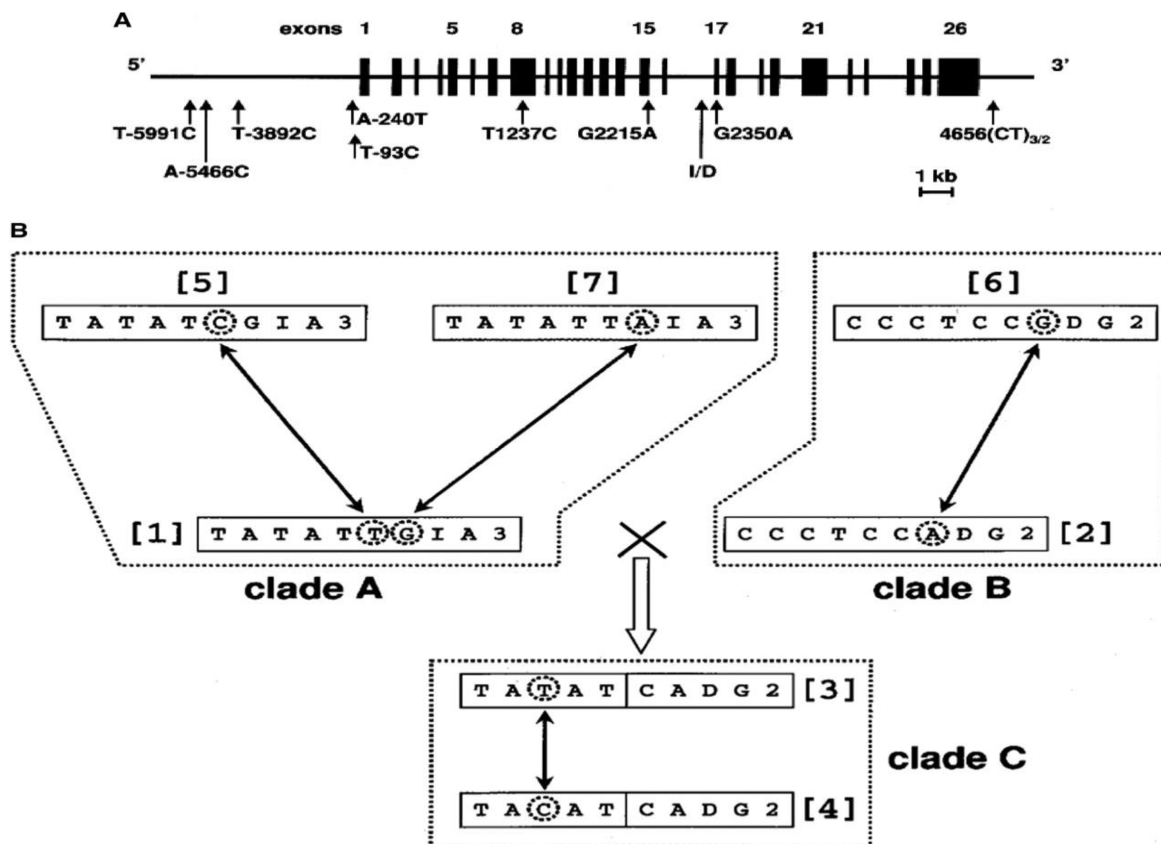



**Fig. 4.26:** Polymorphism of ACE gene

There are many polymorphisms in the ACE gene, but the most common polymorphism is the addition or deletion (I/D) of a segment of 287 nucleotide pairs in intron 16 of this gene, as shown in Fig. 4.27.

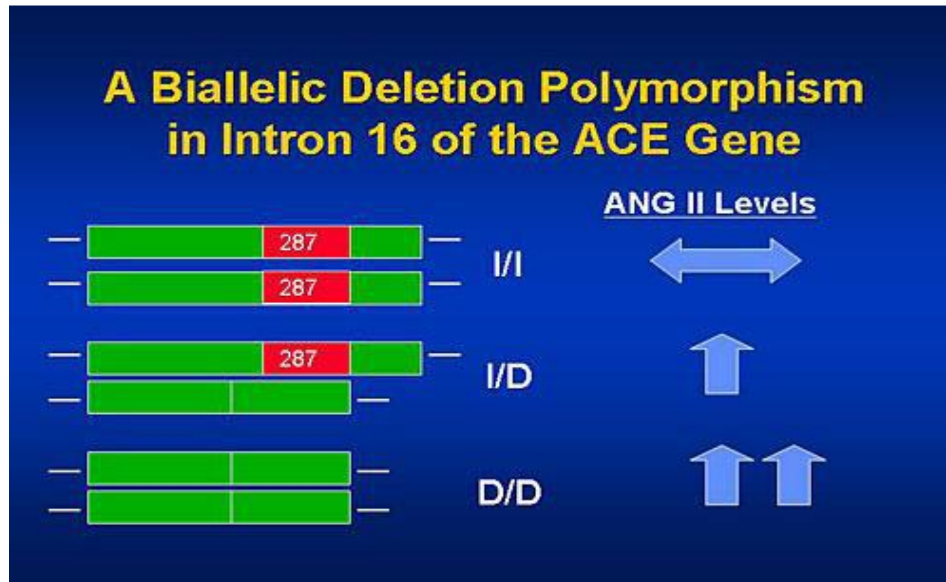



**Fig. 4.27:** Polymorphism (I/D) of a segment of 287 nucleotide pairs in intron 16 of ACE gene

Several studies have shown that this polymorphism affects both the concentration of ACE in the serum and blood pressure [109], where the ACE concentration of carriers of the genotype D/D is twice as high as that of the carriers of the genotype I/I, while the carriers of the genotype I/D are ACE concentrations in their serum are moderate

In many studies conducted on different groups, it has been proven that the presence of the D allele gives a statistically significant relationship with high blood pressure. Therefore, the ACE gene is one of the candidate genes to give a predisposition to high blood pressure.

### 4.9.5. Angiotensin II type 1 receptor gene:

This receptor is a type of G protein-coupled receptor. It is the responsible gene, AGTR1, is located on the third chromosome at 3q21-25, as shown in Fig. 4.28. The length of this gene is more than 55,000 nucleotide pairs, and it consists of 5 exons in addition to 4 introns. A single nucleotide polymorphism (A1166C) has been described,

where there is either the nitrogenous base of the adenine or Cytosine at position 1166 in the 3rd extra translational region of the gene [110].

The A1166C polymorphism is the most studied and evaluated in this gene, and it is the subject of many research studies. It was found that this A1166C polymorphism in the AGTR1 gene is associated with severe forms of hypertension [110 -114], as well as in Caucasian populations with high blood pressure with a family history. It was strong, as it was found that the C allele was present in abundance in these people [115], and it was also present in a high degree in women with gestational hypertension

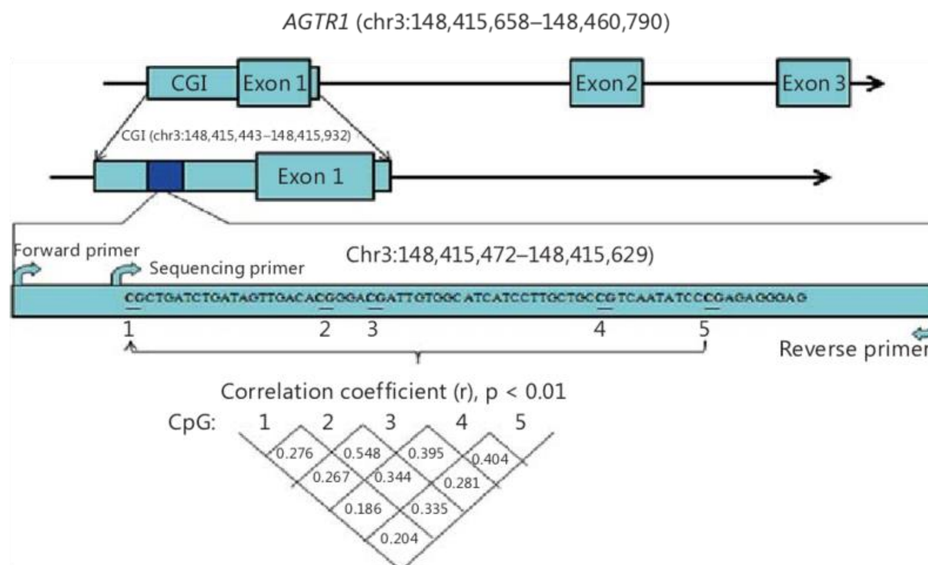



**Fig. 4.28:** Illustration of AGTR1 gene

In addition, a single nucleotide polymorphism (SNP) was detected at site C573T in hypertension and diabetes [112,113]. There are also 9 other single nucleotide polymorphisms (SNPs) [99] that enhance the expression of the AGTR1 gene, as shown in Fig. 4.29. As a result, the A1166C polymorphism in the AGTR1 gene predisposes to hypertension.

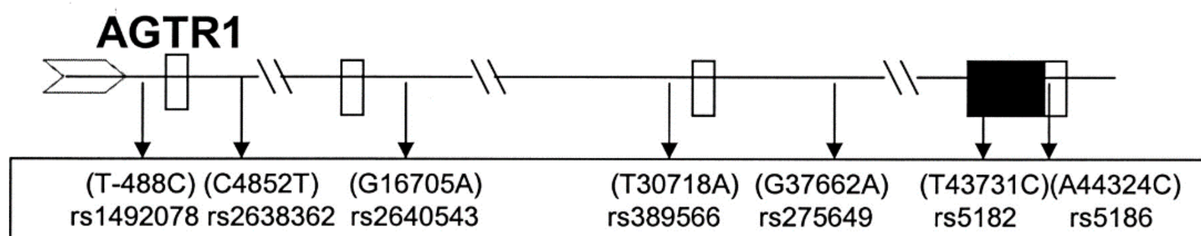



**Fig. 4.29:** Polymorphism of AGTR1 gene

### 4.9.6. Angiotensin II type 2 receptor gene:

The AGTR2 gene is located on the X chromosome and contains 3 exons and 2 introns, where the Open reading frame (ORF) of this gene is located on the third exon [117] (Fig. 4.30). It is believed that the activation of this receptor counteracts the effect of the receptor AGTR1 and contributes to the vasodilator and natriuretic effect

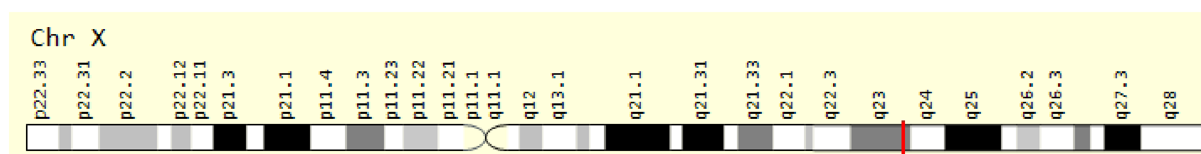



**Fig. 4.30:** Illustration of AGTR2 gene

The most common polymorphisms occurring in intron 1 have been described [118].

Other polymorphisms have also been described, such as (1675+) [111], where it is located before 29 nucleotide pairs of exons 2 close to the region necessary for translation activity [119]. It was also found that the A1675G polymorphism in the AGTR2 gene is among the polymorphisms that interfere with the occurrence of High blood pressure in males [120-122]. The G4599A polymorphism in exon 3 of the AGTR2 gene is associated with high blood pressure in women. It has been suggested that there is a relationship between the T1334C polymorphism and the occurrence of high blood pressure in Chinese [123].

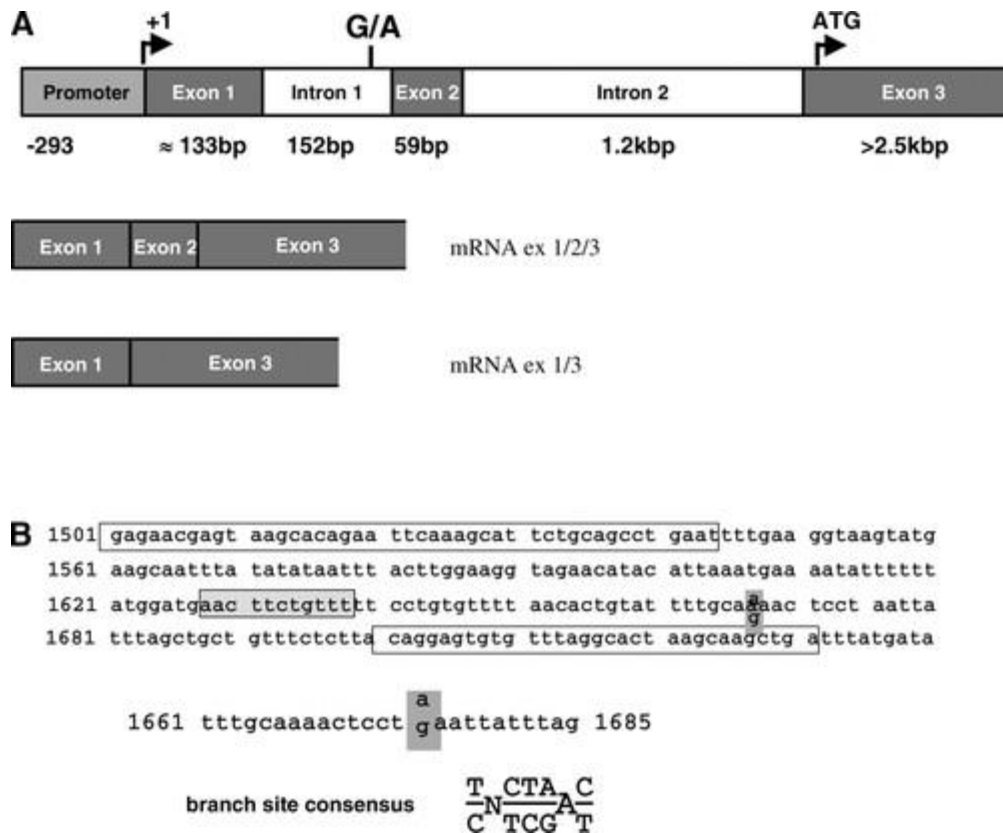



**Fig. 4.31:** Structure of the human ANG II type 2 receptor (AT2) gene and localization of the polymorphism

In Fig. 4.31, A: schematic drawing of the human AT2 gene with its two introns and three exons and its mRNA splice variants. B: localization of the 1675 G/A polymorphism (PM) (highlighted) within the small intron 1 of the AT2 gene. Open boxes mark the flanking exons 1 and 2. The shaded box marks a sequence motif with enhancer activity, which was identified in a previous study. Bottom: comparison between the AT2 sequence surrounding the PM and the splice branch site consensus. Note that the PM localizes to the nucleotide position 5′ adjacent to the highly conserved A residue at which the lariat-splicing intermediate is formed.

### 4.9.7. Aldosterone synthase enzyme gene:

The enzyme that synthesizes aldosterone (CYP11B2) is considered the key to the last step in the process of biosynthesis of aldosterone and is encoded by the gene (CYP11B2) which is located on chromosome VIII at position 8q22 [124, 125] as shown in Fig. 4.32.

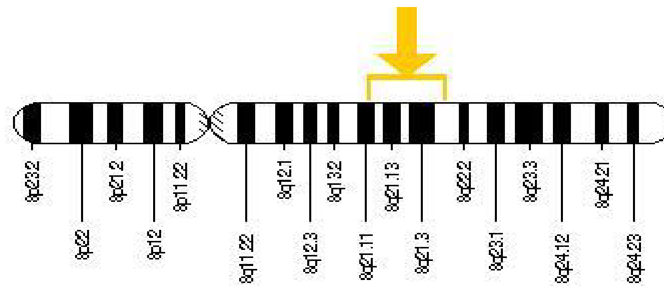


**Fig. 4.32:** Illustration of gene 8 shows where the aldosterone synthase enzyme gene is located

A lot of polymorphisms have been identified in this gene, among which the polymorphism in the promoter region C344T (rs id 1799998) was the most studied and evaluated. The presence of this polymorphism either increases the ratio of aldosterone to renin in patients with high blood pressure or causes a deficiency of aldosterone production leading to Sodium loss and potassium loss [126,127].

In addition, several studies have found that the C344T polymorphism shown in Fig. 4.33 is responsible for the risk of developing high blood pressure [128, 129] as well as cardiovascular diseases. Therefore, the CYP11B2 gene is associated with the development of high blood pressure.

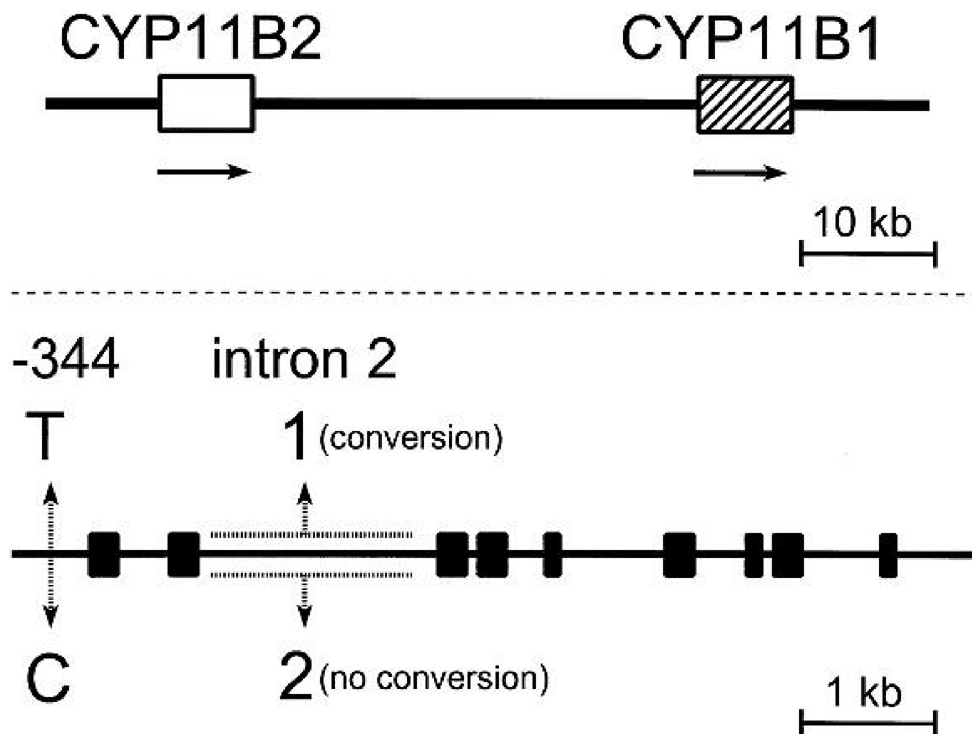



**Fig. 4.33:** Polymorphism of the aldosterone synthase enzyme

# 5. Methodology

## 5.1. Materials:

These materials are specially prepared for use in the field of molecular biology, where the producing company sterilized them and removed the nuclease enzymes from them.

### 5.1.1. Materials for the PCR reaction

PCR-water: It is a nuclease free water that was prepared by sterilizing the water by double distillation with a special device, so that it is necessary to dissolve the primers in order to complete the reaction in the PCR (Fig. 5.1).

**Fig. 5.1:** PCR-Water

Primers used in PCR: These primers are designed according to the studied gene, and the ideal concentration of primers in the amplification solution is (0.1-1 µmolar) [145], as the use of low concentrations of primers results in low amplification yield, while high concentrations cause the formation of a primer dimer after the primer is extended, it is kept at -20° until use (Fig. 5.2).

**Fig. 5.2:** Primers used in PCR

One example of primers is (Custom primers, VBC-Biotech, Austria).

Table 5.1 shows the characteristics of primers Custom

| PRIMER | NUCLEOTIDE SEQUENCE | LENGTH (BP) | DEGREE OF PLASTICITY (T) | GC % | PURIFICATION METHOD |
|---|---|---|---|---|---|
| **FORWARD PRIMER** | 5`-GCACCATGTTTTGAGGTT-3` | 18 | 63° | 44 | HPLC |
| **REVERSE PRIMER** | 5`-CGACTACTGCTTAGCATA-3` | 18 | 56° | 44 | HPLC |

**Table 5.1**: Characteristics of primers Custom

Deoxynucleotides: There is a lot of deoxynucleotides and one example is (dNTP Mix; Fermentas, Lithuania) that is used at a concentration of about (50-200 μmolar) per nucleotide in equal proportions for example Fig. 5.3.

**Fig. 5.3:** Deoxynucleotides (dNTP Mix; Fermentas)

The four types of free nucleotides that serve as substrates for DNA Polymerase are (dATP, dCTP, dTTP and dGTP). It should be noted that the lowest possible amount of nucleotide concentrations should be used to reduce the errors of the polymerase enzyme. But if the concentration is less than the minimum, this will affect the efficiency of the PCR process [146]. These nucleotides are stored at -20°C until use.

Taq DNA polymerase: It is the enzyme necessary for the process of DNA replication, as it adds the nucleotides to the amplified piece, and the amount of polymerase added depends on the number of cycles in the PCR reaction and on the denaturation time in one cycle, as the half-life of the polymerase enzyme at a temperature of 94° is about 40 minutes, and as a result, part of the enzyme is destroyed with each cycle in the phase of separation.

The use of high concentrations of the enzyme polymerase causes an increase in the amplification of by-products and errors in the enzyme polymerase (Fig. 5.4)

**Fig. 5.4:** Invitrogen™ Taq DNA

Magnesium chloride: Magnesium ions, Mg++, play the role of co-enzyme for Taq DNA polymerase, as this enzyme needs the lowest concentration of free magnesium chloride, about 1.2-1.3 mmolar, for maximum effectiveness [145].

The appropriate concentration of magnesium ions is usually determined empirically for each amplification protocol because low concentrations of magnesium ions lead to a decrease in the reaction efficiency, while an increase in the concentrations of magnesium ions leads to:

- Reducing the degree of plasticity of primers and, as a consequence, increasing their non-specific binding to target DANN
- Increased probability of primer dimer formation
- Increased errors of the polymerase enzyme in including the correct nucleotides

**Fig. 5.5:** Magnesium Chloride ($MgCl_2$) Solution, New England Biolabs | VWR

One example of magnesium chloride is (Magnesium chloride solution 25 mM; PCR grade; Thermo, USA).

PCR Buffer: It is a buffer that contains Tris-HCL (Tris is 2-amino 2- hydroxy methyl-propane- 3,1 diol) at a concentration of 100mmolar and potassium chloride at a concentration of 500mmolar and at a pH=8 at a temperature of 25° and it is necessary to maintain a certain pH for the amplification solution

One example of the buffers is (PCR Buffer 10X; PCR grade, Thermo, USA).

**Fig. 5.6:** GeneAmp™ 10X PCR Buffer I, (2 x 75 mL)

DNA Template: The kit used to extract DNA and the volume of the extract is about 100μl and it is kept at -20°C.

Small concentrations of DNA should be used during the PCR reaction, equivalent to 0.001 ng for each reaction, because using high concentrations can lead to the appearance of secondary products.

**Fig. 5.7:** Illustra TempliPhi™ DNA sequencing template

**5.1.2. Materials for Agarose Gel Electrophoresis:**

Restriction enzyme: one example is the following restriction enzyme (DdeI; Thermo, USA) that is used at a concentration of 10 U/μl, and this enzyme specifically cuts double DNA at the sequence C↓TNAG in both directions, and is also called HpyF3I.

**Fig. 5.8:** Restriktionsenzym Thermo Scientific HpyF3I (DdeI)

DAN Loading Dye: It contains hydrochloric acid with a concentration of 10 mmolar, with a pH = 7.6, and two colors, bromophenol blue 0.03%, which behaves like DNA with a length of about 4000 nucleotide pairs, in addition to 60% glycerol.

This dye is used to load the PCR product on an agarose gel, where the dye for loading the DNA solution acquires a colorless color that makes it easy to load it on the gel.

In addition to the presence of a high glycerol percentage, the density of the sample increases and facilitates its entry into the loading hole

As for the dyes, they are visible to the naked eye, and when they migrate on the gel, they give an idea of the progress of the migratory process and the approximate location of the DNA strands.

One example is the following loading dye (Gel Loading Dye 6X, Vivants, USA)

**Fig. 5.9:** DAN Gel Loading Dye, Blue (6X) | NEB

<u>Agarose gel:</u> An agarose gel is prepared at a concentration of 2% due to the short lengths of DNA strands, which is the appropriate concentration to conduct electrophoresis of the PCR product in the research.

In order to verify the integrity of the isolated DNA, an agarose gel is prepared at a concentration of 0.7%. One example is the following agarose gel (TopVision agarose™, Fermentas® Lithuania).

**Fig. 5.10:** TopVision Agarose gel

<u>Tris-Borate-EDTA (TBE):</u> This Tris is used to prepare agarose gel as an electric current carrier in the electrophoresis basin. TBE has been used because it gives greater separation ability for small pieces of PCR products, in contrast to Tris-Acetate-EDTA (TAE) which gives better separation of large pieces of DNA whose size is greater than 1000 nucleotide pairs [147]. One example is the following Tris-Borate-EDTA TBE 10X; SERVA, Germany.

**Fig. 5.11:** Tris-Borate-EDTA Buffer (TBE-10x)

Ethidium Bromide: It is a colored fluorinated substance used to detect DNA strands at a concentration of 1µg/ml, as it enters the DNA helix and is fixed between the nitrogenous base pairs, which leads to DNA fluorescence when exposed to UV rays in the range 302/365 nm. One example is the following solution (Ethidium Bromide 10 mg/ml; Vivantis biomedical, USA).

**Fig. 5.12:** Ethidium Bromide (10 mg/ml)

DNA Ladder: It is necessary in order to know the lengths of DNA segments and distinguish between them. One example is the following DNA Ladder (DNA Ladder, 50 bp, Vivantis, Malaysia).

**Fig. 5.13:** DAN Ladder 50bp

### 5.2. Devices used in the research

Autoclave: For example, Autoclave CL-40L; ALPCo, Japan.

**Fig. 5.14:** Laboratory autoclave - CL series - ALP Co., Ltd. - vertical / floor-standing / with dryer

Autovortex: For example Autovortex; STUART scientific, UK.

**Fig. 5.15:** Stuart Scientific SA6 Autovortex Vortex Mixer Lab Shaking Equipment

<u>Pipette:</u> For example, Pipette of different sizes (10, 20, 50, 100, 200, 1000 µl), Pipettes; Vivantis biomedical, USA.

**Fig. 5.16:** Discovery Comfort Pipettors, Single Channel, HTL

Tubes: For example, Eppendorf tubes; Eppendorf, German, of different sizes (0.2ml and 1.5ml)

**Fig. 5.17:** Eppendorf Tubes

Tips: For example, Eppendorf Tips; Eppendorf, Germany, of different sizes.

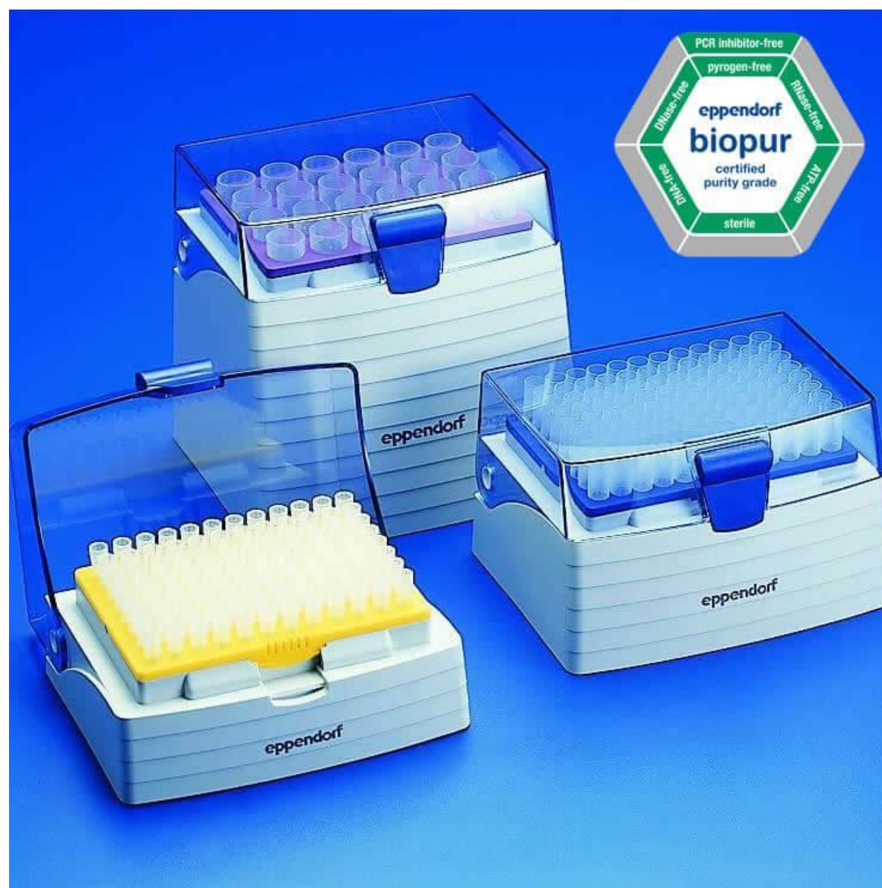



**Fig. 5.18:** Eppendorf™ epTIPS™ Reloads, Pipettenspitzen

Thermal cycler PCR: For example, Thermal cycler PCR; TaKaRa, Japan: to perform a PCR reaction.

**Figure 5.19:** TaKaRa PCR Thermal Cycler TP650

Horizontal-Unit: For example, HU10 Mini-Plus Horizontal; Scie-plas, US: to perform electrophoresis.

**Figure 5.20:** HU10 Mini-Plus Horizontal Unit from Scie-Plas Ltd | SelectScience

Consort: For example, CONSORT; Cleaver Scientific, Belgium: To apply the potential difference needed to conduct electrophoresis.

**Fig. 5.21:** Consort C1010 Benchtop pH Meter - Cleaver Scientific

Tabletop centrifuge: For example, Himac CT15RE tabletop centrifuge; HITACHI, Japan: Especially for Eppendorf 0.2ml, 1.5ml and 2ml tubes.

**Fig. 5.22:** Himac CT15E Tabletop Centrifuge, 15000rpm

Transilluminator: For example, WUV-M10 Transilluminator; Daihan Scientific, Korea: It is a device for displaying DNA bundles equipped with a UV lamp (302/365 nm).

**Fig. 5.23:** DaiHan WUV-M10 UV Transilluminator (365 nm)

AlphaImager: For example, AlphaImager Mini system; ProteinSimple, USA: It is a device for imaging and documenting the gel.

**Fig. 5.24:** The AlphaImager Mini Provides Researchers with a Compact and Economical Digital Imaging

### 5.3. Methods of research:

#### 5.3.1. Sample collection:

Volunteers: The case-control-study includes patients with arterial hypertension, in addition to normal volunteers. All volunteers are from one Society and from different ages and gender.

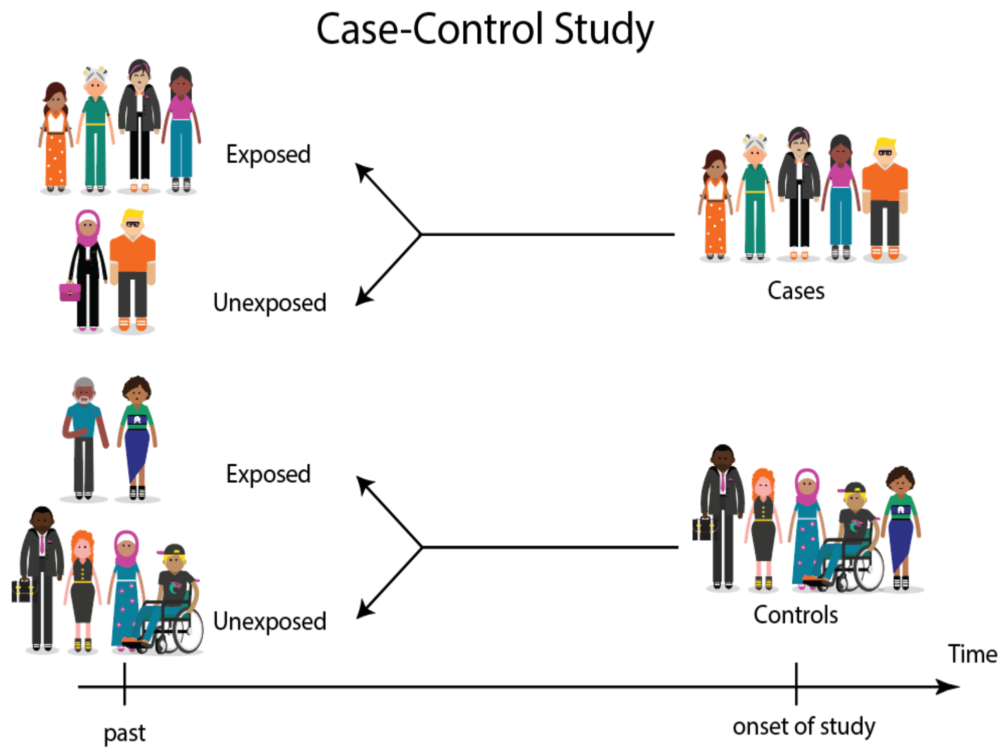



**Fig. 5.25:** Case-Control-Study

<u>Samples:</u> They are venous blood samples taken from patients with arterial hypertension and from healthy volunteers. They are placed in 5ml tubes containing the anticoagulant K3-EDTA. They are serially numbered and stored in a special laboratory at a temperature of -26° until use.

**Fig. 5.26:** Numbered blood samples

The necessary approvals are obtained from the competent authorities, in addition to the consent of the volunteers to take samples from them to conduct the study

<u>Workplace:</u> The PCR reaction preparation area is isolated from the rest of the work areas, in addition to the use of laboratory tools such as Eppendorf tubes, micropipette heads, and other pipettes used in preparing the PCR reaction, and isolated separately from the tools used for other purposes, in order to prevent cross contamination. All laboratory tools used are sterilized by autoclave, filtered pipette heads are also used to ensure the safety of micropipettes from contamination, because any contamination, even of a size of 1 nl before amplification, is sufficient to cause various errors in the PCR reaction.

### 5.3.2. DNA extraction using column chromatography

DNA is isolated from 200µl of human blood using for example the GF-1 Purification Kit; Vivantis, Malaysia
This kit is based in principle on the use of filter membranes designed to bind with DNA with high efficiency in the presence of a high concentration of salts and is extracted according to the standards and directions of the manufacturer by a special protocol.

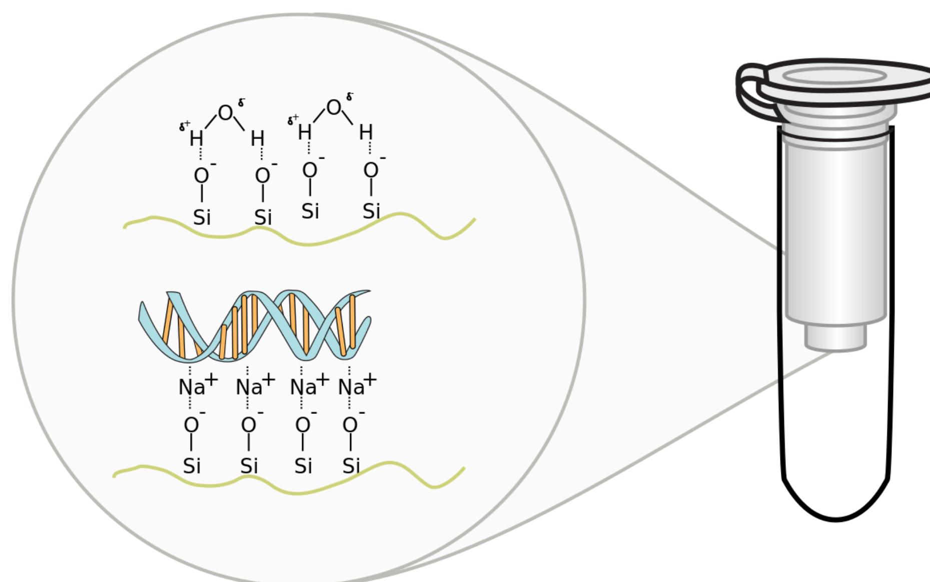



**Figure 5-27:** Spin column-based nucleic acid purification

### 5.3.3. Check Eluted DNA:

The integrity of the extracted DNA is verified by migrating 10µl of the extracted sample after mixing it with 2µl of loading dye solution used to facilitate loading of the sample onto the gel wells at the electrophoresis stage.

Agarose gel is prepared at a concentration of 0.7% with the addition of 10µl/ml of Ethidium Bromide before placing the gel in the mold. An appropriate DNA Ladder is carried along with the extracted samples for comparison and confirmation of the DNA molecular weight.

The electrodes in the electrophoresis basin are connected to a feeding unit so that the electrodes are negatively charged at the sample and positive at the opposite end because DNA has a negative charge.

A difference in latency of 5 vol/cm is applied and the time is determined by about an hour after covering the electrophoresis basin because the electric current raises the temperature and causes evaporation of the liquid in the basin. To show the DNA strands, a special device is used to show the gel electrophoresis provided with an ultraviolet lamp at wavelength (302/365 nm) to show DNA strands.

### 5.3.4. Polymerase Chain Reaction (PCR):

**5.3.4.1. Prepare the nuclease free water:** The water is sterilized by double distillation using an anti-autoclave device, and thus nuclease-free water is obtained. Due to the ease of contamination of the water used with nuclease enzymes present on the skin, in the air and in the dust, the water is distributed in Eppendorf tubes of a capacity of 2 ml and kept at a temperature of -20°.

**Fig. 5.28:** Nuclease-free water

**5.3.4.2. Design and preparation of primers:** A PCR reaction usually requires the design of two primers for each of the two complementary strands of DNA: a forward primer and a reverse primer.

The concentration of primers does not depend on the number of copies resulting from amplification only, but also on the molecular weight of one copy, and the forward primer and reverse primer must be at the same concentration, and the primer concentration is usually (0.1-1 µmolar) i.e. (0.1-1 µmol/l) [145] and this is in the final solution of the amplification reaction containing the template DNA.

The use of low concentrations of the primer leads to a low PCR output, while the high concentrations lead to the formation of a primer dimer, and the possibility of joining the primer at several sites of DNA increases. Therefore, the optimal theoretical concentration in the study is 250ng/50µl, the concentration in the final solution for the amplification reaction is the template DNA contains 5000 µg/l.

To obtain the molecular concentration, we divide by the molecular weight of the primer, which is found in the attached leaflet 5000/5520 = 0.9 µmol/l.

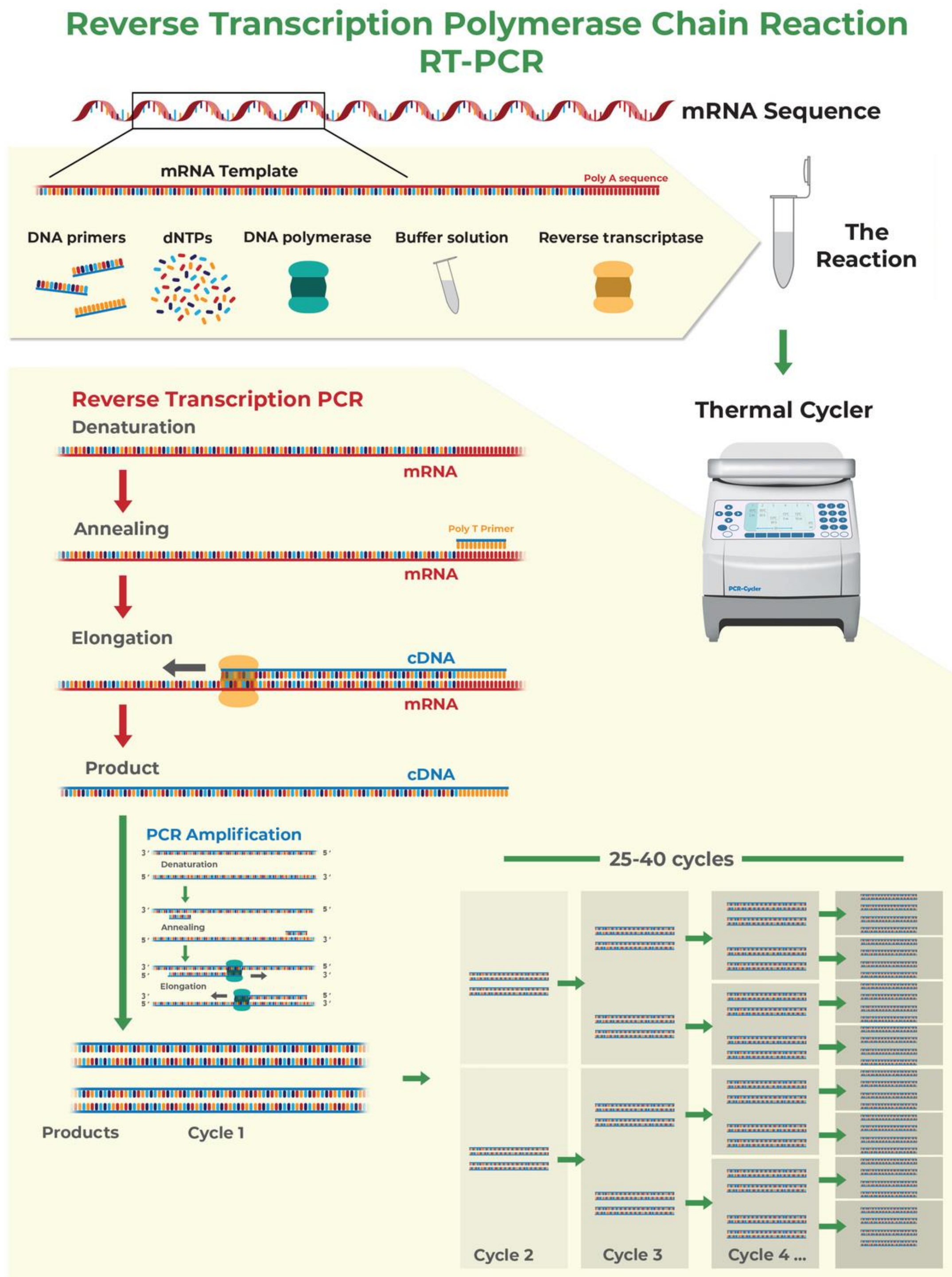



**Fig. 5.29:** Design and preparation of primers

There are many programs for designing a primer. The working solutions of the primers are activated from time to time through a denaturing process by heating to 95° for two minutes and then immediately freezing at -20°.

**5.3.4.3. Amplification using PCR:** Scientist Kary Mullis invented in 1983 a new technique that allows a sequence of DNA to be multiplied in a test tube, and he called this process the term PCR (Polymerase Chain Reaction). It allows pieces of DNA to be copied a billion times in hours. PCR technology relies on DNA replication using one of the DNA Polymerase enzymes.

In the beginning, the Master Mix is prepared, which includes all the components of the PCR reaction, except for the DNA samples that the Template DNA will work on, where the materials are removed from the freezer and placed directly on a cooled stand for Eppendorf tubes that maintains a temperature of +4 in order for the materials to melt slowly.

Subsequently, the materials are added using a micropipette to an Eppendorf tube of 0.2 ml capacity, according to the following sequence: Deoxynuclease water, buffer, deoxynucleotides, magnesium chloride, forward primer, reverse primer, polymerase, template DNA.

Then the tubes are tightly closed and placed in a special microencapsulated Eppendorf tubes for two seconds in order to drop the frozen drops on the walls, then the tubes are placed in a thermal cycler.

The PCR reaction is carried out in a thermal cycler in three main stages, which differ from each other in terms of time and temperature and include these stages:

- Denaturation step: At this stage, the reaction mixture is heated to temperatures ranging from (90-100) ° for 1-2 minutes [148] to break the hydrogen bonds between the nitrogenous bases of DNA and produce a DNA template ready for the reaction.

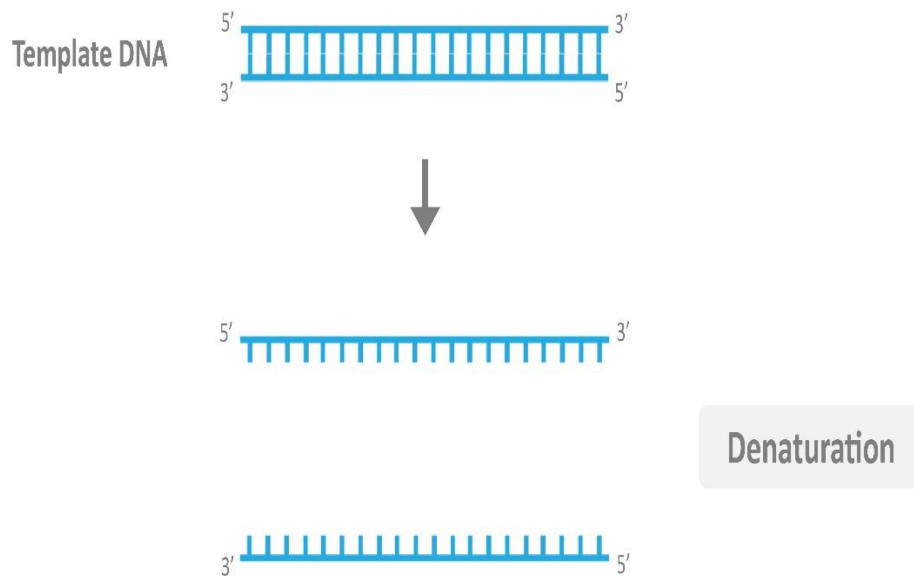



**Fig. 5.30:** Denaturation Step in PCR

- Annealing step: At this stage, the temperature of the reaction mixture is rapidly reduced to a temperature ranging between 45-60°C [148] depending on the special program and the type of initiators, and the mixture is allowed to remain at that temperature for a minute or less.
  Single complete DNA strands cannot, during this short period, re-bind with their complements, while primers, being short, can quickly bind with the target sequences on the template DNA.

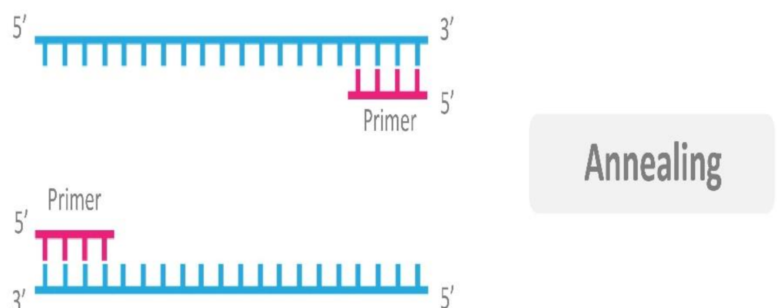



**Fig. 5.31:** Annealing Step in PCR

The optimum temperature for this stage (Tm) is tested based on the number and type of initiator nucleotides, because the nucleotides of guanine and cytosine form three hydrogen bonds among themselves, which requires a greater amount of energy than the bonding of adenine with thymine with only two hydrogen bonds.
A temperature lower than the average annealing temperature of about 3-5 °C is re-selected for the forward and reverse initiators in order to ensure the bonding of the largest possible number of initiators.

- Elongation (Extension) step: The temperature of the reaction mixture is raised to 27 °C, which is the appropriate temperature for the Taq polymerase enzyme to manufacture a new DNA strand using the free nucleotides present in the reaction mixture as shown in the figure (5-32). The time of this stage varies depending on the length of the amplified piece and the speed of the polymerase enzyme's work. For the most widely used Taq polymerase, the time for this stage is one minute per length of 1000 nucleotide pairs [148].

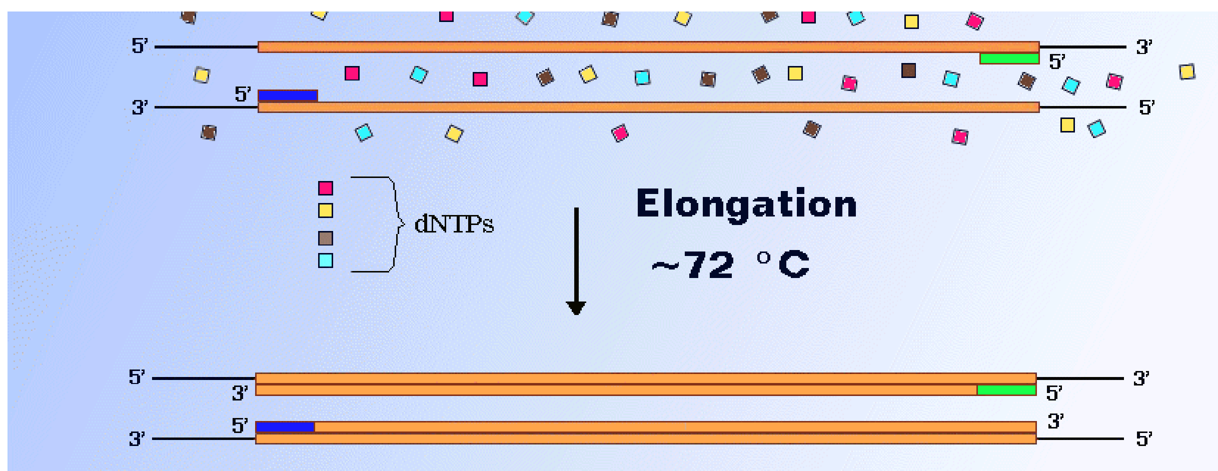



**Fig. 5.32:** Elongation step in PCR

The temperature changes in the three stages by alternating between them for a limited number of cycles ranging between 25-30 cycles.

These three stages are preceded by the initial denaturation step, which is identical to the first stage previously mentioned, except that it takes place during a longer time that ranges from 5-10 minutes and is applied once.
After the end of the specified number of cycles, the program is concluded with a one-time final elongation phase called the final elongation phase and applied for a period of 5-10 minutes.

The exponential increase in the amount of amplified DNA stops after a finite number of cycles ranges often between 20-30 cycles, depending on the amount of DNA present at the beginning of the reaction, as the reaction enters a stage in which the amount of PCR product increases in a manner closer to linearity, then the amplification stops completely or almost completely as a result of the consumption of the reaction components and the amplification product competition for the initiators. This stage is called Plateau, and Fig. 5.33 shows a diagram About the different phases of the PCR process.

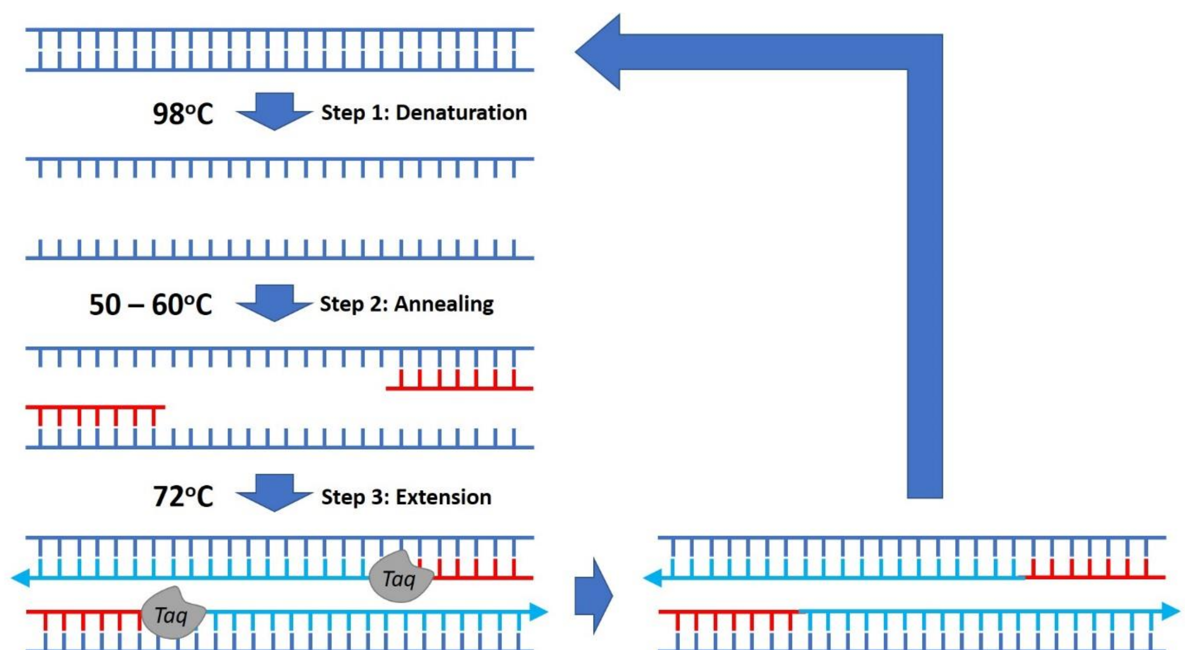



**Fig. 5.33:** The different phases of the PCR process

The device will be programmed and the data will be entered and saved in a special file that includes the reaction conditions in terms of temperature and time for each of the stages as shown in the Table 5.2

| Time | temperature | number of circles | stage |
|---|---|---|---|
| **5 min** | 94°C | One circle | Initial denaturation |
| **30 sec** | 94°C | 35 circles | denaturation |
| **30 sec** | 56.5°C | | annealing |
| **90 sec** | 72°C | | elongation |
| **10 min** | 72°C | One circle | Final elongation |

**Table 5.2:** PCR reaction conditions in terms of temperature and time of each stage

It is chosen based on recognized theoretical data and a summary of the gradual amplification experiments that allow the use of several concentrations of magnesium chloride, which helps to determine the optimal temperature and optimal magnesium concentration.

**5.3.5. Agarose gel electrophoresis for PCR:** Electrophoresis is used on a horizontal gel of 2% Agarose to separate the pieces resulting from the PCR and reveal them according to their lengths. A horizontal migration basin with a capacity of 450 ml of migration buffer is used and the dimensions of the gel casting dish are 6.5 x 23 x 16.5 cm and a capacity of 80 ml of the gel solution to obtain on a gel, its dimensions are 11.5 x 10 cm.

**Fig. 5.34:** Agarose gel electrophoresis (2%) of PCR products

**5.3.5.1. Prepare the buffer solution:** A buffer with PH = 8.3 is usually used as a transfer buffer and to prepare the gel at a concentration of 1X by diluting 100ml of the concentrated buffer solution (10X) to a liter using double distilled water to reduce the conductivity of the medium.

**5.3.5.2. Jelly preparation:** The concentration of the agarose gel varies according to the lengths of the DNA segments to be transferred.

It is required to use an agarose gel with a concentration of 2%, which is the approved concentration to separate DNA segments that range in length within this range.

To prepare a 2% Agarose gel, weigh 1.4 gr of Agarose powder using a metal spoon in a clean, dry container of 250 ml capacity, then add 70 ml of TBE prepared in the previous step at a concentration of 1X and stir gently.

The bowl is then placed in a microwave and heated for a minute. Then the suspension was removed and gently stirred to re-suspend the precipitated Agarose and then heated again for 30 seconds and then repeat the process until the agarose is completely dissolved Then the solution is left to cool for 5-10 minutes, taking care to cover it to prevent evaporation and volume change. Then, Ethidium bromide is added to the gel solution at a concentration of 1 µg/ml by adding 7µl of Ethidium bromide solution with a concentration of 10 mg/ml to the gel solution, which has a volume of 70 ml.

**5.3.5.3. Electrophoresis:** The nomads basin is prepared as follows: After placing the combs in place, the gel solution is poured so that the pouring side is from the corner close to the combs, and the pouring is done quietly to prevent the formation of bubbles, then the gel is left for 45-60 minutes, then the prepared buffer is poured into the side basin stores in a balanced manner, where the entire gel is immersed, then the samples are loaded quickly and load 5µl of DNA, then cover the basin and connect the wires between the feeding unit and the basin and start migration directly to prevent the spread of samples in the gel in all directions, which affects the quality of separation

Then an electric field of 5V/cm is applied for a period of 45 minutes, and after the end of the electrophoresis, the DNA pieces were shown using a gel display device.

**5.3.5.4. Display by UV:** The gel dish is placed on a projector equipped with ultraviolet rays (302/365 nm) and connected to an (Alpha Imager Mini) device, and in a dark room where the Ethidium bromide dye fluoresces associated with DNA, which leads to the appearance of DNA pieces in the form of visible bands that can be photographed.

**5.3.6. DNA Digestion:** The appropriate restriction enzyme is selected so that it cuts the DNA at a nucleotide sequence similar to the nucleotide sequence in the region surrounding the studied SNP on the gene.

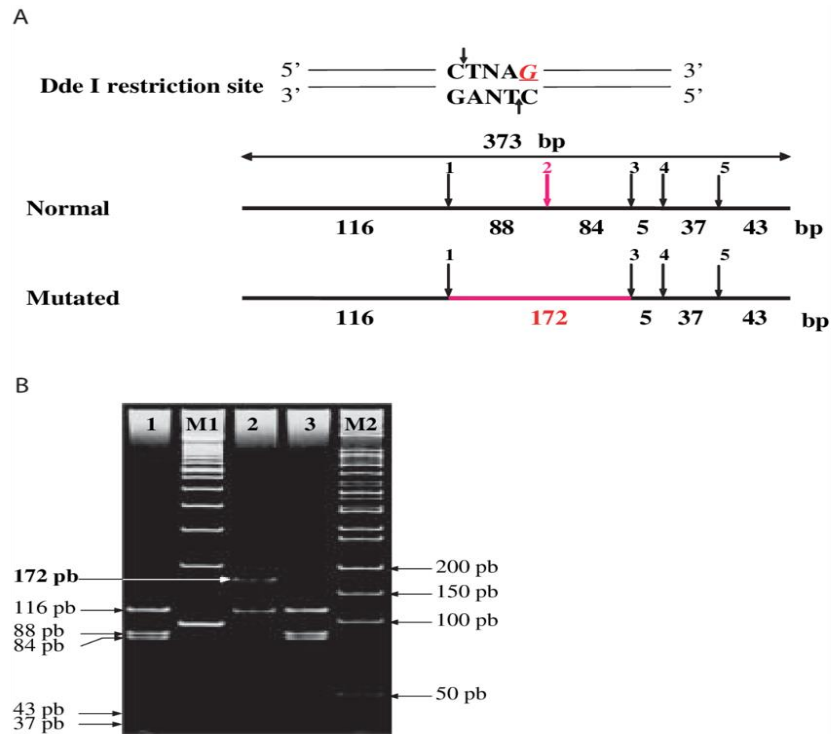



**Fig. 5.35:** Digestion of exon 2 PCR product by DdeI and separation of the fragments by electrophoresis. A) Theoretical restriction cleavages: five restriction sites are present in the normal; the mutation abolishes site 2, generating a 172 bp fragment. B) Electrophoretic pattern obtained on an 8% polyacrylamide gel. Lanes 1 and 3: normal pattern with five visible bands; lane 2: heterozygous carrier of Hb A 2 -Pasteur-Tunis. Abolition of site 2 leads to an additional 172 bp band; M1: 100 bp marker, M2: 50 bp marker.

The truncation enzyme DdeI is used, also called HoyF31, and it distinguishes the nucleotide sequence C\TNAG in both directions. If this nucleotide sequence is present in the studied gene, the complete failure of the enzyme to cut the amplified DNA or its ability to make a complete truncation indicates a homozygous state for one of the two alleles.

5’……taatgtaagctcatccaccaagaagcct**gcaccatgttttgaggtt**gagtgacatgttcgaaacctgtccata
aagtaattttgtgaaagaaggagcaagagaacattcctctgcagcacttcactaccaaatgagcAttagctacttttca
gaattgaaggagaaaatgcattatgtggactgaaccgacttttctaaagctctgaacaaaagcttttctttccttttgcaac
aagacaaagcaaagccacattttgcattagacagatgacggctgctcgaagaacaatgtcagaaactcgatgaatg
tgttgatttgagaaattttactgacagaaatgcaatctccctagcctgcttttgtcctgttattttttatttccacataaaggtattt
agaatatattaaatcgttagaggagcaacaggagatgagagttccagattgttctgtccagtttccaaagggcagtaaa
gttttcgtgccggttttcagctattagcaactgtgctacacttgcacctggtactgcacattttgtacaaaga**tatgctaagc**
**agtagtcg**tcagttgcagatctttttgtg…….3’

**Fig. 5.36:** The figure shows the site of the enzyme DdeI. The complete AGTR1 gene sequence and SNP locus are obtained from the Biotechnology Center's DNA Bank. The shaded sequence is the sequence characteristic of the C\TNAG truncation enzyme, where N stands for nucleotide A, T, C, or G. The capitalized letter represents polymorphism A or C. The letters in bold and underlined represent the forward and reverse indents.

Either make a cut in half of the amplified DNA is indicative of a heterozygous state. The cutoff products are detected after enzymatic digestion using gel electrophoresis. The restriction enzyme can work in a medium similar to that of the PCR reaction in terms of the degree of PH = 8.3 and the concentration of potassium and magnesium ions. Therefore, the digestion reaction can be performed directly without purifying the PCR products by adding the restriction enzyme and a sufficient amount of enzyme buffer that maintains a suitable medium and thus Reducing time, effort and cost. As for the practical digestion method using the restriction enzyme, it is done in the following way:

The digestion mixture is prepared as shown in Table 5.3, which provides a suitable medium for the digestion in terms of pH and ionic strength, taking care that both the product of the digestion and the enzyme remain on the cooled carrier, then it is then incubated at a temperature of 37°C for 3 hours, followed by inactivation of the enzyme by raising the temperature to 65°C for 20 minutes in the incubator.

| the size (µl) | Additive |
|---|---|
| **10** | Amplification product solution |
| **7** | Nuclease-free water |
| **2** | Buffer |
| **1 (10 U/µl)** | truncation enzyme |

**Table 5.3:** The volumes used for the digestion reaction for DNA

**5.3.7. Electrophoresis of the digestion products:** After the amplification process by PCR and the digestion process, all samples of healthy and sick participants are transferred on an Agarose gel in order to compare the images of these samples before digestion with the final images after the digestion process to ensure a correct reading of the results.

**5.3.8. Statistical analyses:** The data are analyzed using cross-tab and logistic regression to find out the association between genotype and allele frequency for the A1166C polymorphism and hypertension. Odd ratios are relied upon with a confidence rate of 95%.

| | | Outcome | |
|---|---|---|---|
| | | Yes | No |
| Predictor | Yes | A | B |
| | No | C | D |

$$OR = \frac{(A*D)}{(B*C)}$$

**Fig. 5.37:** Calculate Odds Ratio with 95% Confidence

The statistically significant P value is also adopted when it is less than 0.05.

# 6. Contents and Results

## 6.1. Studies:

6.1.1. Interactions between gender and the angiotensin type 1 receptor gene polymorphism, Canada, 1997 [139]

**Background:** The cardiovascular effects of angiotensin II are mediated by the angiotensin II type 1 receptor (AGT1R); one polymorphism of the AGT1R gene, A1166→C, has been associated with hypertension. The hemodynamic response to angiotensin II is blunted in women compared to men, but interactions between gender, blood pressure, and AGT1R gene polymorphisms are unclear.

**Methods:** A total of 81 young healthy normotensive individuals maintained regulated sodium and protein intake prior to study. They were divided into four groups based on gender and A1166→C genotype (AA versus AC/CC); serial supine blood pressures were obtained. A subset of 52 individuals received graded infusions of angiotensin II. Inulin and paraaminohippurate clearance techniques were used to measure renal hemodynamic function at baseline and in response to the infusions.

6.1.2. Association between angiotensin II type-1 receptor A1166C polymorphism and the presence of angiographically-defined coronary artery disease in an Iranian population, Iran, 2010 [149]

**Background:** There are reported associations between a polymorphism of the angiotensin II type 1 receptor (AT1 R/A1166C) gene and coronary artery disease (CAD), hypertension, and myocardial infarction in some populations.

Objective: Investigate the association between A1166C polymorphism and CAD in an Iranian population.

**Methods:** Four hundred and thirteen patients with suspected CAD were recruited. Based on coronary angiography, the patients were classified into CAD+ (n=315) and CAD- (n=98) groups defined as >50% and <50% stenosis of any major coronary artery, respectively. One hundred and thirty-five healthy

subjects were also recruited as the control group. The AT1R polymorphism was assessed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based method.

6.1.3. A/C Polymorphism of the Angiotensin II Type 1 Receptor Gene and the Response to Short-Term Infusion of Angiotensin II. Circulation, Germany, 1999. [151]

**Background:** Previous studies reported an association of the 1166 A/C polymorphism of the angiotensin II (Ang II) type 1 receptor gene with high blood pressure and cardiovascular disease. We tested the hypothesis that this polymorphism affects the blood-pressure, renal hemodynamic, and aldosterone response to infused Ang II.

**Methods:** Young, male, white volunteers (n=116) with normal (n=65) or mildly elevated (n=51) blood pressure on a high salt intake were genotyped for the 1166 A/C polymorphism. Two doses of Ang II (0.5 and 3 ng· $kg^{-1}$ · $min^{-1}$ over 30 minutes each) increased blood pressure, plasma aldosterone, glomerular filtration rate, and filtration fraction and decreased renal blood flow.

6.1.4. A/C1166 Gene Polymorphism of the Angiotensin II Type 1 Receptor (AT1) and Ambulatory Blood Pressure: The Ohasama Study, Hypertens, Japan, 2002 [140]

**Background:** We previously investigated the relation between hypertension and each of three major genetic polymorphisms in the renin-angiotensin (AGT)-aldosterone system (R-A-A), AGT M235T, angiotensin converts enzyme (ACE) I/D, and CYP11B2 -344C/T, by means of ambulatory blood pressure (ABP) monitoring in a general Japanese population (the Ohasama Study). A/C1166 gene polymorphism in the 3' untranslated region of the angiotensin II type 1 receptor (AT1) gene is the final remaining major target in R-A-A to be examined in the Ohasama Study population.

**Methods:** In the present study, the AT1 A/C1166 polymorphism was genotyped by the TaqMan polymerase chain reaction (PCR) method or restriction fragment length polymorphism (RFLP) in 802 Japanese subjects aged 40 and over, who were previously genotyped for the AGT M235T, ACE D/I, CYP11B2 -344C/T polymorphisms.

6.1.5 Polymorphism of Angiotensin II type 1 receptor gene in essential hypertension in iranian population, Iran, 2006 [141].

**Background:** Renin angiotensin system (RAS) has an important role in the regulation of hypertension. RAS includes angiotensinogen, Angiotensin Converting Enzyme (ACE), angiotensine II and angiotensin receptors (AGTR). Angiotensin receptors have several types but AT1R is the main subtype.
**Methods:** In this study the effect of A1166→C polymorphism of AT1R gene and the role of possible genetic differences in hypertension was investigated. DNA of the whole blood leukocytes from hypertensive patients and healthy people of Mashhad population as control, were extracted and then PCR was performed on prepared samples followed by amplification of the target fragments which were then digested with the DdeI restriction enzyme. Data were classified on the basis of genotypes and gender and then alleles and genotypes frequencies were analyzed statistically.

6.1.6 Angiotensin II type 1 receptor A1166C gene polymorphism and essential hypertension in San Luis, 2006 [138].

**Background:** Essential hypertension is considered a multifactorial trait resulting from a combination of environmental and genetic factors. The angiotensin II type 1 receptor mediates the vasoconstrictor and growth-promoting effects of Ang II. The A1166C polymorphism of the AT1 receptor gene may be associated with cardiovascular phenotypes, such as high arterial blood pressure, aortic stiffness, and increased cardiovascular risk.
**Methods:** We investigated the association between this A1166C polymorphism and hypertension in hypertense and normotense subjects from San Luis (Argentina) by mismatch PCR-RFLP analysis.

6.1.7 Polymorphism of the Angiotensin II Type 1 Receptor A1166C in Korean Hypertensive Adolescents Korean 2008 [150].

**Background and Objectives:** The renin-angiotensin system (RAS) is a major regulator of blood pressure. The angiotensin II receptor type 1 (AGTR 1)

A1166C has been extensively studied in searching for their involvement in the development of hypertension. The aim of this study was to determine the association of the AGTR 1 A1166C marker with essential hypertension in Korean adolescents.

**Subjects and Methods:** Forty hypertensive adolescents were included in this study. The obesity index (OI) and body mass index (BMI) of the subjects were calculated. Blood pressure was measured at the resting state by oscillometric methods. The serum aldosterone, renin, insulin, angiotensin converting enzyme (ACE), homocysteine, vitamin B12 and folate levels were measured. The carotid intima-media thickness (IMT) and diameter and the brachial-ankle pulse wave velocity (baPWV) were evaluated by ultrasound. Polymerase chain reaction (PCR) was conducted to amplify the DNA of each of the study subjects to analyze the polymorphism of AGTR 1 A1166C.

6.1.8 Association of A1166C polymorphism in AT(1) receptor gene with baroreflex sensitivity, Czech, 2010 [153].

**Background:** The aim of this study was to evaluate the association of A1166C polymorphism in angiotensin II type 1 receptor (AT(1)R) gene with baroreflex sensitivity (BRS in ms/mm Hg; BRSf in mHz/mm Hg) in man.

**Methods:** BRS and BRSf were determined by a spectral method in 135 subjects (19-26 years) at a frequency of 0.1 Hz. Genotypes were detected by means of polymerase chain reaction and restriction analysis using enzyme DdeI. We compared BRS and BRSf among genotypes of this polymorphism.

6.1.9 The association of hypertension with renin-angiotensin system gene polymorphisms in the Lebanese population, Lebanon, 2011 [137].

**Background:** The study objective was to examine the association of hypertension in the Lebanese population with three renin-angiotensin system gene polymorphisms (RAS): angiotensin-converting enzyme (ACE), angiotensinogen (AGT) and angiotensin-receptor type 1 (AT1R).

**Methods:** A total of 270 subjects (124 hypertensive vs 146 normotensive) were genotyped for ACE insertion (I)/deletion (D), AGT (M235T), and AT(1)R (A1166C) gene polymorphisms by polymerase chain reaction and restriction fragment length polymorphism.

6.1.10 AT1 Receptor Gene Polymorphisms in relation to Postprandial Lipemia, Spain, 2012 [152].

**Background:** Recent data suggest that the renin-angiotensin system may be involved in triglyceride (TG) metabolism and Hypertension. We explored the effect of the common A1166C and C573T polymorphisms of the angiotensin II type 1 receptor (AT1R) gene on postprandial lipemia and Hypertension.

**Methods:** Eighty-two subjects measured daytime capillary TG, and postprandial lipemia was estimated as incremental area under the TG curve. The C573T and A1166C polymorphisms of the AT1R gene were determined.

6.1.11 Angiotensin II type 1 receptor A/C1166 polymorphism. Relationships with blood pressure and cardiovascular structure, Italy, 1996 [156].

**Background:** The angiotensin II type 1 (AT1) receptor has a key role in mediating the vasoconstrictor and growth-promoting effects of angiotensin II. It has been reported that a polymorphism of the AT1 receptor gene (an A/C transversion at position 1166) may be associated with cardiovascular phenotypes, such as arterial blood pressure and aortic stiffness, that underlie a condition of increased cardiovascular risk.

**Methods:** We examined a sample of 212 subjects randomly selected from a general population in northern Italy to investigate the role of AT1 receptor gene polymorphism, in the regulation of blood pressure and cardiovascular growth. We measured blood pressure (both clinic and 24-hour ambulatory recording), left ventricular mass (echocardiography), and carotid artery wall thickness (B-mode ultrasound); we assessed the AT1 receptor genotype by polymerase chain reaction and allele-specific oligonucleotide hybridization.

## 6.2. Results:

6.2.1. Interactions between gender and the angiotensin type 1 receptor gene polymorphism, Canada, 1997 [139]

**Results:** Men with the AC/CC genotype exhibited higher blood pressures than men with the AA genotype; however, this relationship was not found among women. Analysis of covariance revealed a significant interaction between gender and AGT1R genotype in the determination of blood pressure. Glomerular filtration rate (GFR) declined variably in the study subjects following infusion of angiotensin II, and a statistical model incorporating gender and genotype best predicted the fall in GFR. There was a trend for females of the AA genotype to have a greater fall in GFR in response to angiotensin II infusion, than any of the other groups.

6.2.2. Association between angiotensin II type-1 receptor A1166C polymorphism and the presence of angiographically-defined coronary artery disease in an Iranian population, Iran, 2010 [149]

**Results:** A higher frequency of the AC and CC genotypes and lower frequency of the AA genotype was observed in both CAD+ and CAD- groups, compared with the control group ($p < 0.05$). CAD+ and CAD- groups also had a higher frequency of the C allele than controls ($p < 0.01$). There was no significant difference in genotype and allele frequencies between hypertensive and non-hypertensive patients ($p > 0.05$). In addition, the AT1R genotype frequencies did not differ significantly among different subgroups of CAD+ patients, based on the number of affected coronary vessels ($p > 0.05$).

6.2.3. A/C Polymorphism of the Angiotensin II Type 1 Receptor Gene and the Response to Short-Term Infusion of Angiotensin II. Circulation, Germany, 1999. [151]

**Results:** The blood-pressure, renal hemodynamic, and aldosterone responses were not significantly different between subjects homozygous for the A allele (n=56) and heterozygous subjects (n=47) or subjects homozygous for the C allele (n=13). Comparison of A allele homozygotes with all C allele carriers

pooled (n=60) or restriction of the analysis to normotensive volunteers also revealed no significant differences between genotypes.

6.2.4. A/C1166 Gene Polymorphism of the Angiotensin II Type 1 Receptor (AT1) and Ambulatory Blood Pressure: The Ohasama Study, Hypertens, Japan, 2002 [140]

**Results:** The AA genotype, AC genotype, and CC genotype were present in 678 (84.5%), 121 (15.1%), and 3 (0.4%) of subjects, respectively. Since the frequency of the C allele was quite low (0.079), the genotypes were classified according to the presence or absence of the C allele. Although daytime blood pressure (BP) was higher in subjects with the C allele, the difference was not statistically significant after adjusting for age, gender, body mass index, and smoking status.

6.2.5. Polymorphism of Angiotensin II type 1 receptor gene in essential hypertension in iranian population, Iran, 2006 [141].

**Results:** There were no significant differences in the genotype, and allele frequencies between hypertensive and normotensive subjects. However, frequency of C allele of AT1R gene in hypertensive women was significantly higher than normotensive women ($P<0.05$).

6.2.6. Angiotensin II type 1 receptor A1166C gene polymorphism and essential hypertension in San Luis, 2006 [138].

**Results:** Hypertensive patients exhibited significant increases in lipid related values and body mass index. The frequency of occurrence of the C1166 allele was higher among patients with hypertension (0.19) than in the control group (0.06).

No significant association was found between this polymorphism and essential hypertension in the study population, although the AC genotype prevalence was higher in patients with hypertension and positive family history of hypertension (32%) than in control subjects (12%). Patients with the A1166C polymorphism

exhibited higher levels of serum total cholesterol, LDL-cholesterol and BMI than in control subjects.

6.2.7. Polymorphism of the Angiotensin II Type 1 Receptor A1166C in Korean Hypertensive Adolescents Korean 2008 [150].

**Results:** The genotypic frequency of AA was 87.5%, that for adenylate cyclase (AC) was 12.5% and no CC type was detected. The serum homocysteine level was higher in the subjects with the AC genotype than that in the subjects with the AA genotype (11.9±2.9 umol/L vs 17.1±4.2 umol/L, respectively). The carotid IMT of the subjects with the AA genotype was greater than that of the subjects with the AC genotype (5.0±0.1 mm vs 8.0±0.2 mm, respectively).

6.2.8. Association of A1166C polymorphism in AT(1) receptor gene with baroreflex sensitivity, Czech, 2010 [153].

**Results:** The frequency of genotypes of AT(1)R A1166C polymorphism was: 45.9 % (AA, n=62), 45.9 % (AC, n=62), 8.2 % (CC, n=11). Differences in BRS ($p<0.05$) and BRSf ($p<0.01$) among genotypes of this single nucleotide polymorphism were found (Kruskal-Wallis: BRS - AA: 7.9+/-3.3, AC: 8.6+/-3.6, CC: 5.9+/-2.3 ms/mm Hg; BRSf - AA: 12.0+/-4.0, AC: 12.0+/-5.0, CC: 8.0+/-3.0 mHz/mm Hg). Compared to carriers of other genotypes (AA+AC) the homozygotes with the less frequent allele (CC) showed significantly lower BRSf (Mann-Whitney: BRSf - AA+AC: 12.0+/-4.0, CC: 8.0+/-3.0 mHz/mm Hg; $p<0.01$) and borderline lower BRS (BRS - AA+AC: 8.2+/-3.5, CC: 5.9+/-2.5 ms/mm Hg; $p=0.07$).

6.2.9. The association of hypertension with renin-angiotensin system gene polymorphisms in the Lebanese population, Lebanon, 2011 [137].

**Results:** The studied genes showed no deviation from Hardy-Weinberg equilibrium. No association could be reported with the ACE I/D polymorphism, although the D allele frequency was high (77%) in patients. AGT TT genotype prevalence was found to be lower in hypertensive versus normotensive subjects

(p<0.0001). AT(1)R CC and AC genotypes were significantly more frequent in hypertensive than normotensive subjects (p<0.0001).

6.2.10. AT1 Receptor Gene Polymorphisms in relation to Postprandial Lipemia, Spain, 2012 [152].

**Results:** Postprandial lipemia was significantly higher in homozygous carriers of the 1166-C allele (mM*h/L) compared to homozygous carriers of the 1166-A allele (mM*h/L) (). Postprandial lipemia was similar for the different C573T polymorphisms.

6.2.11. Angiotensin II type 1 receptor A/C1166 polymorphism. Relationships with blood pressure and cardiovascular structure, Italy, 1996 [156].

**Results:** Blood pressure values were lower in CC homozygotes than in heterozygotes and AA homozygotes; the difference was statistically significant for clinic measurements (mean difference for mean blood pressure, -6.6 mm Hg, P = .01; 95% confidence interval, -1.6 to -11.7 mm Hg) but not for ambulatory blood pressure measurements. CC homozygotes also presented a lower incidence of a positive family history of hypertension (P = .027). No statistically significant differences among AT1 receptor A/C1166 genotypes were observed for left ventricular mass or carotid artery wall thickness.

# 7. Discussion

This section explains and analyses the results of the studies mentioned in Chapter 6.

- Many countries and different races have been interested in determining the genotype of the A1166C polymorphism of the AGTR1 gene. Table 7.1 shows a comparison of the results of studies in some countries in which similar studies were conducted, as it was found that the presence of the C allele differs from one country to another.

| reference number | study year | the presence of the C allele | country |
|---|---|---|---|
| **145** | 2012 | 33.6 | Spain |
| **125** | 2011 | 36.3 | Lebanon |
| **146** | 2010 | 31.2 | Czech |
| **142** | 2010 | 17.5 | Iran |
| **143** | 2008 | 6.3 | South Korea |
| **126** | 2006 | 27.5 | St. Louis |
| **129** | 2006 | 15.8 | Iran |
| **128** | 2002 | 15.9 | Japan |
| **144** | 1999 | 31.5 | Germany |
| **127** | 1997 | 34.7 | Canada |
| **150** | 1996 | 29.7 | Italy |

**Table 7.1:** Percentage of the C allele in different countries

- Several studies showed an association between the A1166C polymorphism and hypertension, and these studies showed a greater frequency of this polymorphism in hypertensive patients [154, 155, 156].
- In other studies, the presence of the CC genotype was associated with a reduced risk of high blood pressure and cardiovascular disease [155, 156].
- For example, in the two studies that were conducted in Iran (Behravan et al, 2006) and (Assalia et al, 2010), it was shown that there was no correlation between the genotype distribution or the distribution of alleles between hypertensive patients and healthy people [141, 149].

- In contrast in a study (Kikuya et al, 2003) in Japan, the results showed that there was no association between the A1166C polymorphism and any clinical evidence of hypertension [140].
- Angiotensin II is a vasoconstrictor and an inducer of vascular hypertrophy and vascular hyperplasia. It is theoretically expected that polymorphisms in the renin-angiotensin system play a role in the incidence of hypertension and vascular diseases.
- One of the explanations for the negative relationship in genotype & phenotype studies is that high blood pressure is a complex disease that does not result from a defect in only one gene, but is affected by different factors. Many genes can interfere with high blood pressure, and it is not necessary that polymorphism One gene in one increased susceptibility to high blood pressure.
- The relationship between the A1166C polymorphism in the AGTR1 gene and hypertension is still not entirely clear, as this nucleotide is located in the 3rd region outside of the 3′-UTR [130].
- It has been suggested that this polymorphism may interfere with the regulation of AGTR1 expression [131].
- Evidence also indicates that there are gender differences in the effectiveness of the RAS device, which is responsible for the differences in the value of blood pressure between men and women [132-134].
- It was suggested that there is an interaction between sex and the A1166C polymorphism that affects hypertension, as it was found that the AC and CC genotypes are associated with high blood pressure in Canadian women compared to the AA genotype of the AGTR1 gene [132].
- Studies of the association between high blood pressure and this polymorphism began in 1994 when the scientist Bonnardeaux and his colleagues indicated that there was a large prevalence of the C allele among people with high blood pressure compared to healthy subjects in France [110] and since then many studies have been conducted around the world to clarify the association between polymorphism AGTR1 and high blood pressure
- Numerous studies have considered the role of polymorphisms in the angiotensin receptor gene AGTR1 as risk factors for high blood pressure.

Studies conducted to determine the relationship between the polymorphism A1166C in the AGTR1 gene and high blood pressure showed conflicting results, as studies in Lebanon [137], France [110], Argentina [138] and Canada [138] showed that there is an important relationship between the A1166C polymorphism and the risk of developing high blood pressure, while other studies did not show any relationship between them as in the studies of Japan [139] and Iran [140].

- According to previous studies, it was predicted that the presence of the A1166C polymorphism is associated with susceptibility to arterial hypertension.
- Indeed, Zhu and his colleagues published in 2006 [141] in his study that included 150 people from the Chinese community that the A1166C polymorphism in the AGTR1 gene is associated with high blood pressure and atherosclerosis, and in another study conducted by Rehman and his colleagues in 2007 in Malaysia [142] it was found that the A1166C polymorphism it is not associated with high blood pressure.
- A 2007 study by Miyama and colleagues showed that the A1166C polymorphism in the AGTR1 gene does not predispose to essential hypertension [143].
- As a result of these discrepancies in previous studies, we tried in this research to supplement the scientific studies regarding the various societies and compare it with each other.

# 8. Conclusions and Prospects

In young healthy subjects in some populations, for example in Canada, there is an important interaction between gender, the AGT1R A1166→C gene polymorphism, and blood pressure. In addition, the renal hemodynamic response to angiotensin II infusion is a function of both gender and the AGT1R genotype.

The frequency of AT1R/A1166C polymorphism was in some Populations, for example in Iran higher among patients with some degrees of coronary stenosis who are candidates of coronary angiography.

The C allele of AT1R gene may be an important risk factor for essential hypertension in women in some populations, for example in Iran.

The 1166 A/C polymorphism does not have a major effect on these actions of Ang II and does not lead to a greater blood-pressure in some populations, for example in Germany.

The AT1 A/C1166 polymorphism was not associated with any clinical parameters associated with hypertension or atherosclerosis in the Japanese population.

Taken together the genotype and biochemical parameters and considering the restrictive selection criteria used, the results suggest in some populations, for example in San Luis a correlation between AT1 A1166C gene polymorphism and risk of cardiovascular disease.

The A1166C mutation group had a significantly greater carotid IMT and higher homocysteine levels than the group with the normal genotype of AGTR 1 in some populations, for example in Korean. The AC genotype of A1166C may be useful to predict the presence of early coronary artery disease in hypertensive adolescents. More investigation is necessary to clarify the relation between the A1166C gene and its involvement with coronary artery disease in hypertensive Korean adolescents.

There is a significant association of A1166C polymorphism in AT(1) receptor gene with baroreflex sensitivity in some populations, for example in Czech. Homozygosity for the less frequent allele was associated with decreased baroreflex sensitivity.

The first conducted study on the RAS gene polymorphisms in Lebanese hypertensive patients demonstrated a possible association of the AGT T and AT(1)R C alleles with hypertension.

The 1166-C allele of the AT1R gene seems to be associated with increased postprandial lipemia and Hypertension in some populations, for example in Spain. These data confirm the earlier described relationships between the renin-angiotensin axis and triglyceride metabolism.

The study in some populations, for example in Italy does not support a major role of the AT1 receptor gene A/C1166 polymorphism as a marker of conditions associated with increased cardiovascular risk.

Because the studies mentioned in our research did not establish a deterministic relationship between AT1 receptor gene A/C1166 polymorphism and between the possibility of high blood pressure, we suggest the following:

In the future, we suggest developing an easier, cheaper and more available technique than PCR to obtain genes based on the continuous development in molecular biology and biotechnology.

We propose in the future to study the relationship between several polymorphisms of the angiotensin receptor gene or several polymorphisms from multiple genes at the same time and the possibility of high blood pressure, because high blood pressure is considered a complex disease and does not result from a defect in only one gene, whereas, a single morphological change in one gene may not contribute to the susceptibility to the occurrence of this disease.

Finally, we suggest conducting joint studies in several countries at the same time, with the same conditions, and for larger numbers of patients, to obtain more reliable results, because hypertension has become a global disease and is widely spread all over the world.

# Appendix

## The method of extracting DNA from a blood sample

In this appendix, we would like to talk about the method of extracting DNA from a blood sample according to the instructions provided by the company (GF-1 Blood DNA Extraction Kit; Vivantis, Malaysia)

The GF-1 blood DNA extraction kit is designed for effective and rapid purification of DNA from 200 microliters of blood by specially treated glass filter membranes placed in columns that bind effectively with DNA when there is a high concentration of salts.

The extraction is based on the principle of columns and the use of optimum buffer to ensure that only DNA has been isolated and that cellular proteins, metabolites, salts and other low molecular weight constituents have been removed during successive washing stages.

At a later stage, the highly pure DNA is dissolved in water or a low-salt buffer and has an absorbance rate at a wavelength of 260/280 nm ranging from 1.7-1.9.

Thus, it is ready for use in molecular biology applications such as restriction enzyme digestion, PCR, or other applications.

### Kit Components:

1. extraction columns
2. collection tubes
3. concentrated washing buffer 1
4. concentrated washing buffer 2
5. removal buffer
6. protease enzyme K

### Preparation and storage of solutions:

Washing buffer 1 and 2 contain a concentrated buffer that must be diluted before use, using absolute ethanol (>95%) specially for molecular biology by adding the volume

mentioned on the box for each of them. The two resulting solutions are kept at room temperature with tight closures.

Protease K is stored at -20°C

BB buffer must be shaken well before each use because it contains a high percentage of salts and stored at room temperature. In the event of salt deposition as a result of cold weather, the container must be heated at a temperature of 55-65° with continuous stirring until the precipitate is completely dissolved.

## The method of work:

1. **Blood lysis:**
   Add 200 microliters of BB buffer to 200 microliters of blood sample in a centrifugation tube and mix using a shaker, then add 20 microliters of protease K and mix immediately, then incubate at 65°C for 10 minutes.

2. **Addition of ethanol:**
   Add 200 microliters of absolute ethanol and mix immediately until a homogeneous solution is obtained. Mixing must be done immediately after adding absolute ethanol to avoid any precipitation of nucleic acids as a result of the high concentration of ethanol.

3. **Loading to column:**
   Transfer the mixture obtained from the previous operations to the extraction column installed on the collection tube, then close the lid, and centrifuge at 5000 x g for 1 minute, then discard the resulting solution in the collection tube.

4. **The first washing of the column:**
   Wash the column with 500 µL of Wash Buffer 1 and then centrifuge at 5000 x g for 1 min and then discard the resulting solution into the collection tube.

5. **The second washing of the column:**
   Wash the column with 500 µL of Wash Buffer 2, centrifuge at 5000 x g for 1 min, and discard the resulting solution into a collection tube.

Wash the column again with 500 µL of Wash Buffer 2 and then centrifuge at a faster rate of 14,000 x g for 3 min. The goal of repeating the wash and centrifugation very quickly is to get rid of the ethanol completely.

6. **DNA removal:**

Install the extraction column on a clean centrifugation tube and add 100 µL of removal buffer or previously heated sterile water making sure to add directly into the center of the membrane for complete removal of the DNA and leave at room temperature for 2 min then centrifuge at 5000 x g for 1 min to remove the DNA Then discard the extraction column.

The extracted DNA can be used directly or preserved at 4°C or -20°C.

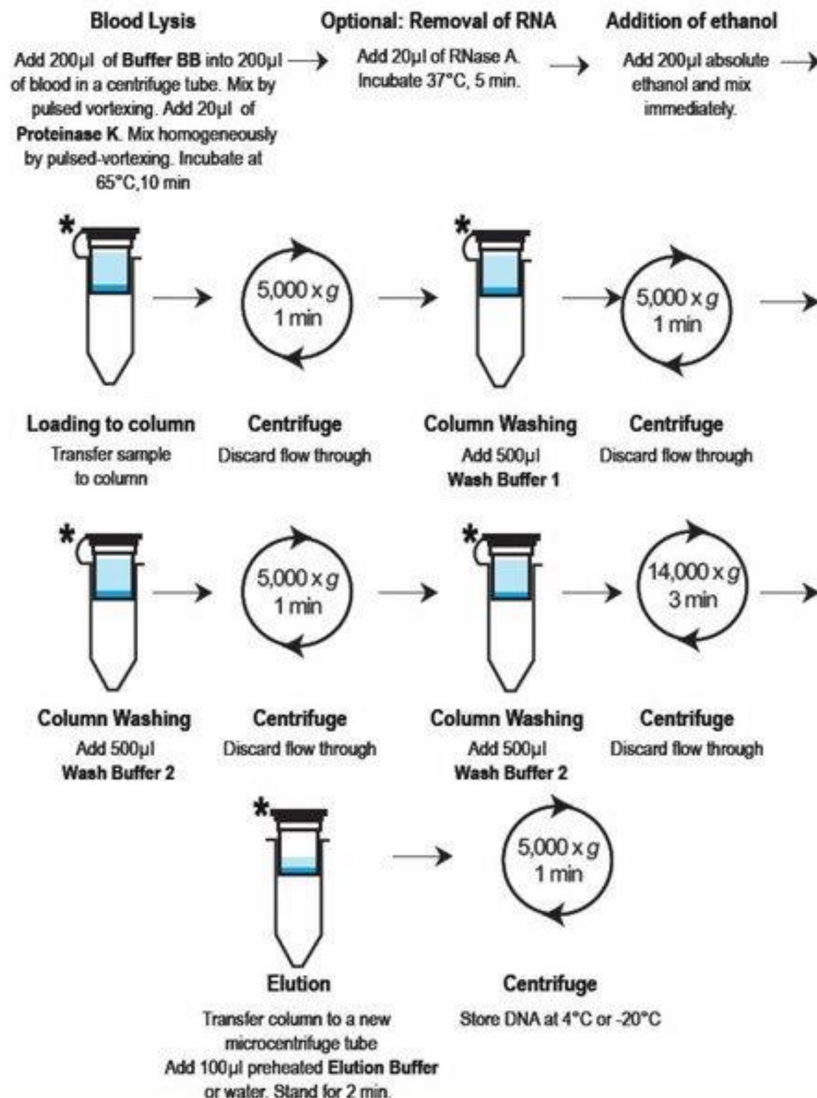



**Fig I:** The method of extracting DNA from a blood sample [158]

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