ANALYSIS OF CYTOCHROME P4501A1 GENETIC POLYMORPHISMS IN PATIENTS WITH ISCHEMIC STROKE

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ABSTRACT

ANALYSIS OF CYTOCHROME P4501A1 GENETIC POLYMORPHISMS IN PATIENTS WITH ISCHEMIC STROKE

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Stroke is the third leading cause of death worldwide and results in serious disabilities. Cytochrome P450 1A1 gene (CYP1A1) is a highly polymorphic gene encoding its corresponding xenobiotic metabolizing enzyme which is responsible from the metabolism of carcinogenic polycyclic aromatic hydrocarbons (PAHs) that are engaged with the formation of free radicals. Atherosclerosis is a major cause of ischemic stroke and this pathology may be associated with the disruption of vascular homeostasis due to the formation of these chemicals. The main objective of this study was to investigate the coding region (A4889G) and non-coding region (T6235C) polymorphisms of the CYP1A1 gene as a risk factor for ischemic stroke.

The study group in Turkish population consisted of 226 unrelated ischemic stroke patients and 113 control subjects. There was no statistically significant difference between the groups with respect to age and gender. Total blood samples were obtained from Gülhane Military Medical Academy Hospital, Neurology Department, Ankara. In stroke patients, hypertension, diabetes mellitus, smoking and obesity were at least 2 times more common and high

density lipoprotein cholesterol (HDL-C) was significantly lower than controls. The frequency of mutant allele 4889G was 0.445 in patients and was nearly the same with controls. The frequency of mutant allele 6235C was 0.151 in patients and was significantly higher in controls (0.226, *P*=0.015). The risk of diabetic, smoker and obese individuals having ischemic stroke was significantly higher in 4889G allele carriers (AG+GG; Odds ratio; OR= 2.1, 2.4 and 3, respectively). The risk of hypertensive and diabetic individuals having ischemic stroke was higher in 6235TT genotypic people (OR= 3 and 2.2, respectively). On the contrary, the risk of smoker and obese individuals having ischemic stroke was significantly higher in 6235 C allele carriers (OR=5.3 and 3.7, respectively).

Logistic regression analysis revealed that hypertension, smoking, levels of low density lipoprotein cholesterol (LDL-C) and HDL-C and 6235C allele were significant predictors of stroke. In this analysis, high level of LDL-C was found to be associated with almost 1.5-fold risk of ischemic stroke. On the other hand, HDL-C and having mutant 6235C allele decreased the risk of ischemic stroke 2.5 and 2-fold, respectively.

This is the first study investigating the relation between A4889G polymorphism and stroke risk. Additionally, in Turkish population A4889G and T6235C polymorphisms were analyzed for the first time in terms of its relation to ischemic stroke. The present study demonstrated that the frequency of mutant 4889G allele was nearly the same in stroke patients and control subjects; whereas the frequency of mutant 6235 C allele was higher in control subjects than in stroke patients. Consequently, we decided that carrying mutant 4889 G allele does not constitute a risk for ischemic stroke and carrying mutant 6235C allele may have a protective effect against stroke.

Keywords: Cytochrome P450 1A1, Ischemic Stroke, Genetic Polymorphism, A4889G, T6235C, Turkish Population.

ÖZ

İSTEMİK İNMELİ HASTALARDA SİTOKROM P4501A1 GENETİK POLİMORFİZMİNİN ANALİZİ

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İnme tüm dünyada üçüncü ölüm sebebidir ve ciddi sakatlıklarla sonuçlanmaktadır. Sitokrom P450 1A1 (CYP1A1) geni, birçok bölgesinde tek nükleotid polimorfizmi (SNP) içermekte olup, zenobiyotik metabolizmasında rol alan CYP1A1 proteinini kodlamaktadır. İlgili protein, sigarada sıklıkla görülen ve kanserojen özelliği olan polisiklik hidrokarbon kimyasallarının ve serbest radikallerin oluşmuşundan sorumludur. Ateroskleroz iskemik inmenin başlıca sebebidir ve bu patoloji damar içi dengenin bozulmasına neden olan bu kimyasallarla ilişkili olabilir. Bu çalışmanın ana amacı CYP1A1 geninin kodlayan bölgesindeki A4889G ve kodlamayan bölgesindeki T6235C polimorfizmlerini iskemik inme için risk faktörü olarak incelemektedir.

Türk popülasyonundaki çalışma grubu, aralarında akrabalık bulunmayan 226 iskemik inme hastası ve 113 kontrolden oluşmuştur. Gruplar arasında yaş ve cinsiyet açısından istatistiksel olarak anlamlı bir fark görülmemiştir. Tam kan örnekleri Ankara Gülhane Askeri Tıp Akademisi Hastanesi Nöroloji Bölümünce temin edilmiştir. Hipertansiyon, diyabet, sigara kullanımı ve obezite, inme hastalarında kontrollerden en az 2 kat fazla ve yüksek dansiteli

lipoprotein kolesterol (HDL-C) hastalarda anlamlı bir biçimde düşük bulunmuştur. 4889G mutant alelinin hastalardaki frekansı 0,445 olup, bu değer kontrollerde neredeyse aynıydı. 6235C mutant alelinin hastalardaki frekansı ise 0,151 olup, bu değer, kontrollerde anlamlı bir biçimde daha fazlaydı (0,226; *P*=0,015). Diyabetik, sigara kullanan ve obez insanlarda iskemik inme riski 4889G aleli taşıyanlarda (eşitsizlik oranı [Odds ratio; OR] sırasıyla= 2,1, 2,4 ve 3) daha yüksekti. Hipertansiyonlu ve diyabetik insanlarda iskemik inme riski 6235TT genotipli bireylerde daha yüksekti (sırasıyla 3 ve 2,2). Buna zıt olarak, sigara kullanan ve obez insanlarda iskemik inme riski 6235 C aleli taşıyanlarda daha yüksekti (sırasıyla 5,3 ve 3,7).

Lojistik regresyon analizi, hipertansiyon, sigara kullanımı, yüksek seviyede düşük dansiteli lipoprotein kolesterol (LDL-C) ve alçak seviyede yüksek dansiteli lipoprotein kolesterolün (HDL-C) inme için önemli tahmin unsurları olduğunu göstermiştir. Bu analize göre, inme riski ile yüksek LDL-C düzeyinin 1,5 kat ilgisi olduğu görülmüştür. Diğer taraftan, HDL-C ve mutant 6235C aleli, iskemik inme riskini sırasıyla 2,5 ve 2 kat azaltmaktadır.

Bu çalışma A4889G polimorfizmi ile inme riski arasındaki ilişkiyi inceleyen ilk çalışmadır. Ayrıca, A4889G ve T6235C polimorfizminin iskemik inme ile ilişkisi Türk popülasyonunda ilk kez çalışılmıştır. Bu çalışma, mutant 4889G alel frekansının inmeli hastalarda ve kontrol grubunda neredeyse aynı olduğunu, mutant 6235C alel frekansının ise kontrol grubunda, inmeli hastalara göre daha büyük olduğunu göstermiştir. Bu sonuçlara göre, mutant 4889 G aleli taşımanın iskemik inme için risk faktörü oluşturmadığına; mutant 6235 C aleli taşımanın ise iskemik inme riskine karşı koruyucu bir etkisi olduğuna karar verilmiştir.

Anahtar Kelimeler: Sitokrom P450 1A1, İskemik İnme, Genetik Polimorfizm, A4889G, T6235C, Türk Popülasyonu.

Dedicated to My Beloved Family,

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CI	Confidence interval
CI	Computed tomography
CAD	Coronary artery disease
CVD	Coronary vascular disease
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
HDL	High density lipoprotein
LDL	Low density lipoprotein
PCR	Polymerase chain reaction
RE	Restriction endonuclease
OR	Odds ratio
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TIA	Transient ischemic attack

CHAPTER 1

INTRODUCTION

1.1. Stroke

A stroke or "brain attack" occurs when a blood clot blocks an artery that carries blood from the heart to the body, or a blood vessel breaks, interrupting blood supply to an area of the brain. The interruption of blood flow or the rupture of blood vessels reduces the delivery of oxygen and nutrients to brain cells which causes serious damage in the brain; as a result, the neurons in the affected area of the brain die (http://www.who.int, last accessed on 23/10/2010).

The effects of a stroke depend on the location of the brain injured, as well as how much damage occurred. The most common symptom of a stroke is sudden weakness or numbness of the face, arm or leg, most often on one side of the body. Other symptoms may differ depending on the area of injury. For instance; while damage in the left or right side of the cerebrum generally results in slow movement, vision problems or trouble on learning and remembering; damage on the brain stem may cause problems in breathing, speaking, body temperature maintenance and loss of balance and coordination. A stroke in the cerebellum causes ataxia, dizziness, headache, nausea and vomiting (http://www.heartandstroke.com, last accessed on 23/10/2010). Figure 1.1 illustrates the parts of the human brain.

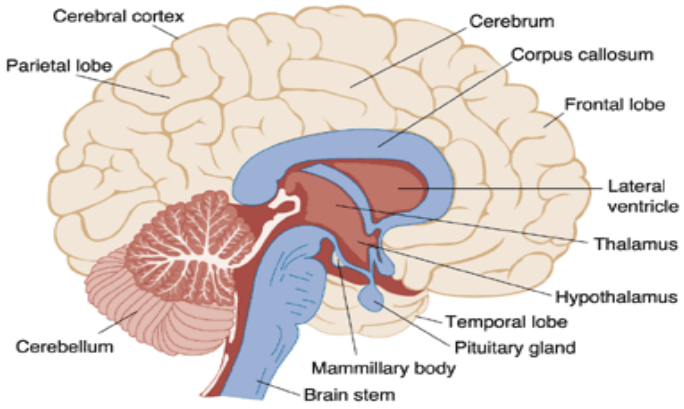

Figure 1.1 Parts of the human brain (taken from http://creationwiki.org, last accessed on 14/01/2011).

According to the 2008 updates of the World Health Organization, after coronary heart disease and other cardiovascular diseases, stroke is the third leading cause of death worldwide. It comprises 9.7% of all deaths leaving behind all kinds of infectious diseases and cancer types. (World Health Organization Fact Sheet 2009; Lloyd-Jones *et al.*, 2010) Each year, 795.000 people experience a new or re-emerging stroke. On average, every 40 seconds, a person experiences stroke in the United States. On average, every 4 minutes, someone dies because of a stroke (Xu *et al.*, 2007). According to the 2010 update of American Heart Association, the estimated direct and indirect cost of stroke for 2010 is $73.7 billion in the United States (Lloyd-Jones *et al.*, 2010).

1.1.1. Classification of Stroke

The pathophysiological symptoms divide stroke into three subtypes namely as ischemic stroke, intracerebral hemorrhage and subarachnoid hemorrhage. In addition, novel imaging techniques for the brain and vascular system has the ability to accurately diagnose phenotype or subtype stroke.

Of all strokes types, 80% are ischemic, 15% are intracerebral hemorrhage, and 5% are subarachnoid hemorrhage strokes among the white population (Warlow *et al.*, 2003). According to a study on Turkish populations, frequency of those stroke types is as follows: ischemic stroke 77%, primary intracerebral hemorrhage 19% and subarachnoid hemorrhage 4% (Kumral *et al.,* 1998).

1.1.1.1. Ischemic Stroke

Ischemic stroke is the most common stroke type. It happens as a result of an occlusion in an artery in the neck or in the brain that leaves a part of the brain deprived of its nutrients, glucose and oxygen (Silverman *et al.,* 2009). The occlusion of the artery may be because of a blood clot that interrupts the blood flow to the brain (Figure 1.2).

Depending on the mechanism, an ischemic stroke can be classified as either "thrombotic" or "embolic". Thrombotic strokes are caused by a thrombus (a blood clot) that clogs an artery directly leading to the brain, which causes narrowing of the artery. As a result the blood flow through the artery to the brain slows down. Without an adequate blood supply, brain cells in that area of the brain stop functioning and can die quickly. The blood clot that triggers a thrombotic stroke usually forms inside an artery that already has been narrowed by atherosclerosis, a condition in which fatty deposits (plaques) build up inside blood vessels. This kind of narrowing is called carotid stenosis (http://www.heartandstroke.com, last accessed on 18/11/2010).

Embolic strokes occur when a clot forms somewhere else in the body, for instance in the heart, and travels through the blood stream to the brain. Roughly 15% of embolic strokes occur in patients with atrial fibrillation which is a kind of abnormal heart rhythm in the upper chambers of the heart (http://www.stroke.org, last accessed on 15/12/2010).

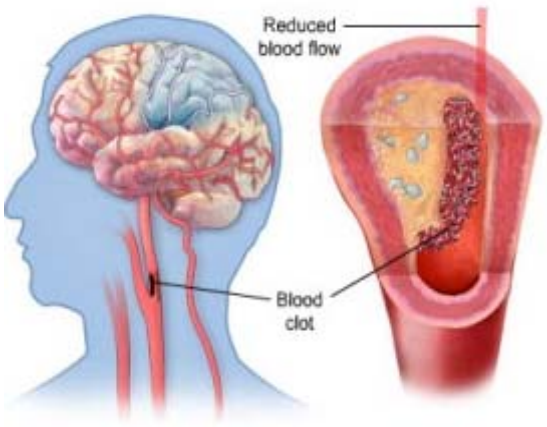

Figure 1.2 Ischemic stroke (taken from http://www.strokeassociation.org, last accessed on 15/12/2010).

The most important contributor of the ischemic stroke is the formation of a plaque around the inner wall of the arteries. This plaque is composed of fat, cholesterol, calcium and other substances found in the blood. Over time, plaque sticks to the artery and causes the narrowing and hardening of it which is called atherosclerosis (http://www.nhlbi.nih.gov, last accessed on 28/10/2010).

When stroke symptoms resolve and do not cause permanent brain damage, they are called a transient ischemic attack (TIA) (Figure 1.3). TIA is a focal neurological problem that develops suddenly and lasts for less than 24 hours. It generally has a vascular origin, and is confined to an area of the brain or eye by a specific artery (Gregory *et al.,* 2002). The duration of most TIAs is between 5 and 30 minutes. TIAs may be referred as 'mini-strokes,' but a TIA is actually a serious warning that a stroke may occur very soon (Johnston, 2002).

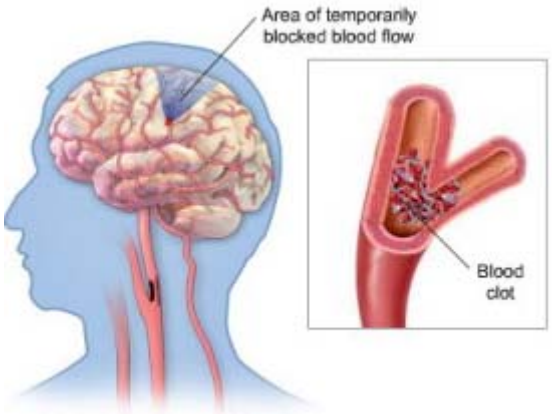

Figure 1.3 Transient Ischemic Attack (TIA) (taken from http://www.strokeassociation.org, last accessed on 15/12/2010).

1.1.1.2. Hemorrhagic Stroke

Hemorrhagic stroke occurs when a weakened blood vessel in the brain ruptures and starts bleeding (Figure 1.4). Leaking blood accumulates and compresses the surrounding brain tissue. Brain hemorrhages can result from a number of conditions that affect the blood vessels like hypertension and weak regions in the blood vessel walls (aneurysms). A less common cause of hemorrhage is the rupture of an arteriovenous malformation (AVM) which is an abnormal tangle of thin-walled blood vessels. There are two types of hemorrhagic strokes:

- Intracerebral hemorrhage: a blood vessel in the brain bursts and spills into the surrounding brain tissue, damaging cells. Brain cells beyond the leak are deprived of blood and are also damaged. High blood pressure is the most common cause of this type of hemorrhagic stroke.
- Subarachnoid hemorrhage: bleeding starts in an artery on or near the surface of the brain and spills into the space between the surface of the brain and skull. This bleeding is often signaled by a sudden, severe headache.

(http://www.mayoclinic.com, http://www.strokeassociation.org, last accessed on 23/10/2010).

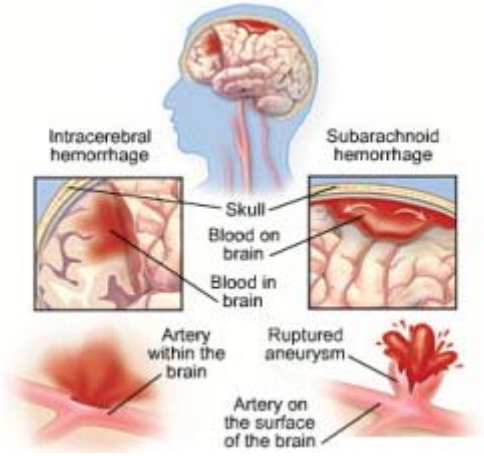

Figure 1.4 Hemorrhagic Stroke (taken from http://www.strokeassociation.org, last accessed on 15/12/2010).

1.1.2. Risk Factors of Stroke

It is possible to classify the risk factors of stroke in two groups. Modifiable risk factors constitute the personal lifestyle choices of a person whereas, nonmodifiable risk factors are the ones that are not related to the lifestyle but predetermined like heredity or natural processes like aging.

In a study done by Wolf *et al.* (1991), 5070 participants were examined after 34 years follow-up in order to determine the impact of nonrheumatic atrial fibrillation, hypertension, coronary heart disease, and cardiac failure on stroke incidence. Compared with the symptom free participants, the age-adjusted incidence of stroke was more than two-fold in the presence of coronary heart disease and more than three-fold in the presence of hypertension. There was

about four-fold risk of stroke in participants with cardiac failure and a near five-fold risk when atrial fibrillation was present (Wolf *et al.,* 1991). According to the study of Kumral and collagues (1998), in Turkish population, the major risk factor of ischemic stroke was found to be hypertension (63%) which was followed by hypercholesterolemia (37%), diabetes mellitus (35%), ischemic heart disease (23%), atrial fibrillation (20%) and smoking (17%).

1.1.2.1. Nonmodifiable Risk Factors

Although stroke can also affect children and young adults, it is mainly a disease of older people. There is an increase in incidence with age, with 75% of all first strokes occurring after the age of 65 years in white populations (Sudlow *et al.,* 1997; Feigin *et al.,* 2003). The incidence of stroke approximately doubles with each successive decade over the age of 55 years (Wolfe, 2000). According to the estimations of Rothwell (2001), by 2020 stroke mortality will almost double due to the increase in the proportion of older people and due to the effects of current smoking patterns.

The incidence rates of stroke differ among populations reflecting the affect of ethnicity on stroke risk (Sacco *et al.,* 1999). Black people have a higher stroke rate than white people, and the rate for Hispanic people is between that of white and black people. There is a high incidence of hemorrhagic stroke in Asian people. Men have a higher incidence of stroke when compared to women but the mortality rate is higher in women (Warlow *et al.,* 2003; United Nations Chronicle Health Watch 2005). Stroke history is also a nonmodifiable risk factor.

1.1.2.2. Modifiable Risk Factors

Based on the stroke risk and prevalence, high blood pressure is the strongest independent risk factor for both ischemic and hemorrhagic stroke which is

followed by tobacco use (Collins and MacMahon 1994). Cardiac morbidity, cigarette smoking, diabetes and waist-to-hip ratio are strongly associated with stroke risk. High blood cholesterol profile and homocysteine levels also increase stroke risk.

Regular physical activity is negatively associated with the risk of stroke. 1–30 servings of alcoholic beverages per month associate with a reduced risk of ischemic stroke, whereas consumption of more than 30 per month increases the risk of both types of stroke (Sacco *et al.,* 1999; O'Donnell *et al.,* 2010).

1.1.2.3. Potential Risk Factors

Stroke is associated with the increased levels of plasma fibrinogen which is partly confounded by smoking. Fibrinogen is also affected by obesity, exercise, alcohol, diabetes, psychosocial factors, inflammation and infection. Due to these dependencies, it is not certain to what extent plasma fibrinogen is the causal factor or an indicator of other risk factors (Sacco, 2005).

A number of studies have investigated the relationship between migraine and stroke risk. These studies have tended to show a positive association with stroke (Tietjen, 2000; Tzourio *et al*., 2000). Oral contraceptive use and drug abuse are other potential risk factors associated with stroke (Sacco, 2005).

1.1.3. Atherosclerosis

Atherosclerosis, the most common cause of stroke, is believed to be a disease of chronic inflammation that specifically affects the medium and large arteries. It occurs as a result building up hard structures called plaques in the inner walls of arteries. Plaques are generally composed of fat, cholesterol, and other substances circulating in the blood stream. The accumulation of these plaques

can eventually cause the narrowing of the artery making it less flexible and brittle (http://www.nlm.nih.gov, last accessed on 24/12/2010).

The formation of atherosclerotic plaques is a cascade of events starting with the oxidation of low-density lipoprotein molecules (LDL) by free radicals, particularly reactive oxygen species (ROS). When oxidized LDL contacts with an artery wall, a series of reactions are triggered to repair the damage of oxidized LDL on the arterial wall. Figure 1.5 represents the formation of an arterial plaque in a diseased artery and its comparison with a healthy artery.

The body's immune system responds to the damage by transporting macrophages and T-lymphocytes to the damaged area in order to clear the oxidized-LDL by forming foam cells in the artery wall. Since these immune cells are not capable of processing the oxidized-LDL, they grow and rupture in the arterial wall causing the deposition of a greater amount of oxidized cholesterol. This triggers more macrophages starting the cycle again. Eventually, the artery becomes inflamed. The cholesterol plaque causes the muscle cells to enlarge and form a hard cover over the affected area. This hard cover causes a narrowing of the artery, which reduces the blood flow and increases blood pressure (http://en.wikipedia.org, last accessed on 24/12/2010). These complications of advanced atherosclerosis are chronic, show a slow progress but eventually lead to death of the tissues fed by the artery (Glagov *et al.*, 1987).

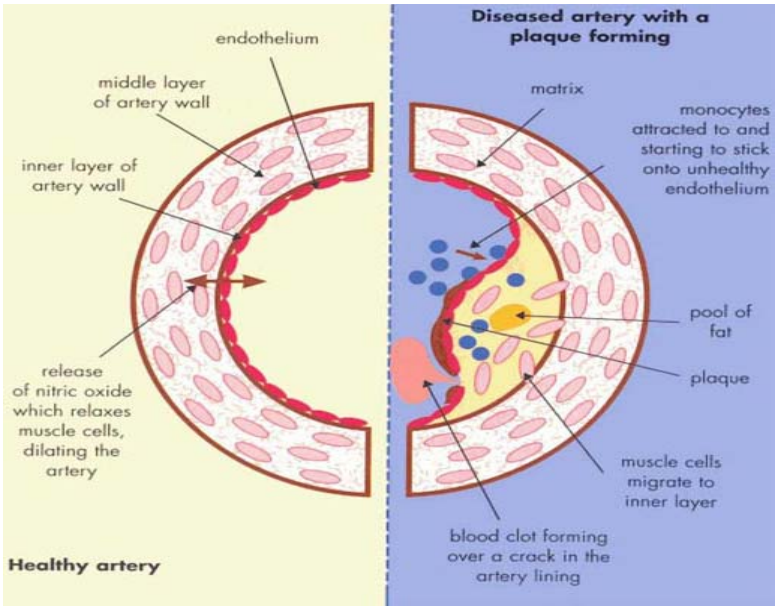

Figure 1.5 The comparison of a healthy artery with a diseased artery (taken from http://www.drugdevelopment-technology.com, last accessed on 25/12/2010).

The intima-media thickness (IMT) of the carotid arteries is a measure of preclinical atherosclerosis. In a study of Chambless *et al.,* (2000), it has been found that having a mean IMT of 0.8-1.0 mm was associated with at least two-fold risk of subsequent ischemic stroke, and having a mean IMT above 1.0 mm was associated with almost four fold increased risk (Chambless *et al.,* 2000).

1.1.4. Prevention of Stroke

Despite the exploitation of novel methods in stroke management and improvement of post stroke recovery, prevention remains the cornerstone of therapy for all devastating outcomes of the disease. Based on a patient's risk assessment profile (Wolf *et al.,* 1991).

It has been confirmed that approximately 30% to 40% stroke risk reduction is possible with blood pressure lowering (Yusuf *et. al.,* 2000; Lawes *et al.,* 2004). Random trials clearly proved that both β-blocker therapy and treatment with high-dose diuretics are effective medications for the prevention of stroke (Psaty *et al.,* 1997).

Tobacco smoke is also a cause for some stroke types. After adjusting for potential confounders (age, sex, history of hypertension, heart disease, and diabetes), Bonita and colleagues (Bonita *et al.,* 1999) found an almost two-fold increase in the risk of stroke among smokers. Wannamethee *et al.* (1995) concluded that quitting smoke provides a considerable and rapid benefit in decreased risk of stroke.

According to a study done by Burchfiel *et al.,* (1994), diabetics have a two to five-fold relative risk of stroke. Glucose intolerance doubles the risk of brain infarction. So, appropriate management of diabetes and glucose intolerance together with other risk factors like hypertension decreases the risk of stroke (Kannel *et al.,* 1979). Since atrial fibrillation is an important risk factor for stroke, antithrombotic therapy with warfarin or aspirin can be considered for patients with nonvalvular atrial fibrillation (Laupacis *et al.,* 1998).

Recent studies clarified the relationship between lipids and stroke and showed that the risk of stroke and degree of carotid atheroma can be reduced with cholesterol-lowering medications. The benefits of statin agents in stroke prevention in patients with coronary heart disease have been supported by several meta-analyses (Blauw et al. 1997; Bucher *et. al.,* 1999). Despite novel management strategies for struggling stroke, significant gaps still exist in current knowledge of the impact of specific factors on stroke risk and it is only possible to reduce the burden of stroke by better identifying and managing the risk factors (Goldstein *et al.,* 2001).

1.2. Xenobiotic Metabolism and Xenobiotic Metabolizing Enzymes

The word “xenobiotic” originates from the Greek words “xenos” meaning “stranger” and “biotic” meaning "related to living beings". Xenobiotics are chemical substances taken from outside like drugs or poisons which are foreign to an organism's normal biochemistry. Xenobiotic metabolism is a series of metabolic reaction pathways which modifies the chemical structure of xenobiotics. These metabolic reaction pathways are a type of biotransformation which is present in all major groups of organisms. They generally have the aim to detoxify poisonous compounds; but in some cases, the intermediates of the xenobiotic metabolism can themselves be the main cause of toxic effects. Xenobiotic metabolism is divided into three phases which are also of vital importance for medicine since it is a part of drug metabolism (http://en.wikipedia.org, last accessed on 23/12/2010):

- Phase I reactions – modification: In phase I reactions, a series of enzymes add reactive and polar groups into their substrates. One of the most common modifications is hydroxylation reactions that are catalysed by the cytochrome P-450-dependent mixed-function oxidase system. These enzyme complexes act to incorporate an atom of oxygen into nonactivated hydrocarbons, which can result in either the introduction of hydroxyl groups or N-, O- and S-dealkylation of substrates (Guengerich, 2001). The reaction mechanism continues with the reduction of cytochrome-bound oxygen and the generation of a highly-reactive oxyferryl species, according to the following scheme (Schlichting *et al.,* 2000):

$$NADPH + RH \longrightarrow NADP^{+} + H_2O + ROH \qquad (1.1)$$

In addition to cytochrome P450 dependent phase I oxidation system, flavin monooxygenases (FMO) also catalyze the NADPH- and oxygen-dependent oxidation of a wide range of nucleophilic nitrogen-, sulfur-, phosphorus-, and

selenium containing chemicals, drugs, and agricultural agents (Adali *et al.,* 1998; Can Demirdogen and Adali, 2005; Aktas *et al.,* 2009)

- Phase II – conjugation: In phase II reactions, activated xenobiotic metabolites are conjugated with charged species such as glutathione (GSH), sulfate, glycine, or glucuronic acid. These reactions are catalyzed by a large group of broad-specificity transferases, which can metabolize almost any hydrophobic compound that contains nucleophilic or electrophilic groups (Jakoby *et al.,* 1990). One of the most important of these groups is the glutathione S-transferases (GSTs). The addition of large anionic groups (such as GSH) detoxifies reactive electrophiles and produces more polar metabolites that cannot diffuse across membranes, and may, therefore, be actively transported.
- Phase III - further modification and excretion: After phase II reactions, the xenobiotic conjugates may be further metabolized. Conjugates and their metabolites can be excreted from cells in phase III of their metabolism, with the anionic groups (Boyland *et al.,* 1969).

Xenobiotic metabolizing enzymes share many common characteristics: (1) they prefer lipophilic compounds; (2) each of them have a broad substrate range; and (3) they are either relatively more concentrated at major points of the body like liver, lung, kidney and intestinal mucosa or they are specific to some organs like the choroid plexus of the brain. A variety of these enzymes known are to be inducible (Arinc *et al.,* 2000a, b, Arslan *et al.,* 2005), in addition, body's response to xenobiotic exposure can trigger the production of more enzymes (Jakoby *et al.,* 1990). Genetic polymorphisms of xenobiotic-metabolizing enzymes (XMEs) have been the subject of molecular-epidemiological studies since they may affect an individual's susceptibility to numerous diseases (Gonzalez *et al.,* 1994; Daly, 1995; Ulusoy *et al.,* 2007, Demirdogen *et al.,* 2008, Turkanoglu *et al.,* 2010).

Cytochrome P450s are the most important components of xenobiotic metabolizing system, as most of the drugs or xenobiotics first encounter with these enzymes in the liver.

1.2.1. Cytochrome P450s

Cytochrome P450s are a super-family of oxygen-reacting hemeproteins which are unique with their remarkable functions and widespread distribution in biological systems. The main function of P450s is to activate the molecular oxygen for the oxidative metabolism of lipophilic organic chemicals. In eukaryotic cells each P450 is composed of roughly 500 amino acids with iron-protoporphoryrin IX as the prosthetic group. These membrane-bound hemeproteins are members of the Cytochrome P450 super-family which contains 58 distinct members in humans (http://drnelson.uthsc.edu, last accessed on 20/01/2011).

The cytochrome P450s were first recognized in 1958 by M. Klingenberg (Klingenberg, 1958) who was studying the spectrophotometric properties of pigments in a microsomal fraction prepared from rat livers. Cytochrome P450 was then identified as a hemeprotein and the name "cytochrome P450" stemmed from the spectrophotometric observation that the reduced state of P450 has an absorption band with maxima at 450 nm after binding to carbon monoxide (Omura and Sato, 1964). This finding was followed by the demonstration of the role of P450 in adrenal cortex in the 21-hydroxylation of progesterone (Estabrook *et al.,* 1963). In 1965, Cooper *et al.* provided evidence that P450s have a pivotal role in the metabolism of drugs and other xenobiotics (Cooper *et al.,* 1965). As shown for the first time by Lu and Coon (1968), in the microsomal system, cytochrome P450 monooxygenase system has three components:

- Cytochrome P450, which catalyzes the monooxygenation reaction;

- FAD and FMN containing NADPH dependent cytochrome P450 reductase, which catalyzes the electron transfer from NADPH to cytochrome P450;
- Lipid which is proposed to facilitate the transfer of electrons from NADPH-cytochrome P450 reductase to cytochrome P450 (Lu and Levin, 1974).

NADPH-cytochrome P450 reductase, cytochrome P450 and lipid are required to reconstitute the full hydroxylation activity (Lu and Coon, 1968; Adali and Arinc, 1990, Adali *et al.,* 1996). The continuous production of reactive oxygen species is considered as the most important result of NADPH consumption by microsomal monooxygenases. This indicates that these enzymes significantly contribute to the cellular production of oxygen-derived free radicals (Davydov, 2001). A schematic representation of the functions of Cytochrome P450 enzymes is provided in Figure 1.6.

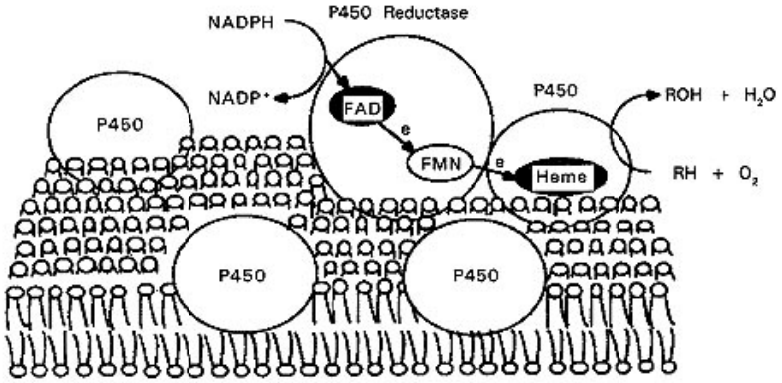

Figure 1.6 The reactions catalysed by the Cytochrome P450 enzymes. As can be seen these enzymes are embedded into the lipid bilayer (Ohkawa *et al.,* 1998).

Cytochrome P450s (CYPs) have been characterized in many species of organisms, including bacteria, fungi, yeast, plants, fish, birds, reptiles, insects and mammalian systems (Lu and Levin, 1974; Arinc and Adali, 1983). Up to date, a total of 11.294 P450s were identified from various organisms (http://drnelson.uthsc.edu, last accessed on 20/01/2011).

CYPs are grouped into families, subfamilies, and individual isozymes based on the similarities in their amino acid sequence. According to this classification system, if two P450 proteins show more than 40% amino acid sequence similarity, then they are classified in the same family. If the percentage of similarity is more than 55%, then they are grouped in the same subfamily (Nebert *et al.,* 1994; Nelson *et al.,* 1996). As of January 24, 2007 18 families with a total number of 58 P450 genes were determined in humans (http://drnelson.uthsc.edu, last accessed on 20/01/2011). The first 3 human P450 families namely CYP1, CYP2 and CYP3 have been identified as the major contributors to phase I metabolism (Hasler *et al.,* 1999; Elbekai *et al.,* 2006). Human families of CYP450s and their functions are given in Table 1.1.

Table 1.1 Members of CYP450 family in humans and the reactions they catalyze (http://en.wikipedia.org, last accessed on 21/01/2011).

Family	Function
CYP1	Drug and steroid (especially estrogen) metabolism
CYP2	Drug and steroid metabolism
CYP3	Drug and steroid (including testosterone) metabolism
CYP4	Arachidonic acid or fatty acid metabolism
CYP5	Thromboxane A_2 synthase activity
CYP7	Bile acid biosynthesis
CYP8	Bile acid biosynthesis
CYP11	Steroid biosynthesis
CYP17	Steroid biosynthesis and 17-alpha hydroxylase
CYP19	Steroid biosynthesis and aromatase activity
CYP20	Unknown function
CYP21	Steroid biosynthesis
CYP24	Vitamin D degradation
CYP26	Retinoic acid hydroxylase activity
CYP27	Bile acid biosynthesis and vitamin D_3 activation
CYP39	7-alpha hydroxylation of 24-hydroxycholesterol
CYP46	Cholesterol 24-hydroxylase
CYP51	Cholesterol biosynthesis

In humans, most CYPs are primarily expressed in the liver, with significantly lower levels of expression in extrahepatic tissues. There are also some CYPs predominantly detected in the heart, vasculature, gastrointestinal tract, kidney and lung (Oyekan *et al.,* 1999; Scarborough *et al.,* 1999); and data have suggested that specific CYPs found in the vascular smooth muscle and endothelium play important roles in the regulation of vascular tone and

homeostasis (Lin *et al.,* 1996). The properties of human P450s are summarized below:

- P450 proteins are composed of approximately 500 amino acids. A cysteine located near the carboxy-terminus of the protein provides the essential thiol-ligand for the heme iron.
- The P450s are responsible for the NADPH and oxygen dependent oxidative transformation of a variety of chemical compounds. Some types of P450 can have a limited and specific substrate acceptance while other P450s can have a broad substrate specificity suggesting a role for a unique "active site geometry" for a P450.
- In some tissues like liver, intestine and the cortex of the adrenal gland, P450s have the highest concentration among other hemeproteins.
- P450s are distributed in almost every organ of the human body although the type of P450 in a tissue appears to be specific.
- The cellular expression of many P450s is regulated by transcription factors which become activated during exposure to various chemicals. (Hasler *et al.,* 1999).

Finalization of human genome sequence provided the evidence that there are 58 different active genes in humans encoding P450 enzymes (http://drnelson.utmem.edu, last accessed on 20/01/2011). It is important to note that all genes encoding P450 enzymes in CYP1, CYP2 and CYP3 families are polymorphic. The functional importance of these variant differs and the frequencies of their distribution in different ethnic groups differ (Ingelman-Sundberg, 2004).

1.2.1.1. Functions of Cytochrome P450s

P450's are of crucial importance in cellular metabolism and the maintenance of cellular homeostasis (Hasler *et al.,* 1999). These enzymes have the ability to oxidize, peroxidize and/or reduce various substances and drugs in an oxygen and NADPH-dependent manner (Capdevila *et al.,* 1981; Schwartzman *et al.,* 1985).

It has been estimated that there may be more than 200,000 substrates that are specific to P450 enzyme system which include the drugs, xenobiotics, saturated and unsaturated fatty acids, eicosanoids, sterols and steroids, bile acids, vitamin D3 derivatives, retinoids, and uroporphyrinogens. In addition, some specific cytochrome P450 enzymes can detoxify secondary metabolites, environmental pollutants, herbicides and pesticides (Gonzalez, 1989).

In humans the P450s play critical roles by catalyzing reactions functional in:

- the biosynthesis of steroid hormones;
- the metabolism of xenobiotics to reactive metabolites called free radicals that interact with cellular macromolecules (DNA, RNA, proteins);
- the stereo- and regio-specific metabolism of fat-soluble vitamins;
- conversion of cholesterol to androgens, estrogens, glucocorticoids and mineralocorticoids;
- the synthesis and degradation of prostaglandins and other unsaturated fatty acids;
- the metabolism of cholesterol to bile acids (Hasler *et al.,* 1999).

1.2.2. Cytochrome P450 1A1 (CYP1A1)

The cytochrome P450 1A1 gene (CYP1A1 gene) is part of a supergene family which is located on the long arm of chromosome 15 (15q22-q24) in human. It is composed of six introns and seven exons with a length of 5810 bp (Drakoulis

et al., 1994). Its corresponding phase I enzyme CYP1A1 is one of the three members of the CYP1 family, which is present mainly in extrahepatic tissues. This enzyme which consists of 512 amino acid residues (Jaiswal *et al.,* 1985) is responsible for the metabolism of a vast number of xenobiotics, as well as a small number of endogenous substrates. It acts as the main enzyme in the bioactivation of polycyclic aromatic hydrocarbons (PAH) such as benzo[a]pyrene (Guengerich *et al.,* 1991) which are considered as important carcinogens because of their DNA-binding, mutagenic and carcinogenic properties (Shimada *et al.,* 1992). CYP1A1 hydroxylates the aromatic ring and this step is the initiation of carcinogenesis, since highly reactive conversion products are formed (Daly, 1995; Androutsopoulos *et al.,* 2009).

Polycyclic aromatic hydrocarbons (PAHs) which consist of merged aromatic rings are potent atmospheric pollutants (Yang *et al.,* 2002). Toxic effects of PAHs are structure dependent and can vary between isomers. One of the PAH compounds, benzo[a]pyrene, is the first chemical carcinogen discovered and it is an important constituent of cigarette smoke. Benzo[a]pyrene, whose metabolites are mutagenic and highly carcinogenic, has a five-ring structure. There are a vast number of studies putting forward a relationship between benzo[a]pyrene and cancers, especially with lung cancer due to tobacco smoking. Benzo[a]pyrene was proved to cause genetic damage in lung cells which is identical to the damage observed in the DNA of most malignant lung tumors (Kawai *et al.,* 2005).

In contrast, a recent study done by Uno *et al.* (2004) has found that CYP1A1 and CYP1B1 are both protective and necessary for preventing benzo[a]pyrene toxicity. Experiments done on CYP1A1 and CYP1B1 knockout mice showed that CYP1A1 primarily acts to protect mammals from low doses of benzo[a]pyrene and when this protection is removed large concentrations of benzo[a]pyrene was observed in blood (Korytina *et al.,* 2003; http://en.wikipedia.org, last accessed on 19/12/2010).

1.2.2.1. Functions of CYP1A1

CYP1A1 is responsible for the metabolism of PAH carcinogens into epoxide intermediates, which are further activated to diol epoxides by the enzyme epoxide hydrolase. The widely accepted demonstration of this process is the activation of benzo[*a*]pyrene, B[*a*]P. The metabolism of this substrate was extensively studied in the mid-1970s in humans (Conney, 1982). B[*a*]P-4,5-epoxide was initially thought to be the ultimate carcinogenic metabolite but following investigations showed that B[*a*]P-7,8-diol-9,10-epoxides were indeed the ultimate carcinogens since they were highly reactive towards DNA. The metabolic activation involves the oxidation of B[*a*]P to B[*a*]P-7,8- oxide by CYP1A1 and subsequent hydrolysis to B[*a*]P-7,8-diol and the two enantiomers (+)-B[*a*]P-7,8-diol and (-)-B[*a*]P-7,8- diol by epoxide hydrolase. A final oxidation of each of these metabolites produces mutagenic diol epoxides which can result in mutations in specific parts of the DNA (Buterin *et al.,* 2000; Shimada *et al.,* 2004). A schematic representation of the chemical reaction is given in Figure 1.7.

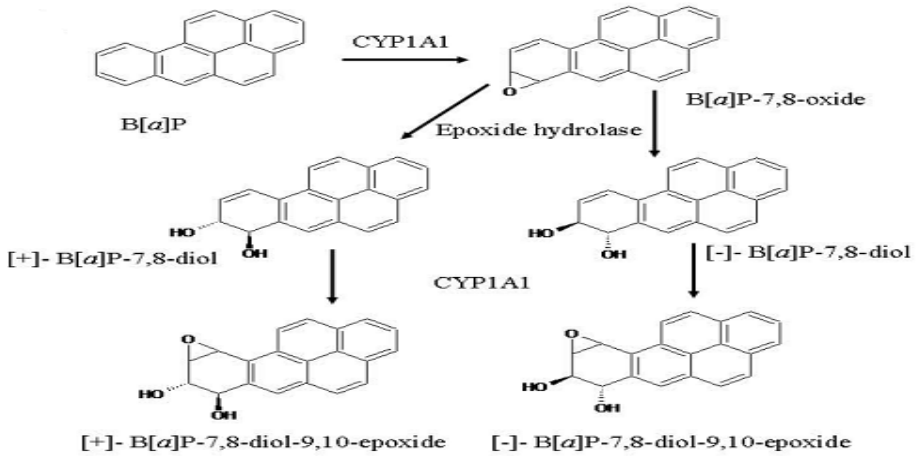

Figure 1.7 The metabolic activation of benzo[a]pyrene. The oxidation of benzo[a]pyrene by CYP1A1 is followed by the hydrolysis of B[a]P-7,8-oxide to (+)-B[a]P-7,8-diol and (-)-B[a]P-7,8-diol. These metabolites are further oxidized to mutagenic diol epoxides (Androutsopoulos et al., 2009).

1.2.2.2. Substrates of CYP1A1

In addition to benzo[*a*]pyrene which is the most common substrate of CYP1A1, benz[*a*]anthracene, benzo[*b*]fluoranthrene, benzo[*c*]phenanthrene, chrysene, benzo[*g*]chrysene and 5,6-dimethylchrysene are other types of PAHs that act as substrates of CYP1A1. The metabolic activation of heterocyclic amines is catalyzed by CYP1A1 and this enzyme has been shown to be involved in the activation of tobacco-related N-nitrosamines, such as Nicotine-derived nitrosamine ketone (NNK) along with CYP1A2 and CYP2A6 (Cheung *et al.,* 2001). Substrates, inducers and inhibitors of CYP1A1 are given in Table 1.2.

Table 1.2 Substrates, inducers and inhibitors of CYP1A1.

Substrates	**Inhibitors**	**Inducers**
Polycyclic Aromatic Hydrocarbons (PAHs) (benzo[*a*]pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthrene, benzo[*c*]phenanthrene, benzo[*g*]chrysene, chrysene and 5,6-dimethylchrysene) 3-methylcholanthrene Heterocyclic amines N-nitrosamines (Nicotine-derived nitrosamine ketone) Phytochemicals belonging to flavonoid subclass (Eupatorin, diosmetin)	hydroxylated flavone derivatives (3-hydroxy-, 5-hydroxy-, 7-hydroxy-, and 3,7-dihydroxyflavone) 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) Ellipticine Alpha-naphthoflavone (alpha NF) Resveratrol 17-allylamino-demethoxygeldanamycin, (17-AAG) Celastrol	Polycyclic Aromatic Hydrocarbons (PAHs) (benzo[*a*]pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthrene, benzo[*c*]phenanthrene, benzo[*g*]chrysene, chrysene and 5,6-dimethylchrysene) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin Omeprazole

A number of phytochemicals belonging to the flavonoid subclass have shown to be CYP1A1 substrates. Eupatorin is another flavonoid which is converted to the structurally similar flavone cirsiliol, by an aromatic demethylation reaction catalyzed by the enzymes CYP1A1 and CYP1B1 (Atherton *et al.,* 2006; Androutsopoulos *et al.,* 2008). Similarly, the flavone diosmetin, present in olive leaves, is activated to the flavone luteolin, mainly by CYP1A1.

1.2.2.3. Induction of CYP1A1

CYP1A1 is induced by 3-methylcholanthrene which is another carcinogenic PAH also present in tobacco smoke. Omeprazole, a benzimidazole derivative, is another inducer of CYP1A1. It is a potent suppressor of gastric acid secretion and it has been used for treating gastro-oesophageal reflux disease and duodenal ulcers (Diaz *et al.,* 1990; Ko *et al.,* 1997).

The transcriptional activation of the *CYP1A1* gene is mediated by the binding of CYP1A1 substrates to the specific cytosolic receptor called Aryl hydrocarbon receptor (AhR). Discovery of cytochrome P4501A1 induction is originated from the observation that PAHs have the ability to induce their own metabolism. Studies demonstrated that induction mechanism involves a novel ligand-dependent mechanism for the regulation of transcription (Whitlock, 1999).

Figure 1.8 represents a schematic overview of pathways of CYP1A1 induction. AhR exists as part of a cytosolic protein complex, which consists of two heat-shock proteins (Hsp-90), an Hsp-90-interacting co-chaperone p23 and an immunophillin- like protein called AIP (Puga *et al.,* 2009). When there is an exogenous ligand such as B[*a*]P or the industrial by product 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the extracellular medium, this receptor moves to the nucleus, and it heterodimerizes with another protein called aryl hydrocarbon nuclear translocator or ARNT. This heterodimer binds to

regulatory sequences such as AhREs (Aryl hydrocarbon response elements); XREs (Xenobiotic response elements) or DREs (Dioxin response elements), which are located in the promoter region of CYP1A1 and CYP1A2. This binding initiates their transcription by recruiting RNA polymerase II (Puga *et al.*, 2009).

AhR-related factor which is also called Aryl hydrocarbon receptor repressor or AhRR acts as an inhibitor that stops CYP1A1 transcription. It is located in the nucleus in the form of a dimeric protein together with ARNT. The AhRR/ARNT heterodimer acts as a repressor both by stopping transcription initiated at the XREs and by competing with AhR for heterodimer formation with ARNT (Okino *et al.*, 2007).

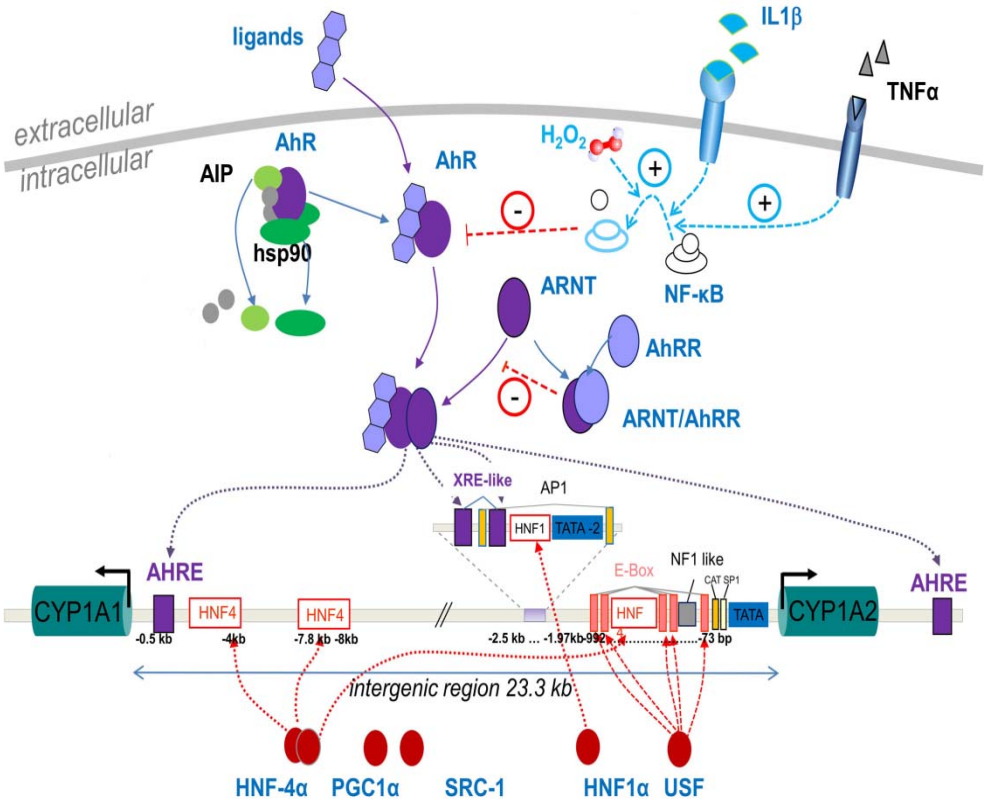

Figure 1.8 Overview of pathways for transcriptional regulation at the CYP1A1 promoter. The scheme shows the 23.3-kb intergenic region between the head-to-head oriented *CYP1A1* and *1A2* genes on chromosome 15q24.1. Binding elements located within the common promoter region may be shared for transcriptional regulation of both genes. Genes selected for the AhR-pathway include the ligand-binding receptor AhR, the AhR nuclear translocator ARNT, and the AhR regulator AhRR. The activated ligand bound form of AhR is complexed with two heat-shock proteins hsp90 and AIP, and co-chaperone p23, which help to correctly fold and stabilize the AhR and prevent inappropriate trafficking to the nucleus. Upon heterodimerization with activated AhR, the ARNT/AhR complex translocates to the nucleus to activate transcription from AHRE- and XRE-motifs. AhRR competes with AhR for ARNT binding, resulting in inhibition of AhR-mediated signal transduction. Under pathophysiological conditions of inflammation, inflammatory cytokines like IL1ß and TNFα or chronic oxidative stress (H_2O_2) activate NFκB which in turn inhibits AhR activity thus leading to reduced CYP1A expression (Klein *et al.*, 2010).

Under pathophysiological conditions of inflammation or chronic oxidative stress (H_2O_2), inflammatory cytokines like IL1ß and TNFα activate NFκB which in turn inhibits AhR activity thus leading to reduced CYP1A1 expression (Tian *et al.,* 1999; Zhou *et al.,* 2008). Figure 1.8 represents the transcriptional regulation pathway of CYP1A1 gene promoter.

Despite the fact that AhR plays an important role in the xenobiotic-activating pathway, this receptor was recently shown to have vital functions in developmental and cell-regulatory processes which include the regulation of cell growth, apoptosis, hypoxia signaling, cell adhesion and matrix metabolism. As a result the exact function of CYP1A1 appears to be a lot more complex than just toxicological roles. Recent *in vivo* investigations suggest that CYP1A1 may function as a carcinogen-detoxification enzyme. In addition, its chemo preventative activity provides further insight into the cancer protecting role of this enzyme (Puga *et al.,* 2009; Kung *et al.,* 2009).

1.2.2.4. Inhibition of CYP1A1

The inhibition of the CYP1A1 generally occurs by the substances that selectively inhibit the Ah receptor. Most effective inhibitors of the CYP1A1 transcription are the alpha-naphthoflavone (alpha NF) and 6-methyl-1,3,8-trichlorodibenzofuran (MCDF). These two inhibitors compete with TCDD for cytosolic Ah receptor binding sites. In addition MCDF may also play role in the inhibition of induction by competing and partially inactivating genomic binding sites of the CYP1A1 (Merchant *et al.,* 1992).

Another substance called ellipticine was originally used as an antitumor drug (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) with anti-HIV activities. This natural compound was also reported to be a strong and selective inhibitor of CYP1A1 and CYP1A2 (Auclair, 1987).

Studies show that resveratrol has an inhibitory action over aryl hydrocarbon-induced CYP1A activity by directly inhibiting CYP1A1 enzyme activity and by inhibiting the signal transduction pathway (Ciolino *et al.,* 1999).

Flavonoids, also known as vitamin P are a class of plant secondary metabolites that bear a similar structure of flavone. They have high potency and selectivity for inhibition of all CYP1A isozymes. Especially four hydroxylated flavone derivatives namely as 3-hydroxy-, 5-hydroxy-, 7-hydroxy-, and 3,7-dihydroxyflavone also act as potent inhibitors of CYP1A1 (Zhai *et al.,* 1998).

As mentioned above, Heat Shock Protein 90 (Hsp90) is part of a cytosolic protein that is important in the proper functioning of AhR. Substances 17-allylamino-demethoxygeldanamycin, (17-AAG) and celastrol act as inhibitors of this protein, which in turn, limits, the inductive effects of PAHs over AhR and CYP1A1 (Hughes *et al.*, 2008).

1.2.2.5. CYP1A1's contribution to disease prevention

Although the majority of the studies have focused on the carcinogenic action of CYP1A1, according to the recent studies it is becoming clear that this enzyme plays important roles in detoxification and chemoprevention. The most significant line of evidence which contradicts the previously proposed mutagen-activating role of CYP1A1 recently came from the study of Uno *et. al.* (2006) which includes the generation of CYP1A1 (-/-) knockout mice. According to this study, mice lacking the CYP1A1 gene died within 30 days of 125 mg/kg/day oral B[*a*]P treatment; whereas CYP1A1 (+/+) mice survived with no overt signs of toxicity. In addition, the clearance rate of B[*a*]P in the CYP1A1 (-/-) strain was 4 times slower when compared to CYP1A1 (+/+) mice. Further pharmacokinetic experiments using TCDD and B[*a*]P suggested that clearance was almost exclusively dependent on inducible CYP1A1 and no other TCDD-B[*a*]P-inducible metabolizing phase I enzyme (Uno *et al.,* 2004).

B[*a*]P treatment caused many toxic effects in the immune system of CYP1A1 (-/-) mice. The data showed for the first time that this enzyme was more important in detoxification of B[*a*]P rather than metabolic activation to its ultimate carcinogenic conversion products (Androutsopoulos *et al.,* 2009).

Moreover, extensive work on the molecular events governing the transcriptional activation of the CYP1A1 gene through the aryl hydrocarbon receptor has revealed the interplay of AhR with various cell signaling pathways, important in normal cell growth, homeostasis and development (Androutsopoulos *et al.,* 2009).

1.3. Polymorphism

Genetic polymorphisms are the locations in the genome that vary among different individuals. In other words, the coexistence of multiple alleles at a specified genetic locus is called genetic polymorphism. Any site in the genome that bears multiple alleles and exists as stable components of the population is called polymorphic. Depending on its nature, polymorphism may affect one or several biological functions of the organism.

The genetic bases of polymorphisms are the mutations that occur by changes in the order or type of nucleotides. These changes may be a deletion of a pre-existing nucleotide in the DNA strand or an addition of a novel nucleotide. Alternatively, the substitution of a pre-existing nucleotide with a novel one can result in polymorphism. Once formed, a polymorphism is also inherited from parent to child like any other DNA sequence (Karp, 2002).

1.3.1. Single Nucleotide Polymorphism (SNP)

The most common type of genetic variability in humans occurs at sites in the genome where single nucleotide differences are found among different

members of the population. These sites are called single nucleotide polymorphisms (SNPs). The vast majority of SNPs occur as two alternate alleles like T or A. Millions of common SNPs have been identified by the comparison of DNA sequences from diverse ethnic populations (Lewin, 2004). Figure 1.9 represents a SNP which includes the substitution of a tymine in place of cytosine.

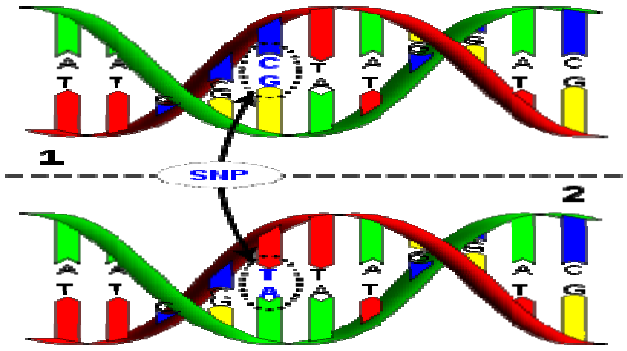

Figure 1.9 A schematic representation of a single nucleotide polymorphism where a cytosine nucleotide in the original DNA strand is mutated and replaced by a tymine (http://www.wikipedia.org, last accessed on 26/12/2010).

On average, two randomly selected human genomes have about 3 million single nucleotide differences between them. According to the estimations, there are almost 60.000 SNPs found within protein coding sequences of human genome which lead to amino acid replacements. This corresponds to about two amino acid substitutions per gene (Karp, 2002). SNPs in a coding region may have two different effects on the resulting protein:

- Synonymous mutation: nucleotide substitution does not result in an amino acid alteration in the protein. This type of mutation is also named as silent mutation.

- Non-Synonymous mutation: nucleotide substitution results in an alteration of the encoded amino acid which in turn may change the secondary or tertiary structure of the protein.

SNPs may also occur in regulatory regions of genes which may be capable of changing the amount of protein production per time. Each person's genetic material contains a unique SNP pattern that is made up of many different genetic variations which can serve as biological markers. They pinpoint a disease on the human genome map since they are usually located near a gene found to be associated with a certain disease (http://www.ncbi.nlm.nih.gov, last accessed on 26/12/2010).

Apart from being biological markers, SNPs play important roles in people's susceptibility to complex diseases that involve the activities of a number of different genes via changing the activity or expression levels of drug metabolizing enzymes.

1.3.2. Polymorphisms of Cytochrome P450 1A1

Several polymorphisms in the CYP1A1 gene have been described so far (Figure 1.10). The first polymorphism (m1) involves the transition of a thymine nucleotide into a cytosine. It is located at position 3801 of genomic DNA, conferring a restriction endonuclease site for cleavage by *Msp*I (Spurr *et al.,* 1987). This alteration occurs in the 3' noncoding region, downstream from exon 7 of the CYP1A1 structural gene and also known as T6235C polymorphism. The second most commonly encountered CYP1A1 polymorphism (m2) involves a substitution of a guanine in place of an adenine at position 2455 of codon 462 at exon 7 and is also known as A4889G polymorphism or Ile462Val polymorphism due to the amino acid change near the heme binding region of the CYP1A1 protein (Hayashi *et al.,* 1991).

Another *Msp*I polymorphism (m3), which has been described by Crofts *et al.* (1993) at nucleotide 3205, was shown to be African black-specific. It includes a base pair replacement of tymine to cytosine. Mutation m4 occurs in the structural part of the gene which causes the replacement of threonine amino acid to asparagine as described by Cascorbi *et al*. (1996).

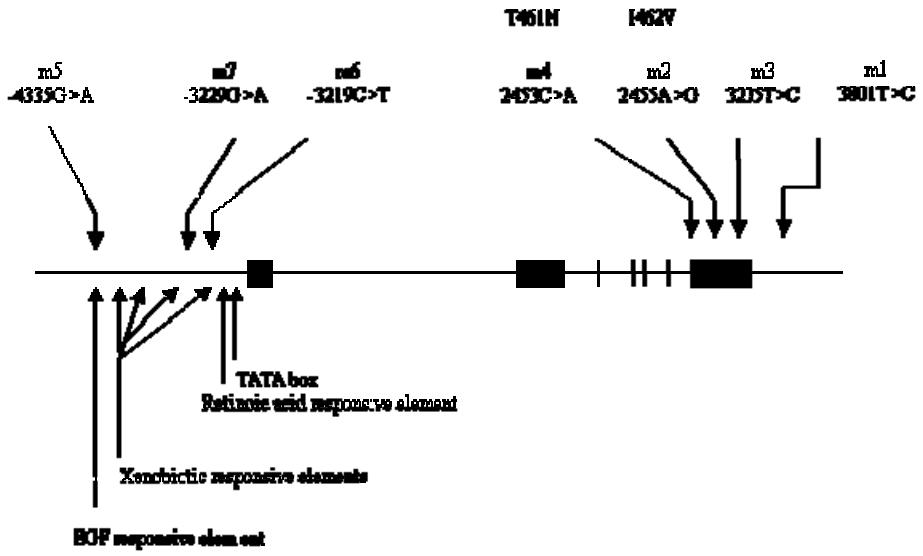

Figure 1.10 Single Nucleotide Polymorphisms in the human cytochrome P450 1A1 (taken from http://edoc.hu-berlin.de, last accessed on 28/11/2010).

In addition to those four SNPs, CYP1A1 promoter region contains at least three polymorphic sites as -4335G>A, -3229G>A and -3219C>T (Smart *et al.*, 2000; Gaikovitch *et al.*, 2003). These polymorphisms are located in the area of the gene which is well conserved between human and mouse (Jaiswal *et al.*, 1985). Figure 1.10 represents the location of these 7 mutations.

There are studies stating that CYP1A1 variants affect the function of the enzyme by altering the level of gene expression or the mRNA stability but these statements are not without opposing views (Tabor *et al.* 2002). For

instance, the A4889G polymorphism (also known as m2 or Ile462Val polymorphism) was found to confer elevated levels of induced CYP1A1 mRNA as the number of Val variants increased in one study (Crofts *et al.,* 1994), whereas in purified *Escherichia coli*, no difference in the metabolism of benzo[a]pyrene between the Ile and Val variants was noted (Zhang *et. al.,* 1996). Similarly, a high activity or lack of correlation has been suggested with the T6235C (m1) and A4889G (m2) polymorphisms regarding the activity of mutant enzymes in lymphocytes (Smart *et. al.,* 2000; Androutsopoulos *et al.,* 2009).

1.3.2.1. A4889G (m2) Polymorphism of CYP1A1

A point mutation in exon 7 (rs 1048943) of the CYP1A1 gene involves an adenine to guanine base transition at position 2455A→G of codon 462 which results in an amino acid substitution from an isoleucine to a valine which is also known as m2 or Ile462Val polymorphism due to this amino acid alteration (Hirvonen *et al.,* 1992; Alexandrie *et al.,* 1994). This mutation occurs in the structural region of the gene which encodes the heme binding motif of the protein, and studies of benzo[a]pyrene metabolism have shown that the mutant valine protein demonstrates increased enzyme activity when compared to the wild type isoleucine protein (Hayashi *et al.,* 1991). On the other hand, it has recently been found that cloned cDNA containing this mutation showed no considerably altered catalytic activity (Zhang *et al.,* 1996).

Ile462Val polymorphism varies among different ethnic groups, with a high prevalence in Asian populations and a low rate in Caucasians (Kawajiri *et al.,* 1990; Hayashi *et al.,* 1991; Cascorbi *et al.,* 1996; Mrozikiewicz *et al.,* 1997a). This polymorphism has been extensively studied in Asian populations (Lee *et al.,* 1994; Zhao *et al.,* 1995). The frequency of the variant G allele in Chinese (28%) and Malays (31%) were similar to those reported in Japanese (Kawajiri *et al.,* 1990) and 1.5-fold higher than that in the Indian (18%). Both the

genotypic and allelic frequencies in the Indian were significantly different from the Chinese and Malay populations (Lee *et al.,* 1994; Zhao *et al.,* 1995). In addition, A4889G transition in exon 7 (Ile462Val) have found to be more frequent in the Japanese population than in the Caucasian population (Hirvonen *et al.,* 1992; Alexandrie *et al.,* 1994)

Ile462Val polymorphism has found to be linked to an *MspI* restriction enzyme fragment length polymorphism in the 3' region of the gene (Hayashi *et al.,* 1991) which is associated with susceptibility to lung cancer in studies of Japanese populations (Kawajiri *et al.,* 1990; Nakachi *et al.,* 1991). The *Msp*I locus (T6235C) is in linkage disequilibrium with the A4889G polymorphism in Japanese (Hayashi *et al.,* 1991) and to a lesser extent in Caucasians, but not in Africans. It is not entirely clear whether the linkage disequilibrium between the two loci is an important risk factor for lung cancer or not, since conflicting results have been obtained in different ethnic groups (Kawajiri *et al.,* 1990; Nakachi *et al.,* 1991; Hirvonen *et al.,* 1992). The strong association between the two loci was postulated to represent increased risk to lung cancer in Asians. However, the linkage disequilibrium between the two loci was found to be rare in Caucasians and insignificant association with lung cancer was found in Caucasians (Hirvonen *et al.,* 1992).

Besides lung cancer, studies found a positive association with mutated CYP1A1 genotypes and several other types of cancers such as breast (Huang *et al.,* 1999a; Diergaarde *et al.,* 2008), prostate (Murata *et al.,* 1998); and ovarian cancer (Aktas *et al.,* 2002). But again, clear conclusion cannot be drawn because of the conflicting results. While some of the studies found increased risk or significant association between the Ile462Val polymorphism and these cancer sub-types, others have found no association at all (Basham *et al.,* 2001; Chen et al., 2007; Yao *et al.,* 2010).

A recent study found a decreased risk for CAD associated with the 4889GG genotype and no association with the wild 4889AA genotype (Yeh *et al.,* 2009). The association between A4889G polymorphism and CAD risk was previously investigated in two Caucasian population studies, but none was significant (Wang *et al.,* 2002; Manfredi *et al.,* 2007).

1.3.2.2. T6235C (m1) Polymorphism of CYP1A1

T6235C polymorphism (rs 4646903) involves the transition of a thymine nucleotide into a cytosine at position 3801 of genomic DNA, conferring a restriction endonuclease site for cleavage by *Msp*I restriction enzyme. This substitution occurs in the 3' noncoding region; downstream from exon 7 of the CYP1A1 structural gene (Spurr *et al.,* 1987).

The noncoding region is important for mRNA stability and expression of gene products. CYP1A1 gene expression is modulated by post-transcriptional and post-translational mechanisms (Tanguay *et al.,* 1996) and *MspI* polymorphism in the noncoding 3'-region seems to control CYP1A1 gene expression. Functional consequences of this polymorphism were compared to wild-type and an increase in inducibility of the enzyme was represented (Crofts *et al.,* 1994). However, it is still unknown whether this mutation itself leads to a functional alteration of the RNA-processing or if linked mutations in regulatory regions of the gene are responsible (Hasler *et al.,* 1999).

There are ethnic differences in the frequency of this polymorphism. For instance; the frequency of C allele was found to be lower than 12% among Caucasians (Tefre *et al.,* 1991; Hirvonen *et al.,* 1992; Cascorbi *et al.,* 1996; Mrozikiewicz *et al.,* 1997a; Krajinovic *et al.,* 2001; Hefler *et al.,* 2004; Li *et al.,* 2004). On the contrary, the frequency of C allele was found to be more than 30% in Indian (Singh *et al.,* 2006), Chinese (Huang *et al.,* 1999a;

Boyapati *et al.,* 2005; Shen *et al.,* 2006) and Japanese (Nakachi *et al.,* 1991; Miyoshi *et al.,* 2002) populations.

Although T6235C polymorphism has been shown to be associated with squamous cell carcinoma in Japan (Nakachi *et al.,* 1991) no such association has been found in Caucasians (Tefre *et al.,* 1991; Hirvonen *et al.,* 1992; Drakoulis *et al.,* 1994). The frequency of T6235C polymorphism in the Japanese population is higher than that in the Caucasian population (Tefre *et al.,* 1991; Hirvonen *et al.,* 1992; Drakoulis *et al.,* 1994; Alexandrie *et al.,* 1994); but it is not associated with gynecological malignancies like endometrial cancer and ovarian cancer in Japanese population (Huang *et al.,* 1999).

T6235C polymorphism of CYP1A1 was found to be associated with lung cancer in some studies (Nakachi *et al.,* 1993; Kawajiri *et al.,* 1996). A number of studies have found that this polymorphism has a positive association with the breast cancer susceptibility (Huang *et al.,* 1999; Chen *et al.,* 2007) but the results were inconclusive. In a meta-analysis study recently done by Yao *et al.,* over 25,087 subjects, no association was found between breast cancer risk and T6235C polymorphism (Yao *et al.,* 2010).

In addition, there are some studies investigating the association of T6235C polymorphism and cardiovascular disease (CVD), hypertension and stroke and their relation with the smoking status since CYP1A1 is the key enzyme in the metabolism of PAHs that are found in cigarette smoke. While some of them reported significant associations with this polymorphism and stroke (Lança *et al.,* 2004); others have found no association at all (Gambier *et al.,* 2006; Manfredi *et al.,* 2007; Yeh *et al.,* 2009).

1.4. The Aim of This Study

Stroke is the third leading cause of death leaving behind other diseases of heart, cancer and infectious diseases. Stroke results in serious disabilities and other life-threatening risks than any other disease. Atherosclerosis, the most common cause of stroke, is actually a disease of chronic inflammation which specifically affects arteries disrupting the vascular homeostasis. The formation of atherosclerotic plaques is a cascade of events starting with the oxidation of low-density lipoprotein molecules (LDL) by free radicals, particularly reactive oxygen species (ROS). Plaques formed in the inner lining of the arteries decreases the diameter of them causing elevated blood pressure. This deleterious cascade results in a vicious circle which gradually increases the risk of stroke.

Evidence is accumulating about the role of CYP metabolites in the maintenance of cardiovascular health, including the regulation of vascular tone, extracellular fluid volume, and heart contractility. CYP metabolites including free radicals (ROS) are also activators of multiple signaling pathways and are involved in inflammation, platelet aggregation, fibrinolysis, and cellular injury, which are quite related to atherosclerosis. Information on genetic polymorphisms and the regulation of CYP expression is of vital importance for the proper regulation of metabolite formation and hence cardiovascular health.

Cytochrome P450 1A1 is a Phase I xenobiotic metabolizing enzyme with a selective affinity over polycyclic aromatic hydrocarbons (PAHs), especially on benzo[a]pyrene which is abundantly found on cigarette smoke. The metabolism of this chemical definitely causes the formation of reactive oxygen species and free radicals. CYP1A1 enzyme is responsible for the bioactivation of PAHs into their ultimate mutagenic and carcinogenic forms which may have deleterious effects on vascular homeostasis. In addition, recent investigations on CYP1A1 activities reflect their positive effect on the prevention of the

formation of these metabolites which cause a debate about CYP1A1s' role in the biological systems.

A number of SNPs in CYP1A1 gene are associated with increased enzyme activity which may result in the accumulation of hazardous metabolites. It is well established that CYP1A1 polymorphisms differ in frequency among populations of different ethnicity. Therefore, identification of CYP1A1 polymorphisms in different populations as well as in Turkish population is crucial. Only a few studies have been carried out in Turkish population including the CYP1A1 A4889G and T6235C polymorphisms. Additionally, according to our knowledge, no study is available in the literature showing the relationship between stroke and these two polymorphisms at the same time.

The aim of this study was to investigate two important CYP1A1 polymorphisms which are A4889G in the structural region and T6235C polymorphism in the non-coding region, in both ischemic stroke patients and healthy volunteers in order to understand whether these mutations are associated with stroke and to determine susceptibility to ischemic stroke in Turkish population. To accomplish this aim, this study was designed to follow the steps given below:

- obtaining total blood samples from ischemic stroke patients and healthy controls,
- isolation of genomic DNA in intact form from blood samples,
- optimization of conditions for amplification of two regions in CYP1A1 gene, one in the coding region (A4889G) and one in the non-coding region (T6235C) by using polymerase chain reaction (PCR),
- determination of the genotype of each individual by using allele specific PCR for the A4889G region,
- digestion of amplified fragments with restriction endonuclease *MspI* to determine the genotype of each individual for T6235C region,

- determination of the genotype and allele frequencies of A4889G and T6235C single nucleotide polymorphisms for Turkish population,
- comparison of genotype and allele frequencies between ischemic stroke and control groups,
- comparison of the results of this study with other studies performed in different ethnicities.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Population and Blood Sampling

The study population was composed of 226 adult Caucasian patients with the diagnosis of acute hemispheric ischemic stroke and 113symptom-free Caucasian controls from the same geographic region (Central Anatolia, Turkey). Whole blood samples of both control and patient group participants were gathered by the Gülhane Military Medical Academy Hospital Neurology Department, Ankara. Before entering the study, all participants were informed about the procedure and their informed consents were obtained. A copy of the informed consent is available in Appendix A and B. Each step in the study had been carried out according to the Decleration of Helsinki principles. In addition, the research study was approved by the Ethical Committee of the Gülhane Military Medical Academy. This is also provided in Appendix C.

Study group was selected among the patients who are suffering atherothrombotic ischemic stroke that are admitted to the neurology service of Gülhane Medical Faculty, Ankara, within 24 hours after onset, from October 2005 to October 2010. Recruitment of the patients was performed consecutively. Definition of stroke was set as clinical designation for a rapidly developing loss of brain functions that lasted at least 24 hours and has no apparent cause other than that of vascular origin.

The cerebral infarction was first diagnosed by a neurological examination and brain computer tomography (CT) scan which is followed by the transthoracic echocardiographic examination, Holter Study and Transcranial Doppler emboli detection procedure to rule out emboli source. In order to be considered eligible, the patients had to meet a number of criteria as:

- They should have anterior circulation stroke
- They should not have other major illnesses, including autoimmune diseases, neoplasms, coagulopathies, hepatic or renal failure,
- There should not be an embolic source known (aortic arch, cardiac or caroid),
- They should not have a family history of myocardial infarction within 3 weeks or of transient ischemic attack or stroke at any time.

The control group was selected from the neurology outpatient clinics in a randomized manner. The same exlusion criteria given above were also applied to the control group members except that the control group should not have antrior circulation stroke. In addition to those criteria, control group should not have had carotid stenosis (lumen narrowing) greater than 50% or ulcerated carotid plaque. In order to ensure that, all subjects underwent bilateral carotid Doppler Ultrasound (CUSG) and transthoracic echocardiographic studies.

A detailed questionniare had been applied to each participant by colleagues from Gülhane Medical Faculty Neurology Department in order to collect the history of conventional vascular risk factors and conditions. The cut-off lines of some vascular risk factors are defined as mentioned below:

- Hypertension: Systolic blood pressure greater than 140 mm Hg and/or diastolic blood pressure greater than 90 mmHg and/or use of antihypertensive drugs.

- Diabetes: Fasting glucose equal or higher than 6.99 mmol/L and/or use of pharmacological treatment.
- Obesity: Body Mass Index (BMI) was 30 or higher.
- Smoking status: Assigned “yes” if the individual is currently smoking or have quitted less than 3 months ago.

In addition, some routine laboratory tests like electrocardiogram, chest X-ray, complete blood count, leukocyte differential, erytrocyte sedimentation rate, routine biochemisty tests including fasting glucose, lipid profile (triglycerides, total cholesterol, LDL, HDL, VLDL-C), creatine, sodium, potassium, bilirubin and liver function tests, routine urine tests and rheumatologic screening tests were carried out for all participants. All laboratory measurements were done blinded to clinical characteristics.

The study population (226 patients and 113 controls) were selected from a larger population of 244 patients and 146 controls. The rationale for selection was as follows:

- 14 controls were not includedto the group since they had either ischemic heart disease or carotid stenosis greater than %50, or both.
- In order to obtain similar mean age of patients and control groups, 18 patients whose age was 86 years and older and 19 controls aged 38 years and younger were excluded from the group.

2.1.2. Chemicals

Agarose (A-9539), bromophenol blue (B-5525), ethidium bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), sodium chloride (NaCI; S-3014), sodium dodecyl sulfate (SDS; L-4390), 2-amino-2 (hydroxymethyl)-1,3-propandiol (Tris; T-1503) were the products of Sigma

Chemical Company, Saint Louis, Missouri, USA. Borate (11607) and absolute ethanol (32221) were purchased from Riedel de Haën, Seelze, Germany. Taq DNA polymerase-supplied together with $MgCI_2$ and amplification buffer – (#EP0407), dNTP mix (#R0191), Gene Ruler TM 50 bp DNA ladder (#SM0371) and restriction enzyme *MspI (HpaII;* #ER0541) supplied with its buffer were ordered from MBI Fermentas, USA.

All chemicals used in this study were molecular grade and were obtained from commercial sources at the highest grade of purity.

2.1.3. Primers

In order to amplify two relevant polymorphic regions of CYP1A1 gene, two sets of oligonucleotide primers were used. Their sequences were derived from the known sequences of human CYP1A1 gene (Hayashi *et al.,* 1991; Wang *et al.*, 2002).

A4889G Single Nucleotide Polymorphism (rs 1048943) was analyzed using two allele specific forward primer which differs in the last nucleotide and a common reverse primer.

Forward primer 1: 5'-GAAGTGTATCGGTGAGACCA-3'
Forward primer 2: 5'-GAAGTGTATCGGTGAGACCG-3'
Reverse primer: 5'-GTAGACAGAGTCTAGGCCTCA-3'

T6235C Single Nucleotide Polymorphism (rs 4646903) was analyzed using the following set of forward and reverse primers:

Forward primer: 5'-CAGTGAAGAGGTGTAGCCGCT-3'
Reverse primer: 5'-TAGGAGTCTTGTCTGATGCCT-3'

2.2. Methods

2.2.1. Preparation of Genomic DNA for PCR

2.2.1.1. Isolation of Genomic DNA from human whole blood samples

Principle:

Salting-out method with slight modifications was used for the isolation of genomic DNA according to the method described by Lahiri and Schnabel (1993). Whole blood samples were collected to the EDTA containing tubes in order to prevent clotting of blood samples. These blood samples were used as the DNA source.

Reagents:

The reagents used for the isolation of genomic DNA from human whole blood samples are given in Table 2.1.

Table 2.1 Name, constituents and the preparation of reagents that are used for the isolation of genomic DNA from human whole blood samples.

Reagent Name	Constituents	Preparation
TKM Buffer	10 mM Tris-HCl at pH 7.6 10 mM KCl 4 mM $MgCl_2$ 2 mM EDTA	242.2 mg Tris, 149,1 mg KCl, 162,6 mg $MgCl_2$ and 148.9 mg EDTA were weighed and dissolved in 100 mL of dH_2O. pH was adjusted to 7.6 with HCl and volume was completed to 200 mL. The solution was autoclaved for sterilization and stored at 4 °C.
10% SDS	Molecular-grade SDS dH_2O	0.1 g molecular grade SDS is dissolved in 1 mL dH_2O. This solution did not require sterilisation and stored in 4°C.
Saturated NaCl (~ 6 M)	NaCl dH_2O	3.51 g NaCl was weighed and dissolved in 10 ml dH_2O. Solution was autoclaved for sterilization and stored at 4°C.
TE Buffer	10 mM Tris-HCl (pH 8.0) 1 mM EDTA (pH 8.0)	10 mL 100 mM Tris-HCl and 0.2 mL 500 mM EDTA was mixed and final volume was completed to 100 mL with dH_2O.
100 mM Tris-HCl	Tris dH_2O HCl	1.21 g Tris was dissolved in 100 mL dH_2O. HCI was used in order to adjust the pH of the solution to 8.0. Afterwards, the solution was autoclaved to prevent potential contamination risk.
500 mM EDTA	EDTA dH_2O HCl	18.61 g EDTA was dissloved in 100 mL dH_2O. HCI was used in order to adjust the pH of the solution to 8.0. Afterwards, the solution was autoclaved to prevent potential contamination risk.

Procedure:

750 µL of whole blood was transferred into eppendorf tube and was treated with the same volume of low-salt TKM buffer. In order to disintegrate the cells, 20 mL of Triton X-100 was added inside the tube and mixed by inversions. This suspension was centrifuged at 1000 g for 10 minutes at room temperature by using Sigma 1-15 benchtop microfuge (Sigma Postfach 1713-D-37507, Osterode). After centrifugation, there were two distinct layers; the top layer, also called the supernatant, was discarded. The bottom layer, which is called the pellet, was containing the genomic DNA. Pellet was washed several times with 750 µL TKM Buffer. The final pellet was resuspended in 200 µL of TKM Buffer by tapping. 10 µL of 10% SDS (Sodium Dodecyl Sulfate) was added and the whole suspension was mixed thoroughly and incubated at 58°C for 10 minutes. Afterwards, 75 µL of cold saturated NaCl (~ 6 M) was added to the suspension and the tube was mixed well. By the centrifugation process with 14000 g for 10 minutes at 4°C, the suspension was again seperated as supernatant and pellet. This time, supernatant was containing the DNA and it was taken to another eppendorf tube. 2 x volume ice-cold ethanol was used in order to precipitate the DNA. The tubes were stored at -20°C for almost one hour and then centrifuged at 10000 g for 10 minutes at 4°C for the precipitation of DNA. Supernatant was removed and DNA containing pellet was solubilised with 100 µL of TE Buffer. Tubes were incubated at 37°C for more than 2 hours to completely dissolve the DNA.

2.2.1.2. Quantification of Genomic DNA Concentration by Spectrophotometry

In order to determine the concentration of DNA in the sample, absorbance values at 260 nm and 280 nm were measured in quartz cuvettes using Shimadzu UV160-A double-beam spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). The nucleic acids gave

maximum absorbance at 260 nm; therefore reading at this wavelenght was used to calculate the concentration of DNA in the sample. Based on the knowledge that an absorbance value of 1.0 corresponds approximately to 50 μg/mL for double-stranded DNA, the concentration of DNA was calculated using the formula below:

$$\text{Concentration } (\mu g/mL) = A_{260nm} \times 50\ (\mu g/mL) \times \text{Dilution Factor} \qquad (2.1)$$

2.2.1.3. Qualification of Genomic DNA by Spectrophotometry

The ratio between absorbance values at 260 nm and 280 nm (A_{260nm}/A_{280nm}) was used to estimate the purity of the nucleic acid. Pure DNA preparations give the ratio of 1.8 while the higher or lower values show either RNA or protein contaminations, respectively.

2.2.1.4. Qualification of Genomic DNA by Agarose Gel Electrophoresis

Determination of the intactness of DNA samples was performed by 0.5% agarose gel electrophoresis using Biogen horizontal agarose gel electrophoresis which gave a gel tray in 8 cm x 9 cm dimensions.

Reagents:

1. TBE (Tris-Borate-EDTA) Buffer, pH 8.3:

- 5x stock solution: 54 g Trizma-base and 27.5 g boric acid were weighed and dissolved in necessary amount of water. 20 mL of 500 mM EDTA (pH 8.0) was added and pH was set to 8.3. Volume was completed to 1 L and solution was autoclaved for sterilization. To prevent precipitation, solution was stored at room temperature.

- 0.5x solution: The 5x stock solution containing 0.45 M Tris-Borate and 10 mM EDTA was diluted 10 times with dH_2O prior to use, to achieve 45 mM Tris-Borate and 1 mM EDTA.

2. Ethidium Bromide (10 mg/mL): 0.1 g ethidium bromide was dissolved in 10 mL dH_2O. Solution was stirred on magnetic stirrer for several hours to ensure that dye was completely dissolved. Because this solution is light sensitive, the bottle was covered with aluminum foil and stored at room temperature.

3. Gel Loading Buffer: 0.25% bromophenol blue, 40% sucrose in dH_2O. This solution did not require sterilization and stored at room temperature.

Procedure:

0.5 % agarose gel was prepared by adding 0.15 g agarose to 30 mL 0.5x TBE buffer, pH 8.3 containing 45 mM Tris, 45 mM Borate and 1 mM EDTA, in an erlenmeyer flask, so the buffer did not occupy more than half of the flask. The sullury was heated in microwave oven till all of the grains of the agarose melted and formed a uniform liquid.

The solution was cooled to approximately 60°C on a magnetic stirrer with continuous stirring for homogenous cooling. When cooled enough, ethidium bromide was added from a stock solution of 10 mg/mL in water to a final concentration of 0.5 µg/mL and the solution was mixed thorougly.

The mold, plastic tray and comb were cleaned with 70% ethanol while the agarose gel is being heated. The plastic tray was settled in the mold and the comb was placed 0.5-1.0 mm above the plate. The warm agarose solution was then poured into the mold and any air bubbles –if present-, especially under or between the teeth of the comb were removed with the help of the pipette tip.

The gel was left for solidifying completely for approximately 20-40 minutes at room temperature.

The gel tank was filled with approximately 300 mL of 0.5x TBE buffer. The comb was carefully removed from the gel. 0.5x TBE buffer was added to the tank until it covered the gel to a depth of 1 mm.

5 µL (0.25-0.5 ng) of DNA sample was mixed with 1 µL of gel loading buffer and slowly loaded into the wells of the gel. The lid of the tank was closed and the electrical leads were attached to the power supply. The power supply was set to the constant voltage of 100 volts. The gel was run for 40 minutes and then examined under UV light and the photograph was taken by using Vilber Lourmat Gel Imaging System (Marre La Vallee, Cedex, France) and Bio-Capture (Version 99.03) computer software.

2.2.2. Genotyping for A4889G and T6235C Single Nucleotide Polymorphisms of CYP1A1

Two different methods of genotyping were applied for the determination of these two polymorphisms in CYP1A1. Allele specific PCR method was used for CYP1A1 A4889G and standard PCR protocols followed by the restriction enzyme digestions were used for genotyping the CYP1A1 T6235C polymorphisms.

Techne Progene Thermocycler (Techne Ltd. Duxford, Cambridge) was used in PCR for both of the regions. Two different forward primers and one common reverse primer were used for the recognition of the SNPs in A4889G region during PCR (Table 2.2). *MspI* restriction endonuclease was used in RFLP analysis of T6235C polymorphism of CYP1A1 after standard PCR protocols (Table 2.5).

2.2.2.1. A4889G Single Nucleotide Polymorphism

2.2.2.1.1 Allele Specific Polymerase Chain Reaction Method for A4889G SNP

CYP1A1 A4889G SNP region (rs 1048943) was amplified using two different SNP-specific forward primer sequences and one common reverse primer sequence as given by Hayashi *et al.* (1991). Two different reaction mixtures were used with different forward primers (Table 2.2). Sequence of the amplified fragment in the exon 7 of CYP1A1 gene that includes the A4889G single nucleotide polymorphism is given in Figure 2.1.

Table 2.2 Genotyping of CYP1A1 A4889G SNP; table showing the type of reaction mixture, primer pairs used for amplification in two different reaction mixtures and size of the PCR products (if any) and the interpretation of the PCR products.

Type of reaction mixture	Primer pairs used	PCR product size	Interpretation
Reaction Mixture 1	Forward Primer 1: 5'-GAA GTG TAT CGG TGA GAC CA-3' Reverse Primer: 5'-GTA GAC AGA GTC TAG GCC TCA-3'	200 bp	If PCR product present; DNA sample bears wild-type A allele If PCR product absent, DNA sample does not bear wild-type A allele
Reaction Mixture 2	Forward Primer 2: 5'-GAA GTG TAT CGG TGA GAC CG-3' Reverse Primer: 5'-GTA GAC AGA GTC TAG GCC TCA-3'	200 bp	If PCR product present; DNA sample bears mutant G allele If PCR product absent, DNA sample does not bear mutant G allele

AGACACAAGTTTGAAAGGCTTTTACATCCCCAAGGGGCGTTGTGTCTTTGTAAACCAGTGGCAGATCAAC
CATGACCAGTAAGTTCAGAGATGCAGAGGAAAGGCTGGGTCCACCCTCTTAAGCTCTTATATATGATTAA
TACAATCATTGCATTGATCCTCCTGTCCATGGGCTGCTTGCCTGTCCTCTATCCTTTGGGGCTGGAGCTCC
ACTCACTTGACACTTCTGAGCCCTGAACTGCCACTTCAGCTGTCTCCCTCTGGTTACAGGAAGCTATGGGT
CAACCCATCTGAGTTCCTACCTGAACGGTTTCTCACCCCTGATGGTGCTATCGACAAGGTGTTAAGTGAGA
AGGTGATTATCTTTGGCATGGGCAAGCGGAAGTGTATCGGTGAGACCA/GTTGCCCGCTGGGAGGTCTTCT
CTTCCTGGCTATCCTGCTGCAACGGGTGGAATTCAGCGTGCCACTGGGCGTGAAGGTGGACATGACCCCCA
TCTATGGGCTAACCATGAAGCATGCCTGCTGTGAGCACTTCCAAATGCAGCTGCGCTCTTAGGTGCTTGAG
AGCCCTGAGGCCTAGACTCTGTCTACCTGGTCTGGTTGGGCAGCCAGACCAGCAGGCTGGCCTATGTGGTC
TAAGGTTCAGCCTGAAACTCATAGACACTGATCTGGCTGCAGTTTTGCTATCTGGGCTGTGGGCAAGCCTA
AGGGATCCTGCCTGCCCCTACCCTGGACTTGCCTCTGCACACCCTCCAGAGACAACAGGTAAAACAGGGCCA
CATAGATGCTGATGGAGCCTTC

Figure 2.1 Sequence of the exon 7 region of CYP1A1 gene that includes the A4889G single nucleotide polymorphism (rs 1048943) in the forward primer region. The yellow highlighted sequences are the forward and reverse primers. The last nucleotide of the forward primer sequence is also showing the A4889G SNP site (The nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov, last accessed on 08/10/2010).

Principle:

It is possible to detect the presence of the A4889GSNP by using the allele specific primers which specifically recognize and bind to the relevant DNA sequence for amplification. Two different reaction mixtures were prepared for all DNA samples:

- Reaction Mixture 1 containing amplification buffer, $MgCl_2$, dNTP mixture, allele specific forward primer 1 for wild type A allele, common reverse primer, template DNA and Taq DNA Polymerase.
- Reaction Mixture 2 containing amplification buffer, $MgCl_2$, dNTP mixture, allele specific forward primer 2 for mutant G allele, common reverse primer, template DNA and Taq DNA Polymerase.

Allele specific forward primer 1 has a specific recognition site at the end (5'-GAA GTG TAT CGG TGA GAC CA-3') for binding to the wild type DNA template for amplification. On the other hand allele specific forward primer 2 has a specific recognition site at the end (5'-GAA GTG TAT CGG TGA GAC CG-3') for binding only to the mutant DNA template for amplification.

If the sample has a wild type AA genotype; only forward primer 1 binds to the DNA and amplifies the DNA template which in turn becomes visible as 200 bp bands. Forward primer 2 can not find a specific recognition site in AA genotype and amplification of the DNA template does not occur.

If the sample has a mutant GG genotype; only forward primer 2 binds to the DNA and amplifies the DNA template which in turn becomes visible as 200 bp bands. Forward primer 1 can not find a specific recognition site in GG genotype and amplification of the DNA template does not occur.

If the sample has a heterozygote AG genotype, both of the forward primers bind to the DNA and amplify the DNA template which in turn becomes visible as 200 bp bands. Schematic representation of the protocol for the determination of A4889G genotypes is given in Figure 2.2.

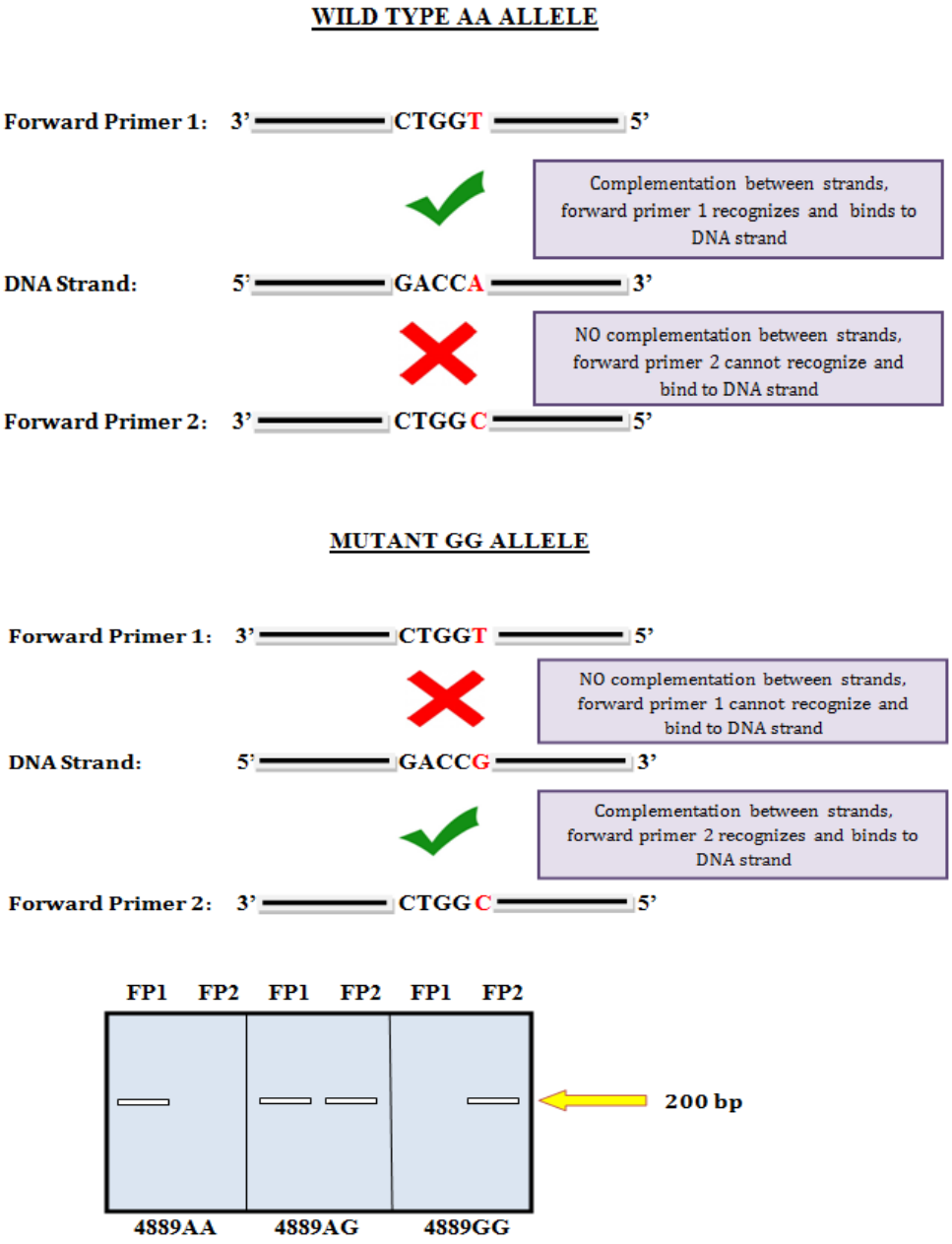

Figure 2.2 Schematic representation of A4889G genotype determination. At the top, the wild allele (4889A) can be seen. Forward primer 1 has a recognition site for wild type A allele, so if there is a wild type allele, only forward primer 1 complements and binds to DNA strand for amplification during PCR. In the middle, the mutant allele (4889G) can be seen. Forward primer 2 has a recognition site for mutant G allele, so if there is a mutant allele, only forward primer 2 complements and binds to DNA strand for amplification during PCR. At the bottom, a representative agarose gel photograph of wild type, heterozygote and mutant alleles are given.

Reagents:

1. 1.25 unit Taq DNA polymerase (5u/μL, stored at -20˚C)

2. PCR amplification Buffer with KCl: 100 mM Tris-HCl (pH: 8.8 at 25°C), 500 mM KCl, 0.8% Nonidet P40. This buffer and 25 mM $MgCl_2$ solution were supplied together with Taq DNA Polymerase. Taq DNA Polymerase, amplification buffer and $MgCl_2$ solutions were stored at -20°C.

3. dNTP Mixture: 10 mM of each dATP, dCTP, dGTP and dTTP in aqueous solution. The solution was stored at -20°C.

4. Forward Primer 1 and common reverse primer for Reaction Mixture 1.

5. Forward Primer 2 and common reverse primer for Reaction Mixture 2.

Procedure:

In order to obtain a single band belonging to the A4889G region of CYP1A1 gene, devoid of non-specific bands, different parameters like $MgCl_2$ concentration, primer and template DNA amounts and amplification program were tested in PCR. The optimized PCR mixture for the amplification of A4889G SNP is given in Table 2.3. Approximately 100 ng DNA, 167 μM dNTPs, 7.5 pmol of each primer, 2 mM $MgCl_2$ and 1.25 unit of Taq Polymerase were included in the PCR reaction (Table 2.3).

Table 2.3 Components of PCR mixtures for CYP1A1 A4889G.

Constituent	**Stock Concentration**	**Volume added**	**Final concentration in 30 µL reaction mixture**
Sterile apyrogen H_2O		Up to 30 µL	
Amplification buffer	10 x	3 µL	1x
$MgCl_2$	25 mM	2.5 µL	2mM
dNTP mixture	10 mM	0.5 µL	167 µM
Forward primer 1 or 2	10 pmol/ µL	0.75 µL	7.5 pmol
Reverse Primer	10 pmol/ µL	0.75 µL	7.5 pmol
Template DNA	varies	varies	~100 ng
Taq DNA Polymerase	5 U/ µL	0.25 µL	1.25 U

The optimized program of thermalcycler used for the amplification of A4889G SNP region of CYP1A1 is given in Table 2.4 (Hayashi *et al*, 1991).

Table 2.4 PCR Program used for the amplification of A4889G SNP region of CYP1A1.

Initial Denaturation	95 °C	5 min	
Denaturation	95 °C	1 min	30 cycles
Annealing	65 °C	1 min	
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	

PCR products were analysed on 2% agarose gel prepared by adding 4 g agarose to 200 mL of 0,5 x TBE buffer as described in section 2.2.1.4. 10 µL of PCR products was mixed with 3 µL of gel loading buffer and applied to the wells of the gel. 6 µL ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V.

2.2.2.2. T6235C Single Nucleotide Polymorphism

2.2.2.2.1 Polymerase Chain Reaction for T6235C SNP

CYP1A1 T6235C SNP region (rs 4646903) was amplified using primer sequences given by Hayashi *et al.* 1991 (Table 2.5). Sequence of the amplified fragment in 3' flanking region of CYP1A1 gene that includes T6235C single nucleotide polymorphism is given in Figure 2.3.

Table 2.5 Genotyping of CYP1A1 T6235C SNP; table showing the region of amplification, primer pairs used for amplification, size of the PCR product, restriction enzyme, and the interpretation of the RE digestion products.

Amplification Region	**Primers**	**PCR Product size**	**RE**	**Size of digestion products and interpretation**
3' flanking (T6235C)	Forward Primer: 5'-CAG TGA AGA GGT GTA GCC GCT-3' Reverse Primer: 5'-TAG GAG TCT TGT CTG ATG CCT-3'	340 bp	*MspI (HpaII)*	**TT**: 340 bp **TC**: 140 bp, 200 bp, 340 bp **CC**: 140 bp, 200 bp

TACCTGAGCTAAATAAAGATATTGTTCAGAAATCCTATAGGTGGAGATTTTTTGAATCATAAATGATTCAT
CACTCGTCTAAATACTCACCCTGAACCCCATTCTGTGTTGGGTTTTACTGTAGGGAGGAAGAAGAGGAGGTA
GCAGTGAAGAGGTGTAGCCGCTGCACTTAAGCAGTCTGTTTGAGGGACAAGACTCTATTTTTTGAGACAGGG
TCCCAGGTCATCCAGGCTGGAGTGCACTGGTACCATTTTGTTTCACTGTAACCTCCACCTCCT/CGGGCTCAC
ACGATTCTCCCACCTCAGCCTCTGAGTAGTTGGGGCCGCAGGCACACGCCACCACAGCTTTTTTTTTTTTTTT
TTTTTTTTTTTGTAGAGATGGGGTTTCACCATGTTGCCCAGGCTGGTCTCAAACTCCTGAGCTCAAGTGATC
CACCTGCCTCAGCCTCCCAAAGTGCTGGGATTACAGGCATCAGACAAGACTCCTAATCACTGTGCTGACTTA
CGCCCTCTCTAACTTATCACAAATTGATGAAGCGTATTCTCCGAATTAGGCAATAAGGGTATCAAGTGAATA
TAGTACAGTCCCTGCTTTCAAGAACCTCCCAAAATGGTTGAGGGAACTGACACATAAACACATCATTCAATT
CATTGTGGTCAGAACTCTACCAGACAGATG

Figure 2.3 Sequence of the fragment in 3' flanking region of CYP1A1 gene that includes the T6235Csingle nucleotide polymorphism (rs 4646903). The yellow highlighted sequences are the forward and reverse primers. Red highlighted nucleotide T/C shows the location of SNP and the green square shows the recognition site of the restriction enzyme *MspI* (The nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov, last accessed on 08/10/2010).

Reagents:

1. 1 unit Taq DNA polymerase (5u/μL, stored at -20˚C).

2. PCR amplification Buffer with KCl: 100 mM Tris-HCl (pH: 8.8 at 25°C), 500 mM KCl, 0.8% Nonidet P40. This buffer and 25 mM $MgCl_2$ solution were supplied together with Taq DNA Polymerase. Taq DNA Polymerase, amplification buffer and $MgCl_2$ solutions were stored at -20°C.

3. dNTP Mixture: 10 mM of each dATP, dCTP, dGTP and dTTP in aqueous solution. The solution was stored at -20°C.

4. 10 pmol/ μL of forward and reverse primers.

Procedure:

In order to obtain a single band belonging to the T6235C region of CYP1A1 gene, devoid of non-specific bands, different parameters like $MgCl_2$ concentration, primer and template DNA amounts and amplification program were tested in PCR. The optimized PCR mixture for the amplification of T6235C SNP is given in Table 2.4. Approximately 100 ng DNA, 0.2 mM dNTPs, 20 pmol of each primer, 2 mM $MgCl_2$ and 1 Unit of Taq Polymerase was included in the PCR reaction (Table 2.6).

Table 2.6 Components of PCR mixtures for CYP1A1 T6235C.

Constituent	Stock Concentration	Volume added	Final concentration in 30 µL reaction mixture
Sterile apyrogen H_2O		Up to 25 µL	
Amplification buffer	10 x	2.5 µL	1x
$MgCl_2$	25 mM	2 µL	2mM
dNTP mixture	10 mM	0.5 µL	0.2 mM
Forward primer	10 pmol/ µL	2 µL	20 pmol
Reverse Primer	10 pmol/ µL	2 µL	20 pmol
Template DNA	varies	varies	~100 ng
Taq DNA Polymerase	5 U/ µL	0.2 µL	1 U

The optimized program of thermalcycler used for the amplification of T6235C SNP region of CYP1A1 is given in Table 2.7 (Hayashi *et al.*, 1991).

Table 2.7 PCR Program used for the amplification of T6235C SNP region of CYP1A1.

Initial Denaturation	95 °C	1 min	
Denaturation	95 °C	30 sec	30 cycle
Annealing	57 °C	1 min	
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	

PCR products were analysed on 2% agarose gel prepared by adding 4 g agarose to 200 mL of 0,5 x TBE buffer as described in section 2.2.1.4. Ten µL of PCR products was mixed by 3 µL of gel loading buffer and applied to the wells of the gel. 6 µL ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V.

2.2.2.2.2 Restriction Endonuclease Digestion of PCR Products for the Determination of T6235C SNP

Principle:

It is possible to detect the T6235C single nucleotide polymorphism by RFLP technique if the polymorphic nucleotide is a part of a restriction endonuclease recognition site, so that with the base substitution from wild type to mutated form, a restriction site is formed. As a result, the genotype of the individual was determined by the digestion of the PCR product with the corresponding restriction enzyme. Schematic representation of the protocol for the determination of T6235C genotype is given in Figure 2.4.

Molecular basis of the T6235C polymorphism is a single nucleotide change in DNA from thymine (T) to cytosine (C). The sequence around cytosine is a recognition site for *MspI* restriction enzyme, which is given below:

5’ …..C↓C G G…..3’
3’…...G G C↑C…..5’

In the mutant allele, digestion with *MspI* cuts the 340 bp PCR product into two fragments of 200 bp and 140 bp which indicate 6235CC genotype. A single undigested 340 bp band in the agarose gel indicated a 6235TT genotype. Three bands (140, 200 and 340 bp) in agarose gel show heterozygote 6235TC genotype which contains two alleles together (Figure 2.4).

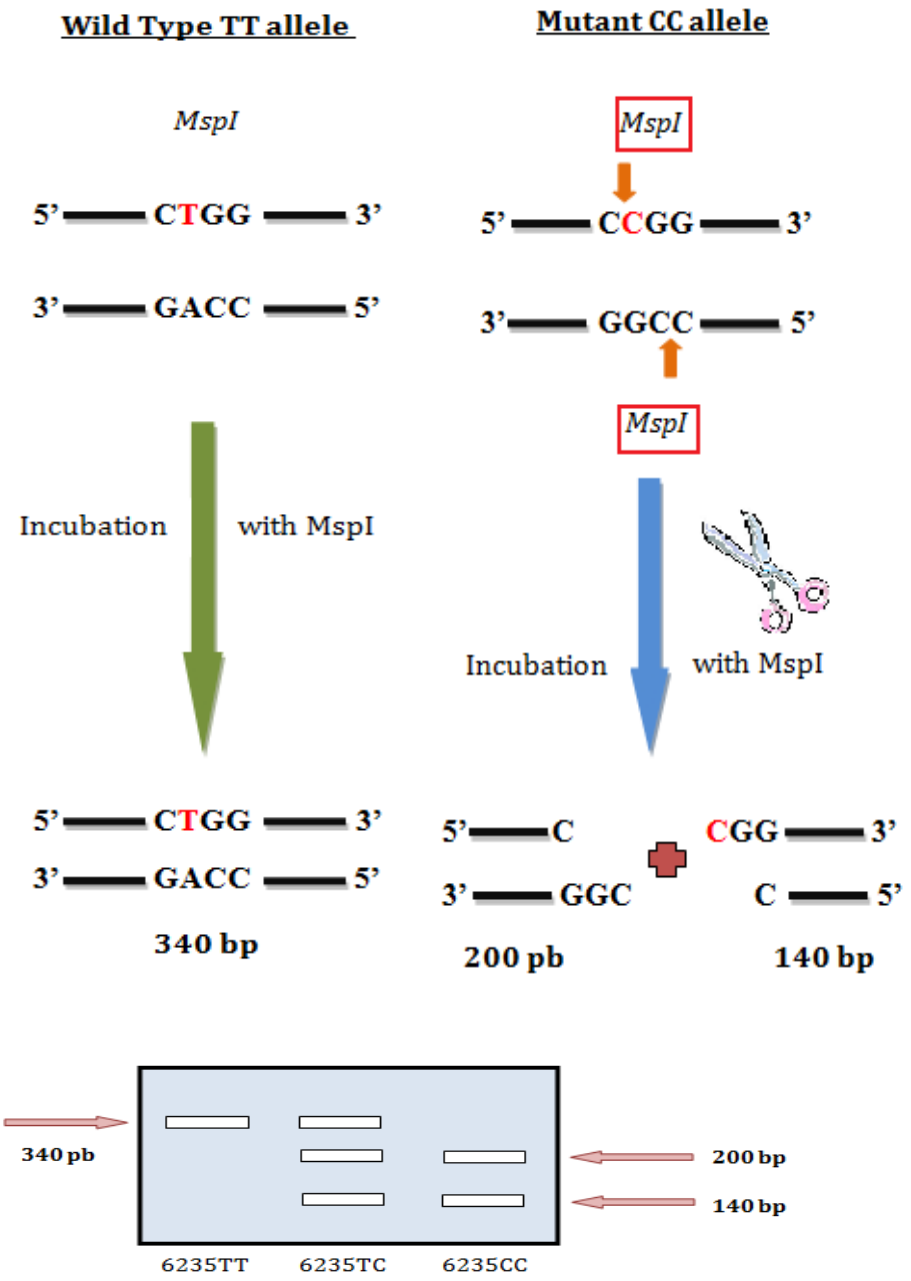

Figure 2.4 Schematic representation of T6235C genotype determination. In the left panel, the wild type allele 6235T can be seen. *MspI* does not recognize the site around wild T allele, thus the unfragmented 340 bp band represents the wild type 6235T allele. In the right panel, the polymorphic 6235C allele can be seen. A recognition site for *MspI* is created within the mutant C allele and MspI cuts the 340 bp PCR product containing the recognition site and results in two fragments of 200 bp and 140 bp. At the bottom, a representative agarose gel photograph of wild type, heterozygous and mutant genotypes are given.

Reagents

1. *MspI (HpaII)* restriction enzyme.

2. 1 X Buffer Tango: 33 mM Tris-acetate (pH 7.9), 10 mM Magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA. This buffer was supplied together with the restriction enzyme MspI. The restriction enzyme and buffer were stored at -20 °C.

Procedure:

Incubation of 10 µL of 340 bp PCR product with 3000 U *MspI* restriction enzyme, at 37°C for at least 20 hours, is the first step of the procedure to determine T6235C single nucleotide polymorphism of CYP1A1 gene. Constituents of the reaction mixture is given in Table 2.8.

Table 2.8 Constituents of reaction mixture for restriction endonuclease (*MspI*) digestion of PCR products for the determination of CYP1A1 T6235C SNP.

Constituent	**Stock Concentration**	**Volume Added**	**Final Concentration in 30 µL reaction mixture**
Sterile apyrogen H_2O		17 µL	
Buffer	10x	2 µL	0.67x
MspI(HpaII)	10 U/ µL	1 µL	10 U
PCR Product		10 µL	

At the end of the incubation period, digestion products were analysed on 2,5% agarose gel (5 g agarose dissolved in 200 mL 0,5X TBE Buffer). 30 µL of

digestion product was mixed with 6 μL of gel loading buffer and applied to the wells of the gel. 6 μL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1,5 hours at 100 V.

2.2.3. Statistical Analysis

SPSS 16.0 statistical software package (SPSS, Chicago, IL, USA) was used for the statistical analysis. Continuous variables were expressed as mean ± SD. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov-Smirnov test. Differences of continuous variables were evaluated by the Independent Samples t-test or Mann-Whitney U test, depending on the shape of the distribution curves. Categorical variables were expressed as proportions and compared using χ^2 test.

In order to determine the effects of vascular risk factors, lipid parameters and CYP1A1 genotypes in the prediction of ischemic stroke, logistic regression analyses with backward selection method was used. Age and sex were also included as covariates. 2-tailed probability values with 95% confidence intervals were estimated for each odds ratio. The Hosmer-Lemeshow goodness of fit test was used for calibration. A *P* value of less than 0.05 was evaluated as statistically significant.

CHAPTER 3

RESULTS

3.1. Study Population

The study population consisted of 226 ischemic stroke patients and 113 healthy controls without symptoms. The two important single nucleotide polymorphisms in Cytochrome P450 1A1 (CYP1A1) gene were studied using the blood samples of participants. In order to determine the genotypes of the participants, PCR/RFLP and allele specific PCR techniques were used. Clinical characteristics like total cholesterol, triglyceride, HLD-cholesterol and LDL-cholesterol levels were measured. In addition, conventional risk factors including hypertension, diabetes, obesity and cigarette smoking were recorded for both groups. Data including all these parameters and the CYP1A1 genotypes of the participants are provided in Appendix D.

The results of the clinical laboratory tests, demographic features and the prevalence of conventional risk factors belonging to patients and control subjects are seperately given in Table 3.1. The age of study population varied between 20 and 85 years in patients and 41 to 90 in the control group. The difference in the mean age of patients (66.7 ± 14.2 years) and the controls (65.5 ±11.9) was not statistically significant (P=0.101). Patient group and control groups contained similar numbers of males and females. The prevalance of all conventional risk factors (hypertension, diabetes, smoking and obesity) were significantly higher in patient group when compared to the control group.

Hypertension was observed in 65.5% of the patients, while 43.4% of the control group were hypertensive (*P*=0.000).

Table 3.1 Clinical characteristics and prevalence of conventional risk factors of ischemic stroke patients and controls.

Parameter	Stroke (n= 226)	Controls (n= 113)	*P*
Age (years)[a]	66.69 ± 14.19	65.48 ±11.88	0.101
Male, n (%)[b]	129 (57.1)	55 (48.7)	0.143
Hypertension, n (%)[b]	148 (65.5)	49 (43.4)	0.000
Diabetes mellitus, n (%)[b]	72 (31.9)	20 (17.7)	0.006
Smokers, n (%)[b]	55 (24.3)	13 (11.5)	0.005
Obesity, n (%)[b]	42 (18.6)	8 (7.1)	0.005
Total cholesterol (mmol/L)[c]	4.82 ± 1.3	4.58 ± 1.3	0.115
Triglycerides (mmol/L)[c]	1.53 ± 0.7	1.41 ± 0.6	0.159
HDL-cholesterol (mmol/L)[c]	1.09 ± 0.3	1.19 ± 0.3	0.008
LDL-cholesterol (mmol/L)[c]	2.94 ± 1.0	2.65 ± 1.0	0.020

Values are either number of subjects, percentage or mean ± SD; [a] Mann-Whitney U test is applied; [b] Chi-square test is applied; [c] Independent Samples T-test is applied

The frequency of diabetic individuals in stroke patients (31.9%) was significantly higher than the frequency of the diabetics in controls (17.7%, *P*=0.006). The frequency of smoking people in the patient group (24.3%) were more than twice the frequency of the smokers in the control group (11.5%, *P*=0.005). Likewise, obesity was observed in 18.6% of the patients which was significantly higher than that of controls (7.1%, *P*=0.005).

Table 3.2 represents the estimations of the effects of those conventional risk factors on ischemic stroke. Four out of all 5 conventional risk factors have significant effects on ischemic stroke. Hypertension, diabetes and smoking

exhibited more than 2-fold relative risk for ischemic stroke. Obesity had almost 3-fold relative risk in patients when compared to the controls. Gender had an insignificant effect on ischemic stroke risk.

Table 3.2 Effects of conventional risk factors on ischemic stroke.

Parameter	**OR (95% CI)**	***P***
Gender (male)	1.402 (0.891-2.207)	0.143
Hypertension	2.478 (1.561-3.934)	0.000
Diabetes mellitus	2.174 (1.244-3.799)	0.006
Smoking	2.474 (1.288-4.753)	0.005
Obesity	2.996 (1.355-6.622)	0.005

According to the clinical laboratory tests given in Table 3.1, the level of LDL-cholesterol was significantly higher in ischemic stroke patients (2.94 ± 1.0 mmol/L) when compared to controls (2.65 ± 1.0 mmol/L, P=0.020); while the level of HDL-cholesterol of patients was significantly lower (1.09 ± 0.3 mmol/L) than that of controls (1.19 ± 0.3 mmol/L, P=0.008). There was an insignificant difference in the total cholesterol levels of patients (4.82 ± 1.3 mmol/L) and controls (4.58 ± 1.3 mmol/L, P=0.115). Likewise, the level of triglycerides in patients (1.53 ± 0.7 mmol/L) was slightly higher than in controls (1.41 ± 0.6 mmol/L, P=0.159).

3.2. Genomic DNA Isolation from Human Whole Blood Samples

In order to ensure the purity of isolated genomic DNA samples, they were tested spectrophotometrically. The absorbance values of DNA samples were measured at 260 nm and 280 nm. The ones that have a A_{260}/A_{280} ratio above 2.0 or below 1.6 were discarded and the DNA isolation procedure was re-

applied to those blood samples . In addition, the quantity of isolated DNA was determined by spectrophotometry and this information was used for calculating the amount of DNA sample required to be added to the PCR reaction mixture.

Obtaining the DNA is a risky procedure; since during the isolation process, DNA may be degraded. It is impossible to use the degraded DNA in the future studies, since it can result in non-specific and unwanted DNA amplification and can give incorrect results and non-specific bands in the agarose gel electrophoresis. In order to test the quality of the isolated DNA, agarose gel electrophoresis technique is used. If the isolated DNA is intact and qualified, one should observe the DNA as an intense single band in the gel. The degradation of the DNA can be understood by the presence of a smear on the gel instead of a dense band. Figure 3.1 shows a representative agarose gel photograph.

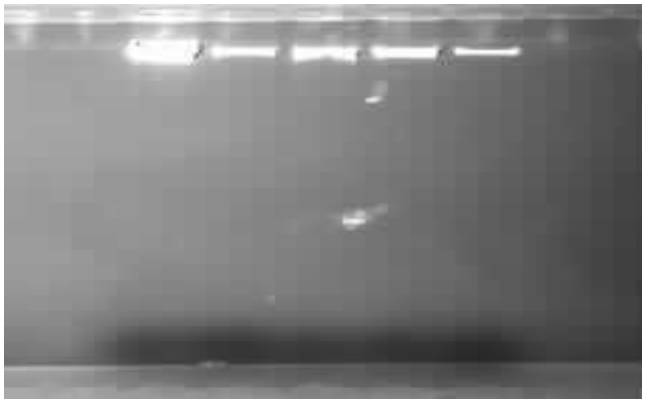

Figure 3.1 Determination of intactness of isolated genomic DNA on 0.7% agarose gel electrophoresis. 5 µL of DNA sample was loaded in each well and run for 1 hour at 100 V.

3.3. Determination of CYP1A1 A4889G Genotype by Allele Specific Polymerase Chain Reaction Method

The A4889G region is in the exon 7 of CYP1A1 gene and this region is amplified by the allele specific PCR method. In order to obtain a single band which belongs to A4889G region of CYP1A1 gene, devoid of non-specific bands, different parameters in the reaction mixture was tested. $MgCI_2$ concentration, primer and DNA template amount and the steps and temperatures of the amplification program were tested in the PCR. Approximately 100 ng DNA, 167 µM dNTPs, 7.5 pmol of each primer, 2 mM $MgCl_2$ and 1.25 unit of Taq Polymerase were included in the PCR reaction.

A4889G single nucleotide polymorphism is at position 2455 in exon 7 of the CYP1A1 gene. This position is occupied with A in the wild type allele; while the same location is G in the mutant allele. This change in the nucleotide results in an Ile to Val substitution at residue 462 which is near the heme binding region of the CYP1A1 protein. This position of the CYP1A1 is a part

of the forward primer's nucleotide sequence and it constitutes the basis of the allele specific PCR method.

There are two types of mixtures used for the same DNA sample; first one including a forward primer (Forward Primer 1) which has a unique DNA sequence that specifically binds to the wild type DNA template and the second one including a forward primer (Forward Primer 2) which has a unique DNA sequence that specifically binds to the mutant DNA template for amplification. Same reverse primer are used for both mixtures. So, if both alleles in the DNA sample are wild type, only forward primer 1 binds to the DNA template for amplification and only the mixture containing forward primer 1 results in the 200 bp PCR product. If both alleles in the DNA sample are mutant, only forward primer 2 binds to the DNA template for amplification and only the mixture containing the forward primer 2 results in the 200 bp PCR product. If there is one mutant and one wild type allele in the DNA sample (heterozygote), both forward primers bind to their complementary DNA strand and both mixtures end up with the 200 bp PCR product.

PCR products were analysed on 2% agarose gel prepared by adding 4 g agarose to 200 mL of 0.5 x TBE buffer as described in section 2.2.1.4. Ten µL of PCR products was mixed with 3 µL of gel loading buffer and applied to the wells of the gel. 6 µL ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V. Figure 3.2 shows a photograph of a representative agarose gel electrophoresis pattern of PCR results for A4889G SNP.

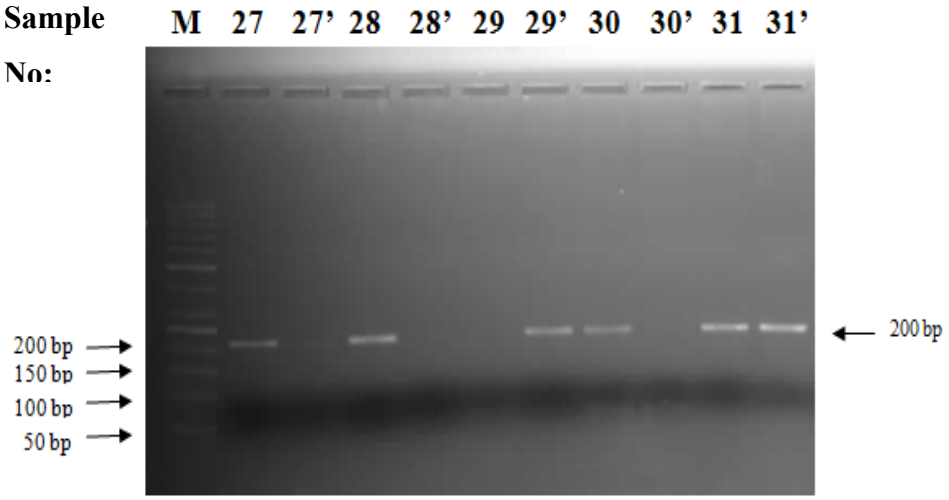

Figure 3.2 2% Agarose gel electrophoresis showing allele specific PCR products of A4889G SNP region of CYP1A1 gene. M means the DNA ladder (50-1000 bp) and the numbers are the PCR products of subjects 27, 28, 29, 30 and 31 respectively. The numbers without quotes are the PCR products of the relevant sample with PCR mixture containing forward primer 1 which amplifies only wild type allele. The numbers with quotes are the PCR products of the relevant sample with PCR mixture containing forward primer 2 which amplifies only mutant allele.

The genotypes of the individuals seen in the figure were decided as;

27-Sample No 27: 4889AA

28-Sample No 28: 4889AA

29-Sample No 29: 4889GG

30-Sample No 30: 4889AA

31-Sample No 31: 4889AG

3.4. Determination of CYP1A1 T6235C Genotype by Polymerase Chain Reaction Followed by Restriction Enzyme Digestion

The T6235C region of the CYP1A1 gene is in the 3' flanking region of the gene. In order to determine the genotypes of stroke patients and controls for T6235C single nucleotide polymorphism, amplification followed by restriction enzyme digestion (with *MspI* restriction endonuclease) procedure was applied.

3.4.1. Polymerase Chain Reaction Results for T6235C SNP

In order to obtain a single band belonging to the T6235C region of CYP1A1 gene, devoid of non-specific bands, different parameters like $MgCl_2$ concentration, primer, template DNA amount and amplification program were tested in PCR. The optimized PCR mixture for the amplification of T6235C SNP is given in "Methods" in Table 2.4. Approximately 100 ng DNA, 0.2 mM dNTPs, 20 pmol of each primer, 2 mM $MgCl_2$ and 1 Unit of Taq Polymerase was included in the PCR reaction.

PCR products were analysed on 2% agarose gel prepared by adding 4 g agarose to 200 mL of 0.5 x TBE buffer as described in section 2.2.1.4. Ten μL of each subject's PCR product was mixed with 3 μL of gel loading buffer and applied to the wells of the gel. 6 μL ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V. Figure 3.3 shows a photograph of a representative agarose gel electrophoresis pattern of PCR results for T6235C SNP.

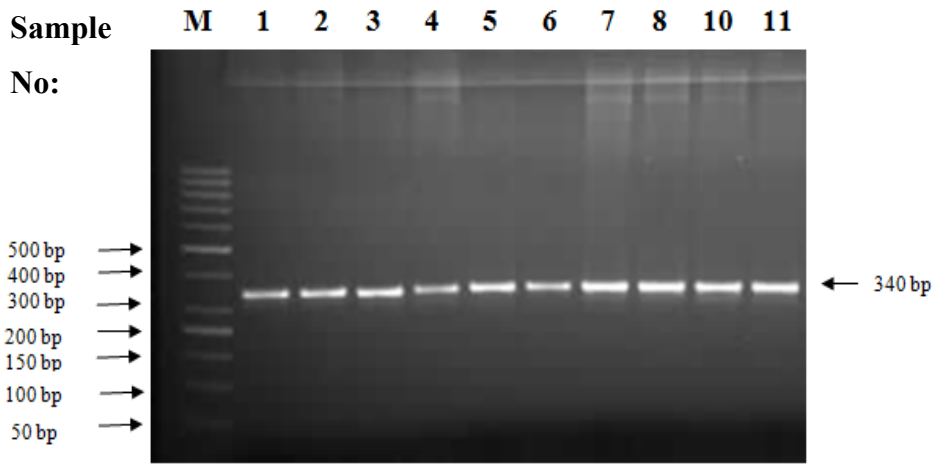

Figure 3.3 2% Agarose gel electrophoresis showing PCR products of T6235C SNP region of CYP1A1 gene. As expected, a single band of 340 bp PCR product was obtained. Lane marked as "M" represents the DNA ladder (50-1000 bp) and lanes marked as 1-11 are the PCR products of the subjects 1, 2, 3, 4, 5, 6, 7, 8, 10 and 11, respectively.

3.4.2. Restriction Endonuclease Digestion Results for T6235C SNP

In the T6235C SNP, there is a transition at position 3801 of genomic DNA, where a cytosine replaces thymidine. This leads to the formation of a restriction endonuclease cleavage site for *Msp*I. This substitution occurs in the 3' noncoding region, downstream from exon 7 of the *CYP1A1* structural gene. Incubation of 10 µL of 340 bp PCR product with 10 U *MspI* restriction enzyme at 37 °C for at least 20 hours efficiently cuts the 340 bp PCR product into two fragments as 200 bp and 140 bp in the mutant allele. However in the wild type allele, there is no recognition site for *MspI*. In this case, digestion with *MspI* yields one fragment of 340 bp undigested PCR product itself.

When analysing the *MspI* restriction endonuclease digestion results in agarose gel, a homozygous wild type individual (6235TT) would yield an undigested single band of 340 bp since *MspI* can not cut the PCR product. On the other

hand, a homozygous mutated individual (6235CC) with Cytosine would yield two bands of 200 bp and 140 bp, as *MspI* would be able to cut the PCR product. In a heterozygote individual (6235TC), both undigested 340 bp band and the digested fragments of 200 bp and 140 bp would be visible.

Digestion products were analysed on 2.5% agarose gel. 30 µL of digestion product was mixed with 6 µL of gel loading buffer and applied to the wells of the gel. 6 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1.5 hours at 100 V. Figure 3.4 shows a representative agarose gel photograph of restriction endonuclease digestion results for T6235C SNP.

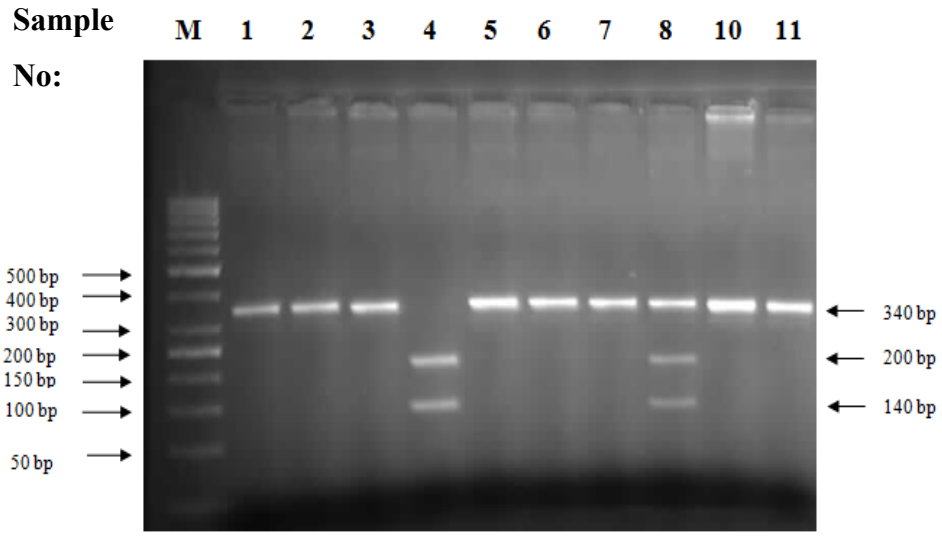

Figure 3.4 2.5% Agarose gel electrophoresis showing restriction endonuclease digestion products of T6235C SNP region of CYP1A1 gene. Lane marked as "M" represents the DNA ladder (50-1000 bp) and lanes marked as 1-11 are the digestion products of the subjects 1, 2, 3, 4, 5, 6, 7, 8, 10 and 11, respectively.

The genotypes of subjects were decided as:

1-Sample No 1: 6235TT
2-Sample No 2: 6235TT
3-Sample No 3: 6235TT
4-Sample No 4: 6235CC
5-Sample No 5: 6235TT
6-Sample No 6: 6235TT
7-Sample No 7: 6235TT
8-Sample No 8: 6235TC
10-Sample No 10: 6235TT
11-Sample No 11: 6235TT

3.5. CYP1A1 Genotypes and Allele Frequencies

The distributon of CYP1A1 genotype and allele frequencies for the A4889G and T6235C single nucleotide polymorphisms within the ischemic stroke patient and healthy control groups are presented in Table 3.3.

Table 3.3 Distribution of genotype and allele frequencies for A4889G and T6235C single nucleotide polymorphisms in stroke patients and controls.

	Stroke (n=226)	**Controls (n=113)**	**OR (95% CI)**	***P***
A4889G				
Genotypes, n(%)				
AA	34 (15.0)	17 (15.0)		
AG	183 (81.0)	96 (85.0)	1.000[a] (0.532-1.881)	1.000
GG	9 (4.0)	0 (0.0)		
Alleles				
A	0.555	0.575	1.084[b] (0.786-1.497)	0.622
G	0.445	0.425		
T6235C				
Genotpes, n(%)				
TT	162 (71.7)	66 (58.4)		
TC	60 (26.5)	43 (38.1)	0.555[c] (0.346-0.690)	0.014
CC	4 (1.8)	4 (3.5)		
Alleles				
T	0.849	0.774	0.607[d] (0.405-0.911)	0.015
C	0.151	0.226		

[a]AG+GG vs. AA, [b]G vs. A, [c]TC+CC vs. TT, [d]C vs. T

In the scope of the study, 226 ischemic stroke patients and 113 healthy control subjects were investigated for the A4889G and T6235C single nucleotide polymorphisms.

For the A4889G polymorphism, 15% of stroke patients and 15% of controls were homozygous wild type (4889AA); while, 81% of stroke patients and 85% of controls were heterozygous (4889AG). It was observed that 4% of the stroke patients had homozygous mutated GG genotype (4889GG). On the other hand, there was not any homozygous mutated individual among controls. Since there was not any individual with GG genotype in the control group, AG and GG genotypes were ascribed to a single group for comparison purposes. The percentage of individuals with GG and AG genotypes did not differ significantly in stroke patients and controls (P=1.000). Similarly, there was not any significant difference between G allele frequencies of patients (0.445) and controls (0.425, P=0.622).

For the T6235C polymorphism, 71.7% of stroke patients and 58.4% of controls had homozygous 6235TT genotype. The percentage of heterozygous subjects (6235TC) were 26.5% and 38.1% in the stroke patients and controls respectively. There was an equal number of homozygous mutant subjects (4) both in the stroke and control group which makes their frequencies 1.8% and 3.5% among stroke patients and controls. When TC and CC genotypes were ascribed to a single group and compared to the wild type TT genotype, the frequency of C allele carriers (individuals with CC and TC genotypes) came out to be significantly different in patients and controls (P=0.014). There was also a significant difference between C allele frequencies of patients (0.151) and controls (0.226, P=0.015).

3.6. CYP1A1 Genotypes in Different Subgroups of Ischemic Stroke Patients and Controls

The CYP1A1 genotypes were analysed in four subgroups having different vascular risk factors. These genotypes were compared between ischemic stroke patients and controls in subgroups of hypertensive versus normotensive, diabetic versus non-diabetic, smoker versus non-smoker and obese versus non-obese.

While making the comparison between genotypes of A4889G polymorphism, the AG and GG genotypes were ascribed to a single group since there was not any GG homozygote in the control group. Similarly, TC and TT genotypes in T6235C polymorphism were also ascribed to a single group against TT genotype.

3.6.1. CYP1A1 Genotypes in Hypertensive-Normotensive subgroup of Ischemic Stroke and Controls

Table 3.4 shows the distribution of the 4889 and 6235 genotypes between patients and controls in hypertensive and normotensive subgroups. For the 4889 genotype, among hypertensives the frequency of risky AG and GG genotypes was almost the same in stroke patients (81.8%) and controls (81.6%). The same genotypes (AG+GG) were slightly higher in the stroke patients (91%) than controls (87.5%) for the normotensive individuals (P=0.496).

For the 6235 genotype, in hypertensives the frequency of TC and CC genotypes was 32.4% in stroke patients which is lower than that of the controls (53.0%). When compared to the wild type TT genotype, the C allele carriers (TC+CC) were significantly higher in controls than in patients (P=0.010). Likewise among the normotensive individuals, the C allele carriers in the

control group were higher than the stroke patient group, but the difference was not significant for the normotensive subgroup (*P*=0.097).

Table 3.4 Distribution of A4889G and T6235C genotypes in hypertensive and normotensive subgroups of stroke patients and controls.

Genotypes n (%)	Hypertensive (n=197)			Normotensive (n=142)		
	Stroke (n=148)	Controls (n=49)	*P*	Stroke (n=78)	Controls (n=64)	*P*
4889 AA	27 (18.2)	9 (18.4)		7 (9.0)	8 (12.5)	
4889 AG	116 (78.4)	40 (81.6)	0.984[a]	67 (85.9)	56 (87.5)	0.496[a]
4889 GG	5 (3.4)	0 (0.0)		4 (5.1)	0 (0.0)	
6235 TT	100 (67.6)	23 (46.9)		62 (79.5)	43 (67.2)	
6235 TC	44 (29.7)	25 (51.0)	0.010[b]	16 (20.5)	18 (28.1)	0.097[b]
6235 CC	4 (2.7)	1 (2.0)		0 (0.0)	3 (4.7)	

[a]AG+GG vs. AA, [b]TC+CC vs. TT

3.6.2. CYP1A1 Genotypes in Diabetic/Non-diabetic subgroup of Ischemic Stroke and Controls

Table 3.5 represents the distribution of A4889G and T6235C genotypes in diabetic/non-diabetic subgroups of stroke patients and controls. For the 4889 genotype, among 92 diabetic individuals, the frequency of AG and GG genotypes (82.3%) in the stroke patients were slightly lower than the frequency of AG and GG genotypes in control group (85.0%). When compared to the wild type AA genotype, the frequency of G allele carriers (AG+GG) were almost the same in both stroke patients and controls (*P*=0.858). This situation was almost the same for the non-diabetic individuals too (*P*=0.868).

For the 6235 genotype, among 92 diabetic individuals, the frequency of TC and CC genotypes were higher in the controls (40%) when compared to stroke patients (26.4%). When compared to the wild type TT genotype, the C allele carriers (TC+CC) were higher in control group than the C allele carriers in the stroke patients group (*P*=0.237). Among 247 non-diabetic individuals, the proportion of TC and CC genotypes were higher in controls (41.9%) when compared to the proportion of same genotypes in ischemic stroke patients (29.2%). The C allele carriers were significantly higher in control group than in stroke patients when compared to the wild type TT genotype (*P*=0.041).

Table 3.5 Distribution of A4889G and T6235C genotypes in diabetic/non-diabetic subgroups of stroke patients and controls.

Genotypes n (%)	Diabetic (n=92)			Non-diabetic (n=247)		
	Stroke (n=72)	Controls (n=20)	*P*	Stroke (n=154)	Controls (n=93)	*P*
4889 AA	12 (16.7)	3 (15.0)		22 (14.3)	14 (15.1)	
4889 AG	59 (81.9)	17 (85.0)	0.858[a]	124 (80.5)	79 (84.9)	0.868[a]
4889 GG	1 (1.4)	0 (0.0)		8 (5.2)	0 (0.0)	
6235 TT	53 (73.6)	12 (60.0)		109 (70.8)	54 (58.1)	
6235 TC	18 (25.0)	7 (35.0)	0.237[b]	42 (27.3)	36 (38.7)	0.041[b]
6235 CC	1 (1.4)	1 (5.0)		3 (1.9)	3 (3.2)	

[a]AG+GG vs. AA, [b]TC+CC vs. TT

3.6.3. CYP1A1 Genotypes in Smoker/Non-smoker subgroup of Ischemic Stroke and Controls

Table 3.6 represents the distribution of A4889G and T6235C genotypes in smoker/non-smoker subgroups of stroke patients and controls. For the 4889 genotype, among 68 smoking individuals, the frequency of AG and GG genotypes in the stroke patients (83.6%) are slightly lower than the frequency of AG and GG genotypes in control group (84.6%). When compared to the wild type AA genotype, the G allele carriers (AG+GG) had almost the same proportion in both stroke patients and controls (*P*=0.931). This situation was nearly the same for the non-smoking individuals (*P*=0.932).

For the 6235 genotype, again among 68 smoking individuals, the proportion of TC and CC genotypes were higher in stroke patients (30.9%) when compared to that of control group (23.1%). When compared to the wild type TT genotype, the C allele carriers (TC+CC) were lower in control group than the C allele carriers in the stroke patients group (*P*=0.577). Among 247 non-smoking individuals, the proportion of TC and CC genotypes were higher in controls (54.0%) when compared to the proportion of TC and CC genotypes in ischemic stroke patients (27.4%). The C allele carriers were significantly higher in control group than stroke patients when compared to the wild type TT genotype (*P*=0.005).

Table 3.6 Distribution of A4889G and T6235C genotypes in smoker/non-smoker subgroups of stroke patients and controls.

Genotypes n (%)	Smokers (n=68)			Non-smokers (n=271)		
	Stroke (n=55)	Controls (n=13)	*P*	Stroke (n=171)	Controls (n=100)	*P*
4889 AA	9 (16.4)	2 (15.4)		25 (14.6)	15 (15.0)	
4889 AG	44 (80.0)	11 (84.6)	0.931[a]	139 (81.3)	85 (85.0)	0.932[a]
4889 GG	2 (3.6)	0 (0.0)		7 (4.1)	0 (0.0)	
6235 TT	38 (69.1)	10 (76.9)		124 (72.5)	56 (56.0)	
6235 TC	17 (30.9)	2 (15.4)	0.577[b]	43 (25.1)	41 (51.0)	0.005[b]
6235 CC	0 (0.0)	1 (7.7)		4 (2.3)	3 (3.0)	

[a]AG+GG vs. AA, [b]TC+CC vs. TT

3.6.4. CYP1A1 Genotypes in Obese/Non-obese subgroup of Ischemic Stroke and Controls

Table 3.7 represents the distribution of A4889G and T6235C genotypes in obese/non-obese subgroups of stroke patients and controls. For the 4889 genotype, among 50 obese individuals, the frequency of AG and GG genotypes in the stroke patients (88.1%) are slightly higher than the frequency of same genotypes in control group (87.5%). When compared to the wild type AA genotype, the G allele carriers had almost the same in both stroke patients and controls (*P*=0.962). This situation was almost the same for the non-obese individuals too (*P*=0.906).

For the 6235 genotype, again among 50 obese individuals, the proportion of TC genotypes were slightly higher in the controls (37.5%) than the stroke patients (31.0%). CC genotype was not observed among the obese individuals. When compared to the wild type TT genotype, the C allele carriers (TC+CC)

were higher in control group than the C allele carriers in the stroke patients group (*P*=0.716). Among 289 non-obese individuals, the proportion of TC and CC genotypes were higher in controls (41.9%) when compared to the proportion of TC and CC genotypes in ischemic stroke patients (27.7%). When compared to the wild type TT genotype C allele carriers were significantly higher in control group than in stroke patients (*P*=0.014).

Table 3.7 Distribution of A4889G and T6235C genotypes in obese/non-obese subgroups of stroke patients and controls.

Genotypes n (%)	Obese (n=50)			Non-obese (n=289)		
	Stroke (n=42)	**Controls (n=8)**	***P***	**Stroke (n=184)**	**Controls (n=105)**	***P***
4889 AA	5 (11.9)	1 (12.5)		29 (15.8)	16 (15.2)	
4889 AG	33 (78.6)	7 (87.5)	0.962[a]	150 (81.5)	89 (84.8)	0.906[a]
4889 GG	4 (9.5)	0 (0.0)		5 (2.7)	0 (0.0)	
6235 TT	29 (69.0)	5 (62.5)		133 (72.3)	61 (58.1)	
6235 TC	13 (31.0)	3 (37.5)	0.716[b]	47 (25.5)	40 (38.1)	0.014[b]
6235 CC	0 (0.0)	0 (0.0)		4 (2.2)	4 (3.8)	

[a]AG+GG vs. AA, [b]TC+CC vs. TT

3.7. Effects of Conventional Vascular Risk Factors in Different CYP1A1 Genotypes of Ischemic Stroke Patients and Controls

The conventional vascular risk factors, hypertension, diabetes, smoking and obesity were examined in terms of proportion of ischemic stroke patients to controls within the risky genotype group and non-risky genotype group. While making the comparison between the genotypes of A4889G polymorphism, the AG and GG genotypes were ascribed to a single group since the sample

number with GG genotype was not statistically satisfactory. For the same reason, TC and CC genotypes were ascribed to the same group for T6235C region.

3.7.1. A4889G Genotype

As can be seen in Table 3.8, in each genotype group of A4889G polymorphism, the proportion of stroke patients to controls was increased in hypertensive, diabetic, smoker and obese group when compared to normotensive, non-diabetic, non-smoker and non-obese group. Within the 4889AG and 4889GG genotype group, the risk of having stroke is 2.385 times higher (*P*=0.0005) in hypertensives when compared to normotensives. Similarly, for individuals carrying 4889AA genotype, the risk of having stroke is 3.428 times higher if they have hypertension when compared to normotensives (*P*=0.050). Overall, having hypertension increases the risk of stroke for all genotypes.

When diabetes is taken into consideration in the 4889AG+GG genotype group, the risk of having stroke is more than two-fold higher in diabetics when compared to non-diabetics (OR= 2.112; 95%CI=1.152-3.874, *P*=0.014). Among the 4889AA genotype, however, the risk of having stroke is insignificantly higher than in non-diabetics (OR=2.545; 95% CI=0.608-10.654, *P*=0.192).

The situation in diabetics also holds for the smoker-non-smoker and obese-non-obese comparisons. We observed that among people with 4889AG or GG genotype, smoking significantly increases the risk of having stroke (OR=2.435; 95% CI=1.197-4.952, *P*=0.012). Among people with 4889AA genotype, the risk of having stroke is higher for smokers when compared to non-smokers, but this is not significant (OR=2.7; 95% CI=0.513-14.208, *P*=0.228). For obesity, among people with risky 4889AG or GG genotype, being obese increases the

risk of stroke for more than three-fold when compared to non-obese people which is a significant risk (OR=3.035; 95% CI=1.299-7.093, *P*=0.008). Among the 4889AA genotype, the risk of stroke is insignificantly higher in obese people than non-obese people (OR= 2.759; 95% CI=0.296-25.709, *P*=0.356).

Table 3.8 Stratification of hypertensive-normotensive, diabetic-non-diabetic, smoker-non-smoker and obese-non-obese individuals according to A4889G genotypes and stroke-control status.

	4889AA (n=51) Stroke / Control	**OR (95%CI)**	***P***	**4889AG+GG (n=288)** Stroke / Control	**OR (95%CI)**	***P***
Hypertensive (n=197)	27 / 9	3.428[a] (0.969-12.137)	0.050	121 / 40	2.385[a] (1.446-3.936)	0.0005
Normotensive (n=142)	7 / 8			71 / 56		
Diabetic (n=92)	12 / 3	2.545[b] (0.608-10.654)	0.192	60 / 17	2.112[b] (1.152-3.874)	0.014
Non-diabetic (n=247)	22 / 14			132 / 79		
Smoker (n=68)	9 / 2	2.7[c] (0.513-14.208)	0.228	46 / 11	2.435[c] (1.197-4.952)	0.012
Non-smoker (n=271)	25 / 15			146 / 85		
Obese (n=50)	5 / 1	2.759[d] (0.296-25.709)	0.356	37 / 7	3.035[d] (1.299-7.093)	0.008
Non-obese (n=289)	29 / 16			155 / 89		

[a]OR calculated against normotensive; [b]OR calculated against non-diabetic; [c]OR calculated against non-smokers; [d]OR calculated against non-obese

3.7.1.1. Effect of CYP1A1 4889 G Allele on Stroke Risk in Risk Groups

The effects of CYP1A1 4889 G allele on stroke risk in hypertensives, diabetics, smokers and obese people is given in Table 3.10. Both in hypertensives (OR=1.075; 95% CI=0.676-1.709, *P*=0.760) and normotensives (OR=1.190; 95% CI=0.744-1.904, *P*=0.467), 4889 G allele carriers were observed to have slightly higher risk of ischemic stroke, but the difference was insignificant for both. Among diabetics, 4889 G allele carriers had almost the same risk of stroke (OR=0.994 95% CI=0.489-2.020, *P*=0.087). Among non-diabetics the risk of ischemic stroke was found to be insignificantly higher for G allele carriers (OR=1.129; 95% CI=0.782-1.629, *P*=0.518). Both for smokers and non-smokers, the G allele carriers had almost the same risk of ischemic stroke. Among obese subjects, the 4889 G allele carriers had higher risk of ischemic stroke (OR=1.225, 95% CI=0.418-3.597, *P*=0.710), whereas among non-obese subjects bearing 4889 G allele did not significantly increase the risk of stroke (OR=1.045, 95% CI=0.742-1.473, *P*=0.798).

Table 3.9 CYP1A1 4889 G allele frequency in risk groups.

Group	4889 G allele frequency		OR (95% CI)	*P*
	Stroke	Control		
Hypertensive	0.425	0.408	1.075 (0.676-1.709)	0.760
Normotensive	0.480	0.437	1.190 (0.744-1.904)	0.467
Diabetic	0.424	0.425	0.994 (0.489-2.020)	0.087
Non-diabetic	0.455	0.425	1.129 (0.782-1.629)	0.518
Smoker	0.436	0.423	1.055 (0.445-2.506)	0.902
Non-smoker	0.447	0.425	1.095 (0.77-1.558)	0.613
Obese	0.488	0.438	1.225 (0.418-3.597)	0.710
Non-obese	0.435	0.424	1.045 (0.742-1.473)	0.798

3.7.2. T6235C Genotype

As shown in Table 3.9; in each genotype group of T6235C polymorphism, the proportion of stroke patients to controls was increased in hypertensive, diabetic, smoker and obese group when compared to normotensive, non-diabetic, non-smoker and non-obese group. The risk of having stroke was significantly higher both in the 6235TT and 6235TC+CC groups in hypertensives relative to normotensives. In hypertensive individuals with 6235TC+CC genotype, the risk of having stroke was 2.423 times higher than normotensives with the same genotype (*P*=0.029). Similarly, for 6235TT genotype, the risk of having stroke was 3.015 times higher than normotensives (*P*=0.0002). Overall, having hypertension significantly increases the risk of stroke for all genotypes.

For diabetics, among individuals with 6235TC+CC genotype, the risk of having stroke was insignificantly higher in diabetic subjects than non-diabetics (OR=2.058; 95% CI=0.812-5.221, *P*=0.124) but for 6235TT genotypes the risk of having stroke was more than two-fold higher in diabetics than non-diabetics (OR= 2.188; 95%CI=1.08-4.434, *P*=0.027).

For obese subjects and smokers, the risk of having stroke was significantly higher in the risky 6235TC+CC genotypes than wild type 6235TT. For smoking, among people with risky 6235TC or CC genotype, we observed that smoking significantly increases the risk of having stroke for more than five-fold (OR=5.304; 95% CI=1.454-19.357, *P*=0.006). Among people with 6235TT genotype, the risk of having stroke is slightly higher for smokers when compared to non-smokers, but this risk was not significant (OR=1.716; 95% CI=0.799-3.687, *P*=0.163). For obesity, again among people with risky 6235TC+CC genotype, being obese significantly increases the risk of stroke for almost four-fold when compared to non-obese people (OR=3.738; 95% CI=1-13.975, *P*=0.038). Among the 6235TT genotype, the risk of stroke is also

significantly higher in obese people than non-obese people (OR= 2.660; 95% CI=0.982-7.204, *P*=0.047).

Table 3.10 Stratification of hypertensive-normotensive, diabetic-non-diabetic, smoker-non-smoker and obese-non-obese individuals according to T6235C genotypes and stroke-control status.

	6235TT (n=228)	OR (95%CI)	*P*	6235TC+CC (n=111)	OR (95%CI)	*P*
	Stroke / Control			Stroke / Control		
Hypertensive (n=197)	100 / 23	3.015[a] (1.659-5.479)	0.0002	48 / 26	2.423[a] (1.081-5.429)	0.029
Normotensive (n=142)	62 / 43			16 / 21		
Diabetic (n=92)	53 / 12	2.188[b] (1.08-4.434)	0.027	19 / 8	2.058[b] (0.812-5.221)	0.124
Non-diabetic (n=247)	109 / 54			45 / 39		
Smoker (n=68)	38 / 10	1.716[c] (0.799-3.687)	0.163	17 / 3	5.304[c] (1.454-19.357)	0.006
Non-smoker (n=271)	124 / 56			47 / 44		
Obese (n=50)	29 / 5	2.660[d] (0.982-7.204)	0.047	13 / 3	3.738[d] (1-13.975)	0.038
Non-obese (n=289)	133 / 61			51 / 44		

[a]OR calculated against normotensive; [b]OR calculated against non-diabetic; [c]OR calculated against non-smokers; [d]OR calculated against non-obese

3.7.2.1. Effect of CYP1A1 6235C Allele on Stroke Risk in Risk Groups

The effect of CYP1A1 6235C allele on stroke risk in hypertensives, smokers and obese people is given in Table 3.11. For both hypertensives and normotensives, 6235 C allele carriers had significantly decreased risk of ischemic stroke (OR= 0.560; 95% CI=0.328-0.957, *P*=0.032 and OR=0.495; 95% CI=0.251-0.979, *P*=0.040 for hypertensives and normotensives respectively). Among smokers, bearing 6235 C allele did not change the risk of ischemic stroke (OR=1.005; 95% CI=0.308-3.286, *P*=0.099), but among non-smokers, 6235 C allele carriers had significantly lower risk of stroke (OR=0.570; 95% CI=0.367-0.888, *P*=0.012). The same situation almost holds for the obese and non-obese subjects. Among obese subjects, 6235 C allele carriers had insignificantly lower risk of stroke (OR=0.793; 95% CI=0.198-3.178, *P*=0.743), but among non-obese subjects, 6235 allele carriers had significantly lower risk of ischemic stroke (OR=0.593; 95% CI=0.385-0.913, *P*=0.017).

Table 3.11 CYP1A1 6235C allele frequency in risk groups.

Group	6235 C allele frequency		OR (95% CI)	*P*
	Stroke	Control		
Hypertensive	0.176	0.275	0.560 (0.328-0.957)	0.032
Normotensive	0.103	0.188	0.495 (0.251-0.979)	0.040
Smoker	0.155	0.154	1.005 (0.308-3.286)	0.099
Non-smoker	0.149	0.235	0.570 (0.367-0.888)	0.012
Obese	0.155	0.188	0.793 (0.198-3.178)	0.743
Non-obese	0.149	0.229	0.593 (0.385-0.913)	0.017

3.8. Logistic Regression Analysis

Logistic regression analyses with backward selection method (Backward likelihood ratio) were used to determine the effects of vascular factors, lipid parameters and CYP1A1 genotypes in the prediction of ischemic stroke. Different binary logistic regression models were established with different combinations of parameters in the overall population or in different subgroups.

Model 1

In model 1, age, sex, hypertension, smoking status, diabetes, obesity, lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP1A1 A4889G and T6235C genotypes were added as covariates and logistic regression analysis revealed hypertension (OR=3.459, 95% CI=2.111-5.667, *P*=0.000), smoking (OR=3.317, 95% CI=1.649-6.672, *P*=0.001) and LDL-cholesterol (OR=1.479, 95% CI=1.175-1.863, *P*=0.001) to be the strongest determinants of ischemic stroke (Table 3.12). In addition, HDL-cholesterol (OR=0.398, 95%CI=0.218-0.729, *P*=0.003) and bearing 6235 mutant C allele (OR=0.480, 95% CI=0.288-0.802, *P*=0.005) are also determinants of stroke with preventive effects. The model predicted 90.3% of cases correctly and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=8.049; 8 degrees of freedom; *P*=0.429).

Table 3.12 Logistic regression analysis of vascular risk factors (age, sex, hypertension, smoking, diabetes and obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP1A1 A4889G and T6235C genotypes (Model 1).

Parameters	OR	95% CI	*P*
Hypertension	3.459	2.111-5.667	0.000
Smoking	3.317	1.649-6.672	0.001
LDL-cholesterol	1.479	1.175-1.863	0.001
HDL-cholesterol	0.398	0.218-0.729	0.003
6235 C allele	0.480	0.288-0.802	0.005

Model 2

In this model, only male subjects were analyzed. Vascular risk factors (age, hypertension, smoking, diabetes and obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP1A1 A4889G and T6235C genotypes were added as covariates and logistic regression analysis revealed hypertension (OR=2.561, 95% CI=1.557-4.214, *P*=0.000), smoking (OR=2.480, 95% CI=1.195-5.145, *P*=0.015) and obesity (OR=4.648, 95% CI=1.033-20.912, *P*=0.045) as significant predictors of ischemic stroke (Table 3.13). The model predicted 100% of cases correctly and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=3.135; 3 degrees of freedom; *P*=0.371).

Table 3.13 Logistic regression analysis of vascular risk factors (age, hypertension, smoking, diabetes and obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP1A1 A4889G and T6235C genotypes in male subjects (Model 2).

Parameters	OR	95% CI	*P*
Hypertension	2.561	1.557-4.214	0.000
Smoking	2.480	1.195-5.145	0.015
Obesity	4.648	1.033-20.912	0.045

Model 3

In this model, only female subjects were analyzed. Vascular risk factors (age, hypertension, smoking, diabetes and obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP1A1 A4889G and T6235C genotypes were added as covariates and logistic regression analysis revealed hypertension, diabetes, smoking, LDL-cholesterol, HDL-cholesterol and bearing 6235 C allele as significant predictors of ischemic stroke where HDL-cholesterol and 6235 C allele were found to have protective effects (Table 3.14). The model predicted 84.5% of cases correctly and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=2.911; 8 degrees of freedom; *P*=0.940).

Table 3.14 Logistic regression analysis of vascular risk factors (age, hypertension, smoking, diabetes and obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP1A1 A4889G and T6235C genotypes in female subjects only (Model 3).

Parameters	**OR**	**95% CI**	***P***
Hypertension	4.154	1.832-9.419	0.001
Diabetes	2.884	1.018-8.168	0.046
Smoking	6.275	1.357-29.017	0.019
LDL-cholesterol	1.779	1.191-2.657	0.005
HDL-cholesterol	0.209	0.072-0.607	0.004
6235 C allele	0.256	0.114-0.575	0.001

It is remarkable that, the stroke risk associated with hypertension and smoking is higher for women when compared to men. While obesity was found to be significantly associated with stroke in male subjects, diabetes and LDL-cholesterol were associated with ischemic stroke risk only for females. HDL-cholesterol levels and bearing 6235 C seems like protective factors against stroke especially for women.

CHAPTER 4

DISCUSSION

A stroke occurs when a blood clot blocks an artery that carries blood from the heart to the body, or a blood vessel breaks, interrupting blood supply to an area of the brain. Stroke is the third leading cause of death worldwide which comprises 9.7% of all deaths leaving behind all kinds of infectious diseases and cancer types (World Health Organization Fact Sheet 2009; Lloyd-Jones *et al.*, 2010). The risk factors of stroke can be modifiable factors that are generally determined by the environmental and lifestyle factors and nonmodifiable factors with genetic bases. Some known risk factors of stroke include the family history of stroke, old age, male sex, hypertension, high blood lipid profile, heart disease, smoking and diabetes mellitus. Stroke causes a great economical burden for patients, families and communities as a whole because of the disability associated with it and consumption of high amount of resources. To reduce the future burden of stroke, the risk factors for this devastating disease require better definition and understanding.

Xenobiotic metabolism is a series of metabolic reaction pathways which modifies the chemical structure of substances foreign to an organism's normal biochemistry. Cytochrome P450s are oxygen-reacting hemeproteins and the most important components of xenobiotic metabolizing system which have the ability to oxidize, peroxidize and/or reduce various substances and drugs in an oxygen and NADPH-dependent manner (Capdevila *et al.,* 1981; Schwartzman *et al.,* 1985). Because of their importance in cellular metabolism and homeostasis (Hasler *et al.,* 1999), these enzyme systems have been

characterized in many species of organisms, including bacteria, fungi, plants, fish, birds, reptiles, insects and mammalian systems (Lu and Levin, 1974) and 58 different P450s have been identified in humans (http://drnelson.uthsc.edu).

The cytochrome P450 1A1 gene (CYP1A1 gene) is part of the cytochrome P450 supergene family. This enzyme is responsible for the metabolism of a vast number of xenobiotics and it acts as the main enzyme in the bioactivation of polycyclic aromatic hydrocarbons (PAH) such as benzo[a]pyrene (Guengerich *et al.,* 1991) which are known as important carcinogens because of their DNA-binding, mutagenic properties (Shimada *et al.,* 1992). Several polymorphisms in the CYP1A1 gene have been described so far and it has been proposed that these polymorphisms affect the function of the enzyme by altering the level of gene expression or the mRNA stability. There is an accumulated literature addressing the presence of an association between some of these mutations and various kinds of cancer and other diseases like CVD (Kawajiri *et al.,* 1990; Nakachi *et al.,* 1991; Tefre *et al.,* 1991; Hirvonen *et al.,* 1992; Drakoulis *et al.,* 1994; Alexandrie *et al.,* 1994; Murata *et al.,* 1998; Huang *et al.,* 1999a; Aktas *et al.,* 2002; Diergaarde *et al.,* 2008; Yeh *et al.,* 2009).

In this study, we aimed to investigate two important CYP1A1 polymorphisms (A4889G and T6235C) as risk factors for ischemic stroke in Turkish population. Conventional risk factors and lipid parameters were also evaluated in stroke patients and healthy control groups. The study population consisted of 226 stroke patients and 113 healthy subjects. While creating the groups, lots of criteria were evaluated in order to avoid significant differences between the patient and control groups. All exclusion criteria were applied to the controls exactly plus not having carotid stenosis (lumen growing) greater than 50% or ulcerated carotid plaque.

Age is one of the most significant nonmodifiable risk factor for stroke. In order to prevent the influence of age on results, the difference in mean age of stroke patients and control group was minimized. The age of study population varied between 20 to 90 with a mean age of 66.7 in patients group and 65.5 in the control group. There was not a statistically significant difference in mean age between the two groups (P=0.101).

Male gender, which is another nonmodifiable risk factor, was reported to increase the stroke incidence rates by 1.25 times (Sacco *et al.,* 1997). In the present study, out of 226 stroke patients, 129 were male showing that they had 1.4 times higher risk of stroke when compared to females. The number of males in the control group (48.7%) was similar with the patient group (57.1%). There was not a statistically significant difference between the groups with respect to gender (P=0.143).

Hypertension, heart disease, cigarette smoking, diabetes, obesity, waist-to-hip ratio, atrial fibrillation, high lipid profile, physical inactivity and over consumption of alcohol are modifiable risk factors for stroke (Collins and MacMahon 1994; Elkind and Sacco, 1998; Sacco *et al.,* 1999; O'Donnell *et al.,* 2010;). In this study, the prevalence of conventional risk factors like hypertension, diabetes, smoking and obesity in the patient group were found to be significantly higher than the control group (Table 3.1). While hypertension and smoking increased the risk of stroke almost 2.5 times, diabetes increased the risk of stroke two-fold. Obesity increased the risk of stroke three-fold (Table 3.2). According to another investigation on Turkish population, hypertension, diabetes and smoking increase the risk of stroke by 63%, 35% and 17%, respectively (Kumral *et al.,* 1998).

There are some studies available in literature investigating the association of blood lipid profile and stroke risk (Iso *et al.,* 1989; Harmesen *et al.,* 1990; Hachinski *et al.,* 1998), yet prospective population-based studies have yielded

controversial results. While some studies have found a positive association between plasma total cholesterol, LDL-cholesterol and triglyceride, (Harmesen *et al.,* 1990; Hachinski *et al.,* 1998) others have found opposing results (Iso *et al.,* 1989). In our study, total cholesterol levels were higher in stroke patients (4.8 ± 1.3 mmol/L) when compared to control group (4.6 ± 1.3 mmol/L), but the difference was insignificant (*P*=0.115). Likewise, the level of triglycerides in patients (1.5 ± 0.7 mmol/L) was slightly higher than in controls (1.4 ± 0.6 mmol/L, *P*=0.159). So, in this study, we found that total cholesterol and triglyceride levels did not constitute a significant risk for stroke.

Most of the studies have found that high levels of HDL-cholesterol decrease the risk of ischemic stroke (Wannamethee *et al.,* 2000; Tanne *et al.,* 2001). According to our findings, the level of HDL-cholesterol of patients was significantly lower (1.1 ± 0.3 mmol/L) than that of controls (1.2 ± 0.3 mmol/L, *P*=0.008); while the level of LDL-cholesterol was significantly higher in ischemic stroke patients (2.9 ± 1.0 mmol/L) when compared to controls (2.6 ± 1.0 mmol/L, *P*=0.020). These results are consistent with the previous similar studies conducted (Tanne *et al.,* 2001). From the current study and previous literature it would be meaningful to state that high levels of LDL-cholesterol increases the risk of stroke while, high levels of HDL-cholesterol exert a protective impact on the vascular health, decreasing the risk of stroke. This statement would be quite reasonable according to the knowledge that LDL-cholesterol is the main constituent of atherosclerotic plaques which in turn triggers the stroke formation. Likewise, the positive effect of HDL-cholesterol has been long known against the vascular plaque accumulation which in turn decreases the risk of stroke.

CYP1A1 polymorphisms show variable frequencies among different ethnic populations. Therefore, it is crucial to identify these polymorphisms even in the healthy subjects. In our study, the CYP1A1 A4889G genotype frequencies of the control subjects were found as 15% AA (wild type genotype) and 85% AG

(heterozygote). There was not any GG mutant subject in the control group. These findings were different from a study done by Aktas *et al.,* on Turkish population where the genotype frequencies were found as 46.7% AA wild type genotype, 47.7% AG genotype and 4.6% GG genotype (Aktas *et al.,* 2002). These results were higher from the genotype frequencies found in other studies on Caucasian populations (Hirvonen *et al.,* 1992; Cascorbi *et al.,* 1996; Taioli *et al.,* 1999). Mutant GG genotype frequency was higher in Indian (6%) (Singh *et al.,* 2006), Chinese (8.2%) (Huang *et al.,* 1999a) and Japanese (8%) (Miyoshi *et al.,* 2002) populations.

The frequencies of 4889 G allele of A4889G polymorphism in healthy subjects from different ethnic populations are given in Table 4.1. In the present study, the frequency of 4889G allele in 113 healthy subjects was found as 0.425. This result is higher than other studies done on Caucasian populations (Hirvonen *et al.,* 1992; Cascorbi *et al.,* 1996; Mrozikiewicz *et al.,* 1997). The frequency of the G allele is lower in Indian (Singh *et al.,* 2006; Chacko *et al.,* 2005), Chinese (Boyapati *et al.,* 2005; Huang *et al.,* 1999a), African-American (Li *et al.,* 2004; Taioli *et al.,* 1999; Garte *et al.,* 1996), American (Li *et al.,* 2004; Taioli *et al.,* 1999; Bailey *et al.,* 1998; Ambrosone *et al.,* 1995), Mestiza (Fragoso *et al.,* 2005) and Japanese (Miyoshi *et al.,* 2002; Kawajiri *et al.,* 1990; Oyama *et al.,* 1995) populations when compared to the results of this study. On the contrary, Teenek population (Fragoso *et al.,* 2005) has higher frequency of 4889 G allele than Turkish population (Table 4.1). A study done by Aynacioglu *et al.* (1998) on Turkish population found 4889 G allele frequency as 0.089 by using restriction enzyme length polymorphism method which is different from the method we used. This may be the reason of different results found and it would not be reasonable to compare Aynacioglu's study with ours since we used allele specific PCR method. Another reason can be the difference between the mean age and the demographic origins of the study populations that are used. It is obvious that differences in genotype and allele frequencies of A4889G polymorphism show the variability of the allele

frequencies from different ethnic populations. Differences of these genotype and allele frequencies even within the same ethnic population suggest that genotype and allele frequencies of A4889G polymorphism should be conducted in groups containing more samples to have more concrete results which will better reflect the allele and genotype composition of the whole population.

Table 4.1 Comparison of CYP1A1 4889 G allele frequency in different populations in healthy subjects.

Ethnicity	**Reference**	**4889 G Allele Frequency**
Indian	Singh *et al.*, 2006	0.220
	Chacko *et al.*, 2005	0.112
Chinese	Boyapati *et al.*, 2005	0.243
	Huang *et al.*, 1999a	0.222
African-American	Li *et al.*, 2004	0.019
	Taioli *et al.*, 1999	0.000
	Garte *et al.*, 1996	0.027
American	Li *et al.*, 2004	0.049
	Taioli *et al.*, 1999	0.086
	Bailey *et al.*, 1998	0.037
	Ambrosone *et al.*, 1995	0.049
Japanese	Miyoshi *et al.*, 2002	0.254
	Kawajiri *et al.*, 1990	0.198
	Oyama *et al.*, 1995	0.202
Finland (Caucasian)	Hirvonen *et al.*, 1992	0.054
German (Caucasian)	Cascorbi *et al.*, 1996	0.028
Poland (Caucasian)	Mrozikiewicz *et al.*, 1997	0.022
Turkey (Caucasian)	This study	0.425
Mestiza (Mexico)	Fragoso *et al.*, 2005	0.344
Teenek (Mexico)	Fragoso *et al.*, 2005	0.654

The frequency of CYP1A1 T6235C polymorphism also shows great difference among different ethnic groups. In the present study, the genotype frequencies of CYP1A1 T6235C polymorphism were found as 58.4% 6235TT, 38.1% 6235TC and 3.5% 6235CC in the control group. These results were also different from the results found for other Caucasian populations (Chen *et al.,* 2007) where the genotype frequencies of 6235TT, 6235TC and 6235CC were 80.6%, 18.4% and 1%; respectively. The genotype frequency of 6235CC found in this study was similar to the ones found in American-African populations (Li *et al.,* 2004; Taioli *et al.,* 1999; Garte *et al.,* 1996; Bailey *et al.,* 1998), while lower than Indian (Singh *et al.,* 2006), Chinese (Shen *et al.,* 2006; Boyapati *et al.,* 2005; Huang *et al.,* 1999a;) and Japanese populations (Miyoshi *et al.,* 2002; Nakachi *et al.,* 1991).

The mutant C allele frequencies of CYP1A1 T6235C polymorphism in different ethnic groups of healthy subjects are provided in Table 4.2. In this study, the frequency of 6235C allele in 113 healthy subjects was found as 0.226. This result was similar to the 6235 C allele frequency found in the Aynacioglu's study on Turkish population (0.181; Aynacioglu *et al.,* 1998). In general, the frequency of this mutant C allele was lower among Caucasians (Hefler *et al.,* 2004; Cascorbi *et al.,* 1996; Li *et al.,* 2004; Tefre *et al.,* 1991; Hirvonen *et al.,* 1992; Mrozikiewicz *et al.,* 1997a; Krajinovic *et al.,* 2001). On the contrary, the frequency of C allele frequency was more than 30% in Indian (Singh *et al.,* 2006), Chinese (Shen *et al.,* 2006; Boyapati *et al.,* 2005; Huang *et al.,* 1999a) and Japanese (Miyoshi *et al.,* 2002; Nakachi *et al.,* 1991) populations.

Table 4.2 Comparison of CYP1A1 6235 C allele frequency in different populations in healthy subjects.

Ethnicity	**Reference**	**6235 C allele frequency**
Indian	Singh *et al.,* 2006	0.306
Chinese	Shen *et al.,* 2006	0.319
	Boyapati *et al.,* 2005	0.389
	Huang *et al.,* 1999a	0.393
Nigerian	Okobia *et al.,* 2005	0.241
German (Caucasian)	Hefler *et al.,* 2004	0.100
	Cascorbi *et al.,* 1996	0.077
American (Caucasian)	Li *et al.,* 2004	0.117
Norway (Caucasian)	Tefre *et al.,* 1991	0.115
Finland (Caucasian)	Hirvonen *et al.,* 1992	0.116
Poland (Caucasian)	Mrozikiewicz *et al.,* 1997a	0.066
Turkey (Caucasian)	Aynacioglu *et al.,* 1998	0.181
	This study	0.226
French (Caucasian)	Krajinovic *et al.,* 2001	0.052
African-American	Taioli *et al.,* 1999	0.229
	Bailey *et al.,* 1998	0.246
	Li *et al.,* 2004	0.229
	Garte *et al.,* 1996	0.220
Japanese	Miyoshi *et al.,* 2002	0.428
	Nakachi *et al.,* 1991	0.332
Mestiza	Fragoso *et al.,* 2005	0.401
Teenek	Fragoso *et al.,* 2005	0.714

Since A4889G polymorphism, which is in the exon 7 of the CYP1A1 gene, causes a Valine residue to be replaced by an Isoleucine in the heme-binding region of the enzyme, studies investigating the effect of this polymorphism on enzyme function have been conducted by several researchers (Crofts *et al.,* 1994; Zhang *et. al.,* 1996). These studies are stating that CYP1A1 G variant increases the activity of the enzyme by altering mRNA stability and induces the enzyme by altering the level of gene expression but these statements are not without opposing views (Tabor *et al.* 2002). For instance, the A4889G polymorphism was found to confer elevated levels of induced CYP1A1 mRNA in one study (Crofts *et al.,* 1994), whereas others found no difference in terms of enzyme activity in wild type and mutant genotypes (Zhang *et. al.,* 1996). In our study, we did not carry out enzyme activity measurements; for this reason we cannot comment on the effects of CYP1A1 4889G allele on enzyme activity.

A4889G polymorphism was studied extensively in relation to different cancer types. A4889G polymorphism was found to be associated with susceptibility to lung cancer in studies of Japanese populations (Kawajiri *et al.,* 1990; Nakachi *et al.,* 1991) but Hirvonen *et al.* found an insignificant association with lung cancer in Caucasians (Hirvonen *et al.,* 1992). In addition, there are some studies in Turkish population investigating the relationship between risk of ovarian cancer, prostate cancer and A4889G polymorphism. In both of these studies, A4889G polymorphism significantly increased the risk of ovarian and prostate cancer risk in Turkish population (Aktas *et al.,* 2002; Aktas *et al.,* 2004).

This polymorphism was also studied in coronary artery disease (CAD). A recent study found a decreased risk for CAD associated with the mutant 4889GG genotype and no association with the wild type 4889AA genotype (Yeh *et al.,* 2009). The association between A4889G polymorphism and CAD

risk was previously investigated in two Caucasian population studies, but none was significant (Wang *et al.,* 2002; Manfredi *et al.,* 2007).

When we compared the CYP1A1 genotypes of stroke patients and controls, we did not observe a significant difference between groups for the CYP1A1 A4889G polymorphism (Table 3.3). 4889AA genotype was 15% for both the patients and controls and 4889AG genotype was similar in patients (85.0%) and controls (81.0%). Mutant 4889GG genotype was 4% in the stroke patients whereas there was not any mutant genotype in the control group (0.0%). There was also no significant difference between mutant G allele frequencies of patients (0.445) and controls (0.425, P=0.622). According to our knowledge, a study investigating the association of CYP1A1 A4889G polymorphism and ischemic stroke is not present in the literature. Thus, this is the first study testing the CYP1A1 A4889G polymorphism as a risk factor for ischemic stroke.

T6235C *(MspI)* polymorphism of CYP1A1 was studied most extensively as a risk factor for cancer. It was found to be associated with lung cancer in some studies (Nakachi *et al.,* 1993; Kawajiri *et al.,* 1996) and a number of studies have found a positive association between this mutation and breast cancer susceptibility (Huang *et al.,* 1999; Chen *et al.,* 2007). On the contrary, in a meta-analysis study recently done by Yao *et al.* over 25,087 subjects, no association was found between breast cancer risk and T6235C polymorphism (Yao *et al.,* 2010).

In addition, there are some studies investigating the association of T6235C polymorphism with CVD and hypertension because of their relation with smoking and PAH exposure. While some of the studies found an association between T6235C polymorphism and CVD (Wang et al., 2002), others have found no association at all (Manfredi *et al.,* 2007; Yeh *et al,* 2009). Gambier *et*

al. investigated the association between this polymorphism and hypertension and could not find a relation between the two (Gambier *et al.,* 2006). Similarly, Cornelis *et al.,* found no association between myocardial infarction and T6235C polymorphism (Cornelis *et al.,* 2004).

In the present study, there was a significant difference between patients and controls both in terms of genotype and allele frequencies for T6235C polymorphism (Table 3.3). When compared to the wild type TT genotype, the frequency of C allele carriers (TC+CC) was significantly higher in controls (41.6%) than the stroke patients (28.3%, *P*=0.014). There was also a significant difference between C allele frequencies of patients (0.151) and controls (0.226, *P*=0.015). Therefore, according to our study, T6235C polymorphism is a significant factor for stroke. It is important to note that in this study, the mutant C allele seems to decrease the risk of stroke since it has higher allele frequency in the control group. In other words, it has a protective effect for stroke. Even though this mutation is in the 3' non-coding region of the CYP1A1 gene, in some studies it has been expressed that the noncoding region is important for mRNA stability and expression of gene products and CYP1A1 gene expression is modulated by post-transcriptional and post-translational mechanisms (Tanguay *et al.,* 1996). This polymorphism in the noncoding 3'-region seems to control CYP1A1 gene expression and functional consequences of this polymorphism were compared to wild-type enzyme and an increase in inducibility of the enzyme was represented (Crofts *et al.,* 1994). However, it is still unknown whether this mutation itself leads to a functional alteration of the RNA-processing or if linked mutations in regulatory regions of the gene are responsible for this alteration (Hasler *et al.,* 1999). As it has been shown by the recent studies (Uno *et al.,* 2004), apart from the PAH metabolism, CYP1A1 enzyme and its related cellular receptor AhR has many important regulatory functions in the body like various cell signaling pathways, normal cell growth, homeostasis and development which may be the causative effect of this protection (Androutsopoulos *et al.,* 2009). Alternatively, this mutation may not

have a direct effect on the enzyme activity or RNA processing but other linked mutations in the regulatory regions of the gene may result in this protective effect.

There is only one study in the literature investigating the association of T6235C polymorphism with stroke and hypertension (Lança *et al.*, 2004). According to this study, T6235C polymorphism constituted a risk factor for stroke and no association was found between this polymorphism and hypertension. In this investigation, cases included only 32 subjects with hypertension and among them only 9 were stroke patients. The control group was composed of 152 controls. Lança and colleagues reported that the 6235C allele was associated with stroke (OR = 5.94; 95% CI = 1.46 - 24.23); but the number of stroke patients were very low (9 subjects) when compared to our stroke patients' amount (226). Thus, the results reported by Lança *et al.* may not be very reliable, when compared to results obtained from larger populations, like ours.

4.1 Effects of vascular risk factors on stroke risk in relation to CYP1A1 genotypes

The CYP1A1 genotypes were analyzed in different subgroups of ischemic stroke patients and controls. Both A4889G and T6235C genotypes were analyzed with respect to hypertension (Table 3.4), diabetes (Table 3.5), smoking (Table 3.6) and obesity (Table 3.7).

We did not detect a significant difference between stroke patients and controls in terms of A4889G genotype frequencies in any of the subgroups (Tables 3.4-3.7). However, when the population was stratified according to the A4889G genotypes, the effect of hypertension on stroke risk was more evident (Table 3.8). The risk of having stroke in hypertensive individuals was 3.4 fold (P=0.050) higher than normotensives within the 4889AA genotype group. For

the 4889AG+GG genotype group, the same risk was 2.4 fold (*P*=0.0005) higher in hypertensives than in normotensives. These results proved that hypertension is a risk factor for stroke and although it could not completely eliminate the risk; mutant G allele decreased the risk of stroke. This may be due to a preventive effect of this mutant allele against stroke. A potential mechanism explaining this preventive effect may be the role of CYP1A1 through estrogen metabolism. CYP1A1, in addition to its role in xenobiotics such as PAHs metabolism, is also responsible for endogen sterol metabolism. CYP1A1 functions in 2-hydroxylation of 17beta-estradiol (E2) and estrone (E1) to the catechol estrogens 2-hydroxy-E2 (2-OH-E2) and 2-hydroxy-E1 (2-OH-E1) in extrahepatic tissues (Lee *et al.,* 2003) and subsequent methylation of catechol estrogens into 2-methoxyestradiol (2-MeOE2) product exhibits antitumorigenic and antiangiogenic effects (Fotsis *et al.,* 1993) and shows cardiovascular protection (Dubey *et al.,* 2001). If mutant G allele increases the activity of CYP1A1 enzyme by altering mRNA stability and induces the enzyme by altering the level of gene expression as suggested by Tabor *et al.* 2002, one can expect an increased estrogen metabolism of CYP1A1 and its cardiovascular protection may have a preventive effect for individuals having G allele. Unfortunately there is no study present in the literature searching the association of A4889G polymorphism and hypertension.

For the T6235C polymorphism, among the hypertensive group, the proportion of TC and CC genotypes was 32.4% in stroke patients, which is significantly lower than that of the controls (53.0%, *P*=0.010).Within each genotype group of T6235C polymorphism, the proportion of stroke patients to controls was increased in hypertensive group when compared to normotensives. The risk of having stroke in hypertensive individuals was 3 fold (*P*=0.0002) higher than normotensives within the wild type 6235TT genotype group. For the 6235TC+CC genotype group, the same risk was 2.4 fold (*P*=0.0005) higher in hypertensives than normotensives. These results showed that hypertension is a risk factor for stroke independently from the T6235C polymorphism but this

polymorphism may have a protective effect against stroke since it decreases the risk of stroke among hypertensives that carry 6235TC or 6235CC genotype. It is well known that estrogen has a protective effect for the arteries and according to the study of Kisselev *et al.,* 2005, 6235 CC genotype had significantly elevated the catalytic activity of CYP1A1 for estrogens. In our study, among 6235TC or CC genotypes, decreased risk of stroke possibly results from the increased protection of arteries due to increased estrogen biosynthesis assisted by CYP1A1 TC or CC genotypes.

Diabetes is another vascular risk factor and for the 4889AG+GG genotypes, it increases the risk of ischemic stroke 2.1 times (*P*=0.014) in diabetics (Table 3.8). Among the individuals with 4889AA genotype, the risk of stroke was insignificantly higher among diabetics when compared to non-diabetics.

For the T6235C polymorphism, among diabetics, the proportion of TC+CC genotypes (40.0%) was higher in the controls when compared to that of the stroke patients (26.4%). Among non-diabetic individuals, the proportion of TC and CC genotypes (41.9%) were significantly higher in controls when compared to the proportion of TC and CC genotypes in ischemic stroke patients (29.0%) (*P*=0.041). Among individuals with 6235TT genotype the risk of having stroke is more than two-fold higher in diabetics than non-diabetics (*P*=0.027). Among 6235TC+CC genotypes, there is no difference in the risk of having stroke for diabetics and non-diabetics. This may also show the protective effect of the heterozygous and homozygous mutant genotypes. While diabetes is a risk factor among individuals with wild type 6235TT genotype, it seems like this risk is eliminated among the people with 6235TC+CC genotype.

Among the individuals having 4889AG+GG genotypes, the risk of having stroke was 2.4 times higher in smokers when compared to non-smokers (*P*=0.012). Within the wild type 4889AA genotype, stroke risk was

insignificantly higher among smokers when compared to non-smokers. These results show that having 4889 AG+GG genotypes is a risk factor for stroke among smoking individuals. It is possible that the effect of A4889G polymorphism on CYP1A1 enzymatic activity becomes more prominent when it is combined with smoking, since cigarette smoke contains PAHs, which are major inducers of CYP1A1.

For the 6235 genotype, among smoking individuals, the proportion of TC and CC genotypes (30.9%) were higher in stroke patients when compared to that of control group (23.1%; *P*=0.577). Among non-smoker individuals, the proportion of TC and CC genotypes (58.0%) were significantly higher in controls when compared to the proportion of TC and CC genotypes in ischemic stroke patients (27.4%; *P*=0.005). The risk of having stroke is 5.3 fold higher (*P*=0.006) in smokers than non-smokers among the 6235TC+CC genotype. However there is not a difference in terms of stroke risk among the smoker and non-smoker subjects having wild type 6235TT genotype (*P*=0.163). A study done by Wang *et al.,* had similar results where light smokers with 6235TC+CC genotype had an increased risk of CVD (Wang *et al.,* 2003). Having mutant C allele causes the induction of CYP1A1 and in our study we found that non-smoker individuals with 6235TC+CC genotypes have a decreased risk of stroke. This may be due to the induction of other protective CYP1A1 mechanisms like homeostasis and signal transduction. However, in the condition of smoking we see that bearing 6235TC+CC genotypes increases the risk of stroke for smokers. This may be due to the additive induction of CYP1A1 by smoking and bearing mutant C allele. As previously mentioned, smokers are over-exposed to high levels of PAHs which are the main inducers of CYP1A1 enzyme. This may be the situation where, the mutation's effect on CYP1A1 enzyme becomes more visible and dominant by smoking habit and this may result in the increased risk of stroke for individuals who smoke and bear mutant C allele at the same time.

The risk of having stroke was 3 times (*P*=0.0008) higher in obese patients when compared to non-obese subjects who carry either 4889AG or 4889GG genotypes. Among the individuals with wild type 4889AA genotype, stroke risk was insignificantly higher among obese individuals when compared to non-obese people. These results show that, having 4889AG or 4889GG genotype is a risk factor for stroke among obese individuals.

For the 6235 genotype, among obese individuals, the proportion of TC genotypes (37.5%) were slightly higher in the controls than the stroke patients (31.0%; *P*=0.716); there was not any CC genotype among the obese individuals. Among non-obese individuals, the proportion of TC and CC genotypes (41.9%) were significantly higher in controls when compared to the proportion of TC and CC genotypes in ischemic stroke patients (27.7%; *P*=0.014). This may be due to the protective effect of mutant C allele and this protection may be more dominant when a factor like obesity does not exert its negative impact on vascular health.

Binary logistic regression analysis in a model containing vascular risk factors (age, sex, hypertension, smoking status, diabetes, obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP1A1 A4889G and T6235C genotypes showed that hypertension, smoking and LDL-cholesterol were significant and the strongest determinants of ischemic stroke. HDL-cholesterol and bearing 6235C allele were also determinants of stroke with preventive effects. When the same analysis was done only on males, hypertension smoking and obesity were significant predictors of ischemic stroke for males. On the other hand, in another analysis done on females, hypertension, diabetes, smoking, LDL-cholesterol, HDL-cholesterol and bearing 6235C allele were significant predictors of ischemic stroke, where HDL-cholesterol and 6235C allele had preventive effects.

Both hypertension and smoking are strong determinants of stroke independently from gender which is a result that one can expect especially when the strong influence of smoking and its resulting PAHs on CYP1A1 enzyme is considered. While obesity is a determinant for stroke among male subjects, it is not for females. This may be due to the estrogen hormone abundantly found in women when compared to men that may mask the negative effect of obesity by its protective impact on vascular flexibility and homeostasis. Other different predictors of stroke in women and men may be due to other hormonal or physiological differences between these two genders.

CHAPTER 5

CONCLUSION

CYP1A1 enzyme is crucial for human beings since it has an important role in oxidation of xenobiotics and also endogen metabolism. CYP1A1 gene is known to be highly polymorphic bearing several single nucleotide polymorphisms that might alter its corresponding enzyme activity. Because of its induction with PAH compounds frequently found in cigarette smoke, a relationship had been postulated between CYP1A1 enzyme and accumulation of mutagenic and carcinogenic ROS. Atherosclerosis, which is the main cause of ischemic stroke, may be associated with variability of PAH and ROS levels due to CYP1A1 polymorphisms. The frequencies of these polymorphisms show great variability in different populations that are mostly studied with various types of cancer and cardiovascular diseases. Therefore, in the present study CYP1A1 A4889G and T6235C polymorphisms were investigated in Turkish population for the first time as risk factors for ischemic stroke.

The study population consisted of 226 ischemic stroke patients and 113 healthy controls with no differences between the two groups with respect to age and gender. Hypertensives, diabetics, smokers and obese individuals in patient group were found to be significantly higher when compared to the control group.

The G allele frequency of CYP1A1 A4889G polymorphism was found to be almost the same among stroke patients and control group. Therefore, we might say that presence of the G allele is not a risk factor for having stroke. The

frequency of this allele in healthy subjects was lower in Chinese, Japanese and Caucasian populations than this population. This is the first study investigating the relation between A4889G polymorphism and stroke. The frequency of the C allele in T6235C polymorphism was found to be significantly lower in stroke patients when compared to controls which demonstrate that mutant 6235C allele may have a preventive effect for risk of stroke.

Both A4889G and T6235C polymorphisms were also analyzed with respect to subgroups of hypertension, diabetes, smoking and obesity. For A4889G polymorphism, our results proved that hypertension is a risk factor for stroke and although it could not completely eliminate the risk; mutant G allele decreased the risk of stroke. Thus we conclude that this may be due to a preventive effect of this mutant G allele against stroke. For the T6235C polymorphism, we conclude that hypertension is a risk factor for stroke independently from the T6235C polymorphism but this polymorphism may have a protective effect against stroke since it decreases the risk of stroke among hypertensives that carry 6235TC+CC genotypes.

In the condition of smoking, we found that having 4889 AG+GG genotypes is a risk factor for stroke among smoking individuals and concluded that the effect of A4889G polymorphism on CYP1A1 enzymatic activity may become more prominent when it is combined with smoking since PAHs found in cigarette smoke are major inducers of CYP1A1. Similarly, we observed that bearing 6235TC+CC genotypes increases the risk of stroke for smokers and concluded that this may be due to the additive induction of CYP1A1 by smoking and bearing mutant C allele. In this condition; mutation's effect on CYP1A1 enzyme may become more visible and dominant by smoking habit and this may result in the increased risk of stroke for individuals who smoke and bear mutant C allele at the same time.

For obesity, we concluded that having 4889AG or 4889GG genotype is a risk factor for stroke among obese individuals.

According to our findings, we conclude that high levels of HDL-cholesterol has a protective effect against stroke and high levels of LDL-cholesterol increases the risk of ischemic stroke. According to our binary logistic regression analysis results; we concluded that hypertension, smoking and LDL-cholesterol were significant and the strongest determinants of ischemic stroke. HDL-cholesterol and bearing 6235C allele were also determinants of stroke which exert preventive effects.

A4889G and T6235C genetic polymorphisms of CYP1A1 gene and effects of these polymorphisms on stroke risk were determined in the present study. The investigation of CYP1A1 polymorphisms came into prominence in Turkish population due to the variations of these allele frequencies in different populations and limited number of studies within CYP1A1 polymorphisms and ischemic stroke risk.

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APPENDIX A

INFORMED CONSENT FOR PATIENTS

BİLGİLENDİRİLMİŞ ONAM (RIZA) FORMU

İnme-felç hastalığı için risk oluşturan faktörleri bulmak üzere yeni bir araştırma yapmaktayız. Araştırmanın ismi **"İstemik İnmeli Hastalarda Sitokrom P4501A1 Genetik Polimorfizminin Analizi"**dir.

Sizin de bu araştırmaya katılmanızı öneriyoruz. Bu araştırmaya katılıp katılmamakta serbestsiniz. Çalışmaya katılım gönüllülük esasına dayalıdır. Kararınızdan önce araştırma hakkında sizi bilgilendirmek istiyoruz. Bu bilgileri okuyup anladıktan sonra araştırmaya katılmak isterseniz formu imzalayınız.

Araştırmaya davet edilmenizin nedeni sizde bu hastalığın bulunmasıdır. Size gerekli tetkikleri yaptıktan sonra bu hastalık için kabul görmüş klasik bir tedavi başlayacağız.

Eğer araştırmaya katılmayı kabul ederseniz Prof.Dr. Okay Vural, Doç.Dr. Şeref Demirkaya ve Uz. Öğ.V. Semai BEK veya onların görevlendireceği bir hekim tarafından muayene edilecek ve bulgularınız kaydedilecektir. Bu çalışmayı yapabilmek için kolunuzdan 10 ml (2 tüp) kadar kan almamız gerekmektedir. Bu kandan çalışmada kullanılacak olan tetkikler çalışılacaktır.

Bu çalışmaya katılmanız için sizden herhangi bir ücret istenmeyecektir. Çalışmaya katıldığınız için size ek bir ödeme de yapılmayacaktır. Kan alımı sizin hastalığınız klinik takibi sırasında alınacak kanlar alınır iken 2 tüp fazladan alınacaktır. Dolayısı ile size ek bir işlem yapılmayacaktır.

Yapılacak araştırmanın getireceği olası yararlar: Böyle bir analiz hastalığınıza sebep olan beyin damarlarınızın tıkanmasına yol açan veya damarınızın tıkanması için risk oluşturan faktörleri tespit edilmesinin öğrenilmesinde yararlı olacaktır. Şu anda bu çalışmanın hemen size bir fayda olarak dönüp dönmeyeceğini bilmiyoruz. Ancak ilgili hastalığın temelinde yatan nedenlerin öğrenilmesinde ve gelecekte yeni tedavi yaklaşımlarının geliştirilmesi, bu hastalık geçirme riski olan hastaların önceden tespit edilmesi ve belki de hastalık geçirmeden önce önlem alınmasında fayda sağlayacaktır.

Bu çalışmaya katılmayı reddedebilirsiniz. Bu araştırmaya katılmak tamamen isteğe bağlıdır ve reddettiğiniz takdirde size uygulanan tedavide ya da bundan sonra kliniğimizde size karşı davranışlarımızda herhangi bir değişiklik olmayacaktır. Yine çalışmanın herhangi bir aşamasında onayınızı çekmek hakkına da sahipsiniz.

Hastanın Beyanı

Sayın Prof Dr. Okay Vural, Doç.Dr. Şeref Demirkaya ve Uz.Öğ.V. Semai BEK tarafından Gülhane Askeri Tıp Akademisi Nöroloji Anabilim Dalı'nda tıbbi bir araştırma yapılacağı belirtilerek bu araştırma ile ilgili yukarıdaki bilgiler bana aktarıldı. Bu bilgilerden sonra böyle bir araştırmaya "katılımcı" olarak davet edildim.

Eğer bu araştırmaya katılırsam hekim ile aramda kalması gereken bana ait bilgilerin gizliliğine bu araştırma sırasında da büyük özen ve saygı ile yaklaşılacağına inanıyorum. Araştırma sonuçlarının eğitim ve bilimsel

amaçlarla kullanımı sırasında kişisel bilgilerimin ihtimamla korunacağı konusunda bana yeterli güven verildi.

Araştırma için yapılacak harcamalarla ilgili herhangi bir parasal sorumluluk altına girmiyorum. Bana da bir ödeme yapılmayacaktır.

İster doğrudan, ister dolaylı olsun araştırma uygulamasından kaynaklanan nedenlerle meydana gelebilecek herhangi bir sağlık sorunumun ortaya çıkması halinde, her türlü tıbbi müdahalenin sağlanacağı konusunda gerekli güvence verildi. (Bu tıbbi müdahalelerle ilgili olarak da parasal bir yük altına girmeyeceğim).

Bu araştırmaya katılmak zorunda değilim ve katılmayabilirim. Araştırmaya katılmam konusunda zorlayıcı bir davranışla karşılaşmış değilim. Eğer katılmayı reddedersem, bu durumun tıbbi bakımıma ve hekim ile olan ilişkime herhangi bir zarar getirmeyeceğini de biliyorum.

Bana yapılan tüm açıklamaları ayrıntılarıyla anlamış bulunmaktayım. Kendi başıma belli bir düşünme süresi sonunda adı geçen bu araştırma projesinde "katılımcı" olarak yer alma kararını aldım. Bu konuda yapılan daveti büyük bir memnuniyet ve gönüllülük içerisinde kabul ediyorum.

Katılımcı

Adı, soyadı:

Adres:

Tel:

İmza:

Görüşme tanığı

Adı, soyadı:

Adres:

Tel:

İmza:

Katılımcı ile görüşen hekim

Adı soyadı, unvanı:

Adres:

Tel:

İmza:

APPENDIX B

INFORMED CONSENT FOR CONTROLS

GÖNÜLLÜ BİLGİLENDİRİLMESİ

Araştırma beyin damar tıkanması sonucu oluşan felç-inme hastalığına sebep olan veya katkıda bulunan durumların ortaya konmasına yönelik bir çalışmadır. İnme-felç için risk oluşturan birçok hastalık ve durumu şu an için biliyoruz. Bizim yapacağımız çalışma bunların dışında da bu hastalık için risk oluşturabilecek faktörlerin olup olmadığının araştırılmasıdır. Bu amaçla kanda yüksek yoğunluktaki yağ proteinine (HDL) bağlı olarak bulunan ve eksikliğinde damar sertliği ve sonuçta damar tıkanmasına sebep olabilen paraoksonaz 1 ve benzeri enzimlerin aktivitesi ve genetik durumu incelenecektir. Yapacağımız çalışma daha önce temelde aynı mekanizmaya dayanan kalp krizi için yapılmış ve anlamlı sonuçlar bulunmuştur. Bu işlem için sizden 2 tüp 10 ml kan alınacak ve çalışmalar buradan yapılacaktır. Kan alımı sizin hastalığınızın klinik takibi sırasında alınacak kanlar ile birlikte alınacak ve size ek bir işlem yapılmayacaktır. Sizden 2 tüp kan alımı dışında her hangi bir işlem veya bu çalışmayla ilişkili ek bir tedavi yapılmayacaktır. Araştırma sırasında oluşabilecek herhangi bir zararlı durumu yoktur. Sizden sadece kan alınacaktır. Araştırmaya gönüllü olarak katılmaktasınız ve araştırmaya katılmakta tamamen serbestsiniz. Çalışmada yer alacak gönüllü sayısı yaklaşık 150 hasta ve 150 sağlıklı kişi olacaktır.

Çalışmada yer aldığınız ve bilimsel gelişmelere katkılarınızdan dolayı teşekkür ediyoruz.

Açıklamaları Yapan Araştırmacının

Adı, Soyadı:

Görevi:

İmzası:

Açıklamayı başından sonuna kadar tanıklık eden kişinin

Adı, Soyadı:

Adresi:

İmzası:

Çalışmaya katılan gönüllünün

Adı, soyadı:

Adres:

İmzası:

APPENDIX C

ETHICAL COMMITTEE APPROVAL FORM

HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
A N K A R A

22 Şubat 2008

Y. ETİK KRL. : 1491 - 547 - 08
KONU : GATA Etik Kurulu

Doç. Dr. Şeref DEMİRKAYA

20 Eylül 2005 tarihli 43. Oturumda GATA Etik Kurulu'ndan onay almış olan **"Paraoksonaz 1'in Aktivite ve Gen Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** başlıklı çalışmanın adının **"HMG-Co Redüktaz, Lesitin Kolesterol Asetil Transferaz, GST Transferazlar, Lipoproteinler ve Sitokrom P450 Enzimlerinin Genetik Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** olarak değiştirilmesi ile ilgili protokol değişikliği başvurunuz ile ilgili, GATA Etik Kurulu'nun kararı EK'tedir.

Rica ederim.

Ali Uğur URAL
Prof. Tbp. Kd. Alb.
GATA Etik Kurulu Başkanı

EK :
1 Adet Etik Kurul Raporu

HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
ETİK KURUL TOPLANTI RAPORU

OTURUM NO : 103
OTURUM TARİHİ : 15 Şubat 2008
OTURUM BAŞKANI : Prof. Tbp. Kd. Alb. Ali Uğur URAL
OTURUM SEKRETERİ : Doç. Dr. Ecz. Kd. Alb. Adnan ATAÇ

GATA Etik Kurulu'nun 15 Şubat 2008 günü yapılan 103. oturumunda; GATA Nöroloji AD'dan Doç.Dr. Şeref Deirkaya'nın sorumlu araştırmacılığını yaptığı 20 Eylül 2005 tarihli 43. Oturumda GATA Etik Kurulu'ndan onay almış olan **"Paraoksonaz 1'in Aktivite ve Gen Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** başlıklı çalışmanın adının **"HMG-Co Redüktaz, Lesitin Kolesterol Asetil Transferaz, GST Transferazlar, Lipoproteinler ve Sitokrom P450 Enzimlerinin Genetik Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** olarak değiştirilmesi ile ilgili protokol değişikliği değerlendirildi.

Protokol değişikliğinin amaç, yöntem ve yaklaşım bakımından etik ilkelere UYGUN olduğuna karar verildi.

BAŞKAN	**ÜYE**	**ÜYE**	**ÜYE**
Ali Uğur URAL Prof.Tbp.Kd.Alb.	Ali İhsan UZAR Prof.Hv.Tbp.Kd.Alb.	Ayhan KUBAR Prof.Tbp.Alb.	Adnan ATAÇ Doç.Dr.Ecz.Kd.Alb.
ÜYE	**ÜYE**	**ÜYE**	
Mükerrem SAFALI Doç.Tbp.Kd.Alb.	K. Melih AKAY Doç.Tbp.Kd. Alb.	Mustafa ÖZER Doç.Tbp.Alb.	
ÜYE	**ÜYE**	**ÜYE**	
Ergün TOZKOPARAN Doç. Tbp. Alb.	Nalân AKBAYRAK Prof. Dr. Sağ. Yb.	Muharrem UÇAR Yrd.Doç.J.Tbp.Yb.	

APPENDIX D

LIST OF STUDY POPULATION

Table D.1 List of study population composed of 226 stroke patients and 113 controls.

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
1	Patient	75	M	Y	Y	Y	N	N	3.09	1.28	1.85	0.62	AA	TT
2	Patient	57	F	Y	Y	N	N	N	5.46	1.53	3.38	1.31	AA	TT
3	Patient	41	M	N	N	Y	N	100%	4.32	1.57	2.67	0.87	AA	TT
4	Patient	73	M	Y	N	N	N	N	3.72	0.72	1.9	1.46	AA	CC
5	Patient	53	M	Y	Y	N	N	N	10.43	2.6	7.59	1.51	AA	TT
6	Patient	66	M	Y	Y	Y	N	N	3.38	1.42	2.18	0.51	AA	TT
7	Patient	84	F	Y	N	N	N	N	3.77	0.66	1.79	1.62	AA	TT
8	Patient	56	F	Y	Y	N	N	N	3.59	2.38	1.79	0.97	AG	TC
10	Patient	67	F	Y	N	N	N	N	5.43	1.19	3.56	1.26	AG	TT
11	Patient	76	M	Y	N	Y	N	N	4.03	0.76	2.33	1.28	AG	TT
13	Patient	75	F	Y	N	N	N	N	4.73	1.17	3.26	0.87	AG	TT
14	Patient	74	F	Y	Y	N	N	50%	4.34	0.7	2.74	1.23	AG	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
15	Patient	68	F	Y	N	Y	N	N	3.64	2.27	1.92	0.64	AG	TT
16	Patient	72	F	N	N	N	N	N	6.66	2.84	4.23	1.03	AG	TT
17	Patient	84	M	Y	N	N	N	N	4.13	1.94	2.38	0.79	AG	TC
18	Patient	81	F	Y	N	N	N	70%	4.89	1.75	2.95	1.08	AA	TC
19	Patient	73	F	Y	Y	N	N	N	5.38	2.27	3.21	1.08	AA	TT
20	Patient	73	F	Y	N	N	Y	N	4.68	0.99	2.74	1.41	AA	TT
21	Patient	67	F	Y	N	N	N	N	5.41	1.39	3.67	1.03	AA	TC
23	Control	71	M	Y	N	N	N	N	3.85	1.34	2.1	1.08	AA	TC
24	Patient	61	M	Y	Y	N	N	100%	4.39	1.37	2.77	0.95	AA	TT
25	Patient	40	M	N	N	Y	N	N	3.77	1.17	2	1.21	AA	TT
26	Control	61	F	Y	N	N	N	N	3.38	0.93	1.18	1.74	AA	TC
27	Patient	60	F	Y	Y	N	Y	90%	4.99	1.69	3.05	1.1	AA	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
29	Patient	75	M	N	N	N	N	50%	4.55	1.08	1.82	2.18	GG	TC
30	Patient	76	F	Y	N	N	N	N	3.22	1.52	1.28	1.18	AA	TT
31	Control	76	M	Y	Y	N	N	50%	3.43	1.04	1.9	1.00	AG	TC
32	Control	51	M	N	N	Y	N	N	3.69	1.29	2.13	0.9	AG	TT
33	Control	50	F	N	N	N	N	N	3.61	2.02	1.87	0.77	AA	TT
34	Control	42	F	N	N	N	N	N	2.7	1.27	1.46	0.62	AA	TT
35	Control	45	F	N	N	Y	N	N	3.67	0.6	1.87	1.46	AG	TT
36	Patient	70	M	Y	N	N	N	40%	3.02	0.82	1.51	1.08	AA	TT
38	Control	63	M	Y	Y	Y	N	40%	3.04	1.12	1.54	0.95	AA	TT
39	Patient	83	F	N	N	N	N	70%	7.25	2.36	4.67	1.41	AA	TT
40	Control	63	F	N	N	N	N	N	5.2	2.48	3.13	0.87	AG	TT
42	Control	75	M	Y	Y	N	N	N	4.73	1.72	2.56	1.31	AA	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
43	Control	58	F	Y	N	N	N	N	2.7	1.09	1.1	1.08	AA	TC
44	Control	78	F	Y	N	N	N	30%	3.61	1.07	1.82	1.26	AG	TT
46	Control	74	M	N	N	N	N	50%	4.34	0.92	2.56	1.31	AA	TT
47	Patient	71	M	Y	N	N	N	50%	5.38	2.61	3.23	0.90	AG	TT
50	Control	61	M	N	N	N	N	N	5.93	1.87	3.97	1.03	AG	TT
51	Control	85	M	Y	N	N	N	50%	3.09	1.8	0.62	1.62	AG	TT
52	Control	65	F	N	Y	N	N	50%	6.11	1.42	3.23	2.15	AG	TC
53	Control	65	M	N	N	N	N	N	4.97	0.64	3.28	1.33	AG	TT
54	Control	58	M	N	Y	Y	N	N	5.95	1.2	4.33	1.00	AG	TT
55	Control	61	F	N	N	N	Y	N	6.81	1.83	4.36	1.51	AG	TT
57	Control	80	F	N	N	N	N	N	3.35	0.71	1.18	1.82	AG	TT
59	Control	76	M	N	N	N	N	50%	6.01	2.31	3.67	1.21	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
62	Control	69	F	Y	N	N	N	N	3.93	1.76	1.9	1.18	AG	TT
63	Control	66	M	N	Y	Y	N	N	6.97	3.92	4.1	0.97	AG	TT
64	Control	60	F	Y	N	N	N	N	4.97	1.38	3.1	1.15	AA	TC
66	Patient	64	F	N	N	Y	Y	50%	4.32	1.06	2.54	1.23	AG	TT
67	Patient	58	F	Y	Y	N	N	N	9.1	4.04	5.85	1.28	AG	TT
68	Patient	74	F	Y	N	N	Y	50%	6.27	1.73	4.15	1.23	AG	TT
69	Control	71	M	N	N	N	N	50%	4.29	1.57	2.05	1.41	AG	TC
71	Patient	80	F	Y	N	N	Y	100%	3.77	0.97	2.31	0.97	AG	TT
72	Patient	62	M	Y	Y	N	N	70%	4.89	1.2	3.28	1.00	AG	TC
73	Control	68	M	N	N	N	N	N	5.46	1.29	3.15	1.64	AG	CC
74	Control	65	F	N	N	N	N	N	5.2	1.76	3.15	1.18	AG	TT
76	Control	72	F	Y	N	N	N	50%	6.08	1.67	4.05	1.18	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
77	Control	65	M	N	N	N	N	N	3.04	0.9	1.64	0.95	AG	TT
79	Control	70	F	N	N	N	N	50%	4.86	1.48	3.03	1.10	AG	TT
80	Control	70	F	Y	N	N	N	50%	5.25	1.18	3.33	1.31	AG	TT
81	Control	65	F	Y	N	N	N	N	5.95	1.76	3.77	1.31	AG	CC
82	Patient	63	M	Y	N	N	N	N	3.41	1.00	1.85	1.05	AG	TT
83	Control	65	F	Y	N	N	N	N	4.52	1.27	2.18	1.69	AG	TC
85	Patient	68	M	Y	N	N	N	N	4.45	0.9	2.44	1.54	AG	CC
86	Control	47	M	N	N	N	N	N	6.27	3.06	3.33	1.46	AA	TT
87	Patient	77	F	Y	N	N	N	90%	5.07	1.07	3.51	1.00	AG	TC
88	Control	77	F	Y	Y	N	N	N	4.06	0.98	2.64	0.92	AG	TT
89	Patient	80	F	Y	N	N	N	N	5.23	1.06	3.08	1.59	AG	TT
90	Control	71	M	N	N	N	N	N	4.37	1.47	2.31	1.33	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
92	Control	73	M	Y	N	N	N	50%	3.64	2.47	1.95	0.51	AG	TT
93	Control	61	M	N	N	Y	N	N	4.68	0.39	2.92	1.51	AG	CC
95	Patient	62	M	N	N	N	N	50%	3.69	1.01	1.38	1.10	AG	TT
96	Patient	77	M	N	N	N	N	50%	3.82	0.61	1.74	1.74	AG	TT
97	Patient	24	M	N	N	N	N	N	6.11	2.87	3.67	1.05	AG	TT
98	Patient	53	F	N	N	Y	N	N	2.5	2.16	0.59	0.90	AG	TT
99	Patient	61	M	N	N	N	N	N	4.84	1.6	3.1	0.95	AG	TT
101	Control	52	M	N	N	N	N	N	6.94	1.16	4.69	1.62	AG	TT
102	Patient	78	M	Y	N	Y	N	50%	5.23	2.06	3.08	1.13	AG	TT
103	Control	65	M	N	N	N	Y	N	4.16	0.85	2.9	0.82	AG	TC
105	Patient	81	M	Y	N	N	N	70%	3.46	3.03	0.95	1.08	AG	TC
106	Patient	80	F	Y	N	N	Y	70%	2.73	1.27	1.46	0.64	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
107	Control	50	F	Y	N	N	N	N	3.2	0.85	2.15	0.62	AG	TT
108	Control	87	F	Y	N	N	Y	50%	2.76	1.02	1.38	0.87	AG	TT
109	Control	67	M	N	N	N	N	N	4.37	1.15	2.1	0.15	AG	TC
110	Patient	84	M	Y	N	N	N	70%	3.72	1.1	1.9	1.26	AG	TT
112	Control	50	M	N	N	N	N	N	3.82	0.62	2.03	1.46	AG	TT
114	Patient	26	M	N	N	N	N	N	4.21	1.07	2.05	1.62	AG	TT
115	Patient	55	M	Y	N	N	N	N	5.04	0.93	3.15	1.38	AG	TT
117	Patient	73	F	Y	N	N	N	70%	4.00	1.13	2.54	0.9	AG	TT
119	Control	80	F	N	Y	N	N	N	3.98	1.38	1.97	1.31	AG	TC
120	Patient	36	M	N	N	N	N	N	4.86	1.57	2.97	1.10	AG	TT
121	Patient	56	M	N	N	N	N	N	4.34	1.22	2.56	1.15	AG	TT
122	Patient	47	F	N	N	N	N	N	4.78	1.38	3.15	0.92	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
123	Patient	21	M	N	N	Y	N	N	2.63	0.43	1.18	1.21	AG	TT
125	Patient	84	M	Y	Y	N	N	N	4.76	0.98	3.03	1.23	AG	TT
126	Patient	73	M	Y	Y	N	N	N	5.15	2.52	3.03	0.90	AG	TC
127	Patient	73	F	Y	N	N	N	70%	4.00	1.13	2.54	0.90	AG	TT
128	Patient	66	F	N	N	N	N	70%	3.35	2.79	1.49	0.54	AG	TC
129	Patient	74	F	Y	N	N	N	70%	5.98	1.43	3.95	1.31	AA	TT
130	Control	44	M	N	N	Y	N	N	5.2	2.38	2.46	1.59	AG	TT
131	Control	51	F	N	N	N	N	N	4.37	0.57	2.82	1.23	AG	TC
132	Control	67	M	Y	N	N	N	N	4.47	1.66	2.59	1.05	AG	TT
133	Control	73	F	Y	Y	N	N	N	5.9	2.31	3.67	1.10	AG	TC
134	Control	88	F	Y	N	N	N	50%	4.00	0.8	2.56	1.03	AG	TC
136	Patient	66	M	Y	Y	N	N	N	3.35	2.85	1.26	0.74	AG	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
138	Patient	61	F	Y	Y	N	N	50%	3.22	2.00	1.62	0.64	AG	TT
139	Patient	78	M	N	N	Y	N	N	3.38	1.24	1.79	1.03	AG	TC
140	Control	59	M	N	N	N	N	N	4.08	1.24	2.31	1.15	AG	TT
142	Patient	80	M	Y	N	N	N	N	7.90	0.83	6.13	1.28	AG	TT
143	Patient	76	F	Y	Y	N	N	90%	4.11	1.24	2.51	0.97	AG	TT
144	Patient	79	F	Y	N	N	N	N	5.25	1.53	3.31	1.18	AG	TT
146	Control	51	F	N	N	N	N	N	2.86	1.01	1.38	0.97	AG	TC
147	Patient	21	M	N	N	N	N	N	3.87	0.93	2.56	0.82	AG	TC
148	Patient	76	M	N	N	N	Y	N	5.02	1.19	3.38	1.03	AG	TC
151	Patient	28	M	N	N	Y	N	N	3.93	1.64	2.00	1.13	AG	TT
152	Patient	20	M	N	N	Y	N	N	4.32	0.90	2.56	1.28	GG	TC
154	Patient	64	F	Y	N	N	N	N	3.77	1.62	1.97	1	AG	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
157	Patient	58	F	Y	Y	N	N	N	7.12	2.88	4.33	1.38	AG	TT
158	Control	77	M	N	N	N	N	N	4.65	1.88	2.05	1.18	AG	TC
161	Patient	78	F	N	N	N	N	50%	4.63	0.99	2.64	1.46	AG	TT
162	Patient	65	M	N	N	N	N	100%	4.00	1.84	2.03	1.08	AG	TT
163	Patient	75	M	Y	N	N	N	90%	5.07	1.60	3.33	0.95	AG	TT
164	Patient	79	M	N	N	Y	N	50%	2.73	0.90	1.38	0.90	AG	TT
165	Patient	73	F	Y	Y	N	N	90%	7.57	3.54	5.26	0.59	AG	TC
166	Patient	84	F	Y	N	N	N	90%	6.71	0.88	4.36	1.85	AG	TC
167	Patient	25	M	N	N	N	N	N	4.6	1.57	2.74	1.1	AG	TT
168	Patient	73	M	Y	N	N	N	N	5.07	0.89	3.05	1.54	AG	TT
169	Patient	74	M	Y	N	N	N	90%	3.33	1.33	1.79	0.87	AG	TC
172	Patient	56	M	Y	Y	N	N	N	4.6	1.76	2.69	1.05	AG	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
173	Patient	67	M	Y	N	N	N	N	4.29	1.73	2.28	1.1	AA	TC
174	Patient	74	M	Y	N	N	N	N	4.39	2.26	2.03	1.28	AG	TT
175	Patient	64	M	Y	Y	N	N	N	4.37	1.91	2.38	1.05	AG	TT
176	Patient	73	M	Y	Y	N	N	50%	4.63	1.94	2.56	1.1	AG	TC
177	Control	52	F	N	N	N	N	50%	3.82	1.12	1.87	1.38	AG	TT
179	Patient	57	M	N	N	Y	N	N	4.86	1.67	2.97	1.05	AG	TT
180	Patient	76	M	N	N	N	N	50%	4.19	1.92	2.41	0.85	AG	TC
181	Patient	61	M	N	N	Y	N	100%	4.86	1.39	2.97	1.18	AG	TT
182	Patient	85	M	N	N	Y	N	N	2.26	0.82	0.79	1.05	AG	TT
184	Control	77	F	Y	N	N	N	50%	4.21	2	2.03	1.21	AG	TC
185	Patient	62	F	N	Y	N	N	N	4.08	1.63	2.33	0.95	AA	TT
186	Patient	73	M	N	N	Y	N	N	7.9	2.98	5.23	1.21	AA	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
188	Patient	52	M	N	N	Y	N	100%	4.11	1.1	2.77	0.79	AG	TC
189	Control	79	M	N	N	N	N	N	5.25	0.87	3.85	0.95	AG	TT
190	Patient	61	F	Y	N	N	N	70%	3.15	1.39	1.59	0.87	AG	TT
191	Control	46	M	Y	N	Y	N	N	7.90	2.83	5.31	1.21	AG	TC
193	Patient	45	M	N	N	Y	N	N	3.93	3.74	1.31	0.85	AG	TC
195	Patient	56	F	N	Y	N	N	N	7.02	3.19	4.33	1.13	AG	TT
196	Patient	67	M	Y	Y	N	N	70%	6.92	2.84	4.38	1.13	AG	TT
197	Patient	53	M	Y	N	N	N	N	7.38	3.39	4.51	1.23	AG	TT
202	Patient	80	M	N	N	N	N	70%	5.72	1.34	3.74	1.28	AG	TT
203	Patient	62	M	Y	Y	N	N	50%	5.56	1.24	3.67	1.18	AG	TC
204	Patient	83	F	Y	N	N	N	100%	3.64	1.10	1.97	1.10	AG	TT
207	Control	41	F	N	N	N	N	N	3.9	1.46	2.15	1.03	AA	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
209	Patient	80	M	N	N	N	N	N	5.28	1.69	3.15	1.28	AG	TT
210	Patient	61	M	N	N	Y	N	50%	3.41	1.35	1.92	0.87	AG	TC
211	Patient	67	F	N	Y	N	N	N	4.94	1.52	3.21	0.97	AG	TT
212	Patient	64	M	N	N	Y	N	90%	4.32	1.09	2.64	1.13	AA	TT
213	Control	48	F	N	N	N	Y	N	2.6	0.97	1.41	0.72	AG	TT
214	Patient	65	F	N	N	Y	N	50%	5.77	1.18	3.82	1.33	AG	TT
216	Patient	79	F	Y	Y	N	N	50%	4.26	1.69	2.64	0.79	AA	TT
217	Patient	61	M	Y	N	Y	N	50%	4.71	1.2	3.05	1.05	AG	TC
218	Patient	36	F	N	N	Y	N	70%	6.4	1.94	3.79	1.62	AG	TT
219	Patient	80	M	Y	Y	N	N	70%	7.51	4.24	4.44	1.05	AG	TT
221	Patient	61	M	N	Y	Y	N	N	3.72	1.15	2.15	1.00	AG	TT
222	Patient	69	M	Y	N	N	N	100%	4.06	0.87	2.69	0.92	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
223	Patient	76	F	Y	Y	N	N	70%	4.11	0.76	2.67	1.03	AG	TT
224	Patient	69	F	N	N	N	N	50%	3.2	1.11	1.79	0.85	AG	TT
225	Control	43	F	N	N	Y	N	N	5.77	1.84	3.51	1.33	AG	TC
226	Patient	77	M	Y	Y	Y	N	100%	3.85	0.82	2.62	0.79	AG	TT
227	Patient	82	M	N	N	N	N	50%	3.77	1.66	2.05	0.9	AG	TT
229	Control	45	F	N	N	N	N	N	5.23	2.75	2.59	1.31	AG	TT
232	Patient	78	F	Y	N	N	N	N	4.89	0.7	3.18	1.33	AG	TT
233	Control	64	F	Y	N	N	Y	N	6.99	1.49	4.77	1.44	AG	TC
234	Patient	58	F	N	N	N	N	N	4.32	1.1	2.41	1.33	AG	TT
235	Control	66	M	N	N	N	N	50%	5.3	0.7	3.46	1.46	AG	TT
236	Patient	59	F	Y	Y	N	N	70%	5.2	1.88	3.41	0.85	AG	TT
237	Control	69	F	Y	N	N	N	N	4.86	1.17	2.00	1.21	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
239	Patient	53	M	N	N	Y	N	50%	3.61	0.37	2.21	1.18	AG	TT
240	Control	42	M	N	N	Y	N	N	4.63	2.74	2.46	0.85	AG	TT
241	Control	65	M	Y	N	N	N	N	3.85	1.29	1.95	1.26	AG	TC
242	Control	87	M	Y	Y	N	N	50%	2.7	1.24	0.97	1.13	AA	TC
244	Control	75	F	N	N	Y	Y	50%	4.78	0.91	2.9	1.41	AA	TT
245	Patient	54	M	N	N	N	N	N	4.42	0.66	3.1	0.95	AG	TT
246	Control	77	M	Y	N	N	N	N	3.61	0.67	2.44	0.82	AA	TT
247	Control	69	F	N	N	N	N	N	4.63	1.29	2.72	1.26	AG	TC
250	Patient	84	F	N	N	N	N	100%	4.55	0.8	2.62	1.51	AG	TT
252	Patient	40	F	N	N	Y	Y	N	4.99	1.06	3.26	1.18	AG	TT
254	Patient	80	F	Y	Y	N	N	N	4.78	0.87	2.87	1.38	AG	TT
255	Patient	41	M	N	N	N	N	N	3.77	1.35	2.26	0.85	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
256	Patient	77	F	Y	Y	N	N	70%	4.99	1.45	3.15	1.1	AG	TT
257	Patient	63	F	Y	Y	N	Y	50%	3.93	0.78	2.56	0.95	AG	TT
258	Patient	55	M	Y	N	Y	N	50%	7.2	1.83	5.31	0.95	AG	TC
259	Patient	79	M	Y	Y	N	N	70%	3.12	0.76	1.56	1.15	AG	TC
261	Patient	63	M	Y	Y	N	N	70%	3.77	1.76	2.13	0.79	AG	TC
262	Patient	75	F	Y	N	N	N	50%	5.93	1.9	3.9	1.08	AA	TT
263	Control	65	M	N	N	N	N	50%	4.13	0.7	2.64	1.13	AA	TC
264	Control	78	M	N	N	N	N	N	4.11	0.92	2.23	1.41	AG	TT
265	Control	81	M	Y	N	N	N	N	2.81	0.61	1.23	1.26	AG	TC
266	Control	56	M	N	Y	N	N	N	4.6	1.37	2.82	1.10	AG	TT
267	Control	64	F	Y	Y	N	N	N	4.32	2.76	2.21	0.79	AG	TT
268	Control	79	F	Y	N	N	N	N	4.71	1.24	2.82	1.26	AG	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
269	Control	67	F	N	N	N	N	N	7.75	1.15	5.85	1.28	AG	TT
270	Control	76	F	Y	N	N	N	50%	4.32	1.24	2.18	1.51	AG	TC
271	Control	64	M	N	N	Y	N	N	3.38	1.02	2.21	0.67	AG	TT
272	Control	77	M	N	Y	N	N	N	8.79	1.78	6.23	1.62	AG	CC
273	Control	75	M	N	N	N	N	N	4.97	1.67	2.79	1.33	AG	TT
274	Patient	54	F	N	N	N	N	100%	5.23	3.38	2.92	0.69	AG	TT
275	Patient	61	F	Y	N	N	N	50%	4.68	1.24	3.15	0.9	AA	TC
276	Patient	71	M	Y	N	N	N	90%	3.25	0.8	1.87	0.97	AG	TT
277	Control	68	F	Y	N	N	Y	N	4.84	1.91	2.49	1.41	AG	TC
278	Patient	74	F	Y	N	N	N	70%	5.49	1.85	3.15	1.41	AA	TT
280	Patient	62	M	N	N	Y	N	N	4	0.79	2.64	0.95	AG	TT
281	Patient	82	F	Y	Y	N	N	50%	4.65	1.18	3.08	0.97	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
282	Control	71	F	Y	N	N	N	50%	3.87	1.09	2.31	1.03	AG	TC
283	Control	52	F	N	N	N	N	50%	4.00	0.8	2.56	1.03	AG	TC
284	Control	78	M	Y	N	N	N	N	4.13	1.39	2.49	0.95	AA	TC
285	Patient	70	M	Y	N	N	N	50%	5.15	0.85	3.44	1.26	AG	TC
286	Patient	69	M	Y	N	N	N	50%	4.42	0.93	2.85	1.08	AG	TT
287	Control	80	M	Y	N	N	N	50%	5.12	0.97	3.23	1.38	AG	TC
288	Patient	57	F	N	Y	N	N	100%	5.46	1.73	3.46	1.13	AG	TT
289	Patient	58	F	Y	N	N	N	N	3.2	1.26	1.54	1.05	AG	TC
292	Patient	80	M	Y	N	N	N	70%	3.54	0.7	2	1.18	AG	TT
293	Patient	84	M	Y	N	N	N	50%	3.43	1.84	1.82	0.72	AG	TT
294	Control	78	F	N	N	N	N	50%	4.08	0.94	2.51	1.05	AA	TT
296	Control	57	F	N	N	N	N	N	2.44	1.04	0.49	1.44	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
297	Control	60	F	Y	N	N	N	50%	5.36	2.22	2.87	1.38	AG	TC
299	Patient	74	M	N	Y	N	N	50%	3.38	0.73	1.67	1.33	AG	TT
300	Patient	50	M	Y	N	N	N	50%	2.00	0.84	0.62	0.97	AG	TT
301	Control	58	M	Y	N	N	N	50%	3.9	2.25	2.05	0.77	AG	TC
302	Control	54	M	Y	N	N	N	N	4.32	1.16	2.41	1.31	AG	TC
304	Control	78	M	N	N	N	N	50%	3.69	0.67	2.05	1.28	AG	TT
305	Patient	57	M	N	Y	N	N	100%	5.02	1.43	3.56	0.74	AG	TT
306	Control	75	M	Y	Y	N	N	50%	4.81	0.8	2.95	1.44	AG	TT
307	Control	77	M	Y	N	N	N	50%	3.07	0.99	1.64	0.92	AG	TC
308	Patient	85	M	N	N	N	N	50%	4.84	1.35	3.03	1.13	AG	TT
309	Patient	62	M	Y	N	N	N	50%	5.07	2.62	3.05	0.74	AG	TC
310	Patient	81	F	Y	Y	N	N	70%	4.19	0.96	2.46	1.23	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
311	Patient	77	F	Y	Y	N	N	50%	3.02	2.1	1.56	0.46	AG	TT
312	Patient	54	M	Y	Y	N	N	50%	4.13	1.34	2.85	0.62	AG	TC
315	Patient	82	F	Y	Y	N	N	70%	4.34	1.01	2.62	1.21	AG	TT
316	Patient	71	F	Y	Y	N	N	50%	4.24	1.93	2.33	0.97	AG	TC
317	Patient	84	M	Y	Y	N	N	100%	2.89	1.51	1.49	0.67	AG	TC
318	Patient	80	F	Y	N	N	N	50%	4.29	1.56	2.31	1.21	AG	TT
319	Control	59	F	N	N	N	N	N	5.54	1.42	3.23	1.59	AG	TT
320	Control	57	F	N	N	N	N	N	4.37	1.87	2.56	0.9	AG	TC
321	Control	79	F	Y	Y	N	N	50%	4.24	1.65	2.21	1.23	AG	TC
322	Control	52	F	N	N	N	N	N	6.06	1.28	4.18	1.21	AG	TC
323	Control	79	F	Y	N	N	N	N	1.53	1.11	0.59	0.41	AG	TT
324	Patient	69	M	Y	Y	N	N	50%	5.38	2.51	2.79	1.36	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
325	Patient	84	M	N	N	N	N	N	4.16	1.64	2	1.36	AG	TT
326	Control	54	M	N	Y	N	N	N	7.33	2.47	4.9	1.21	AG	TT
327	Patient	54	F	Y	Y	N	N	50%	5.15	1.2	2.59	1.38	AG	TT
328	Patient	70	F	Y	N	N	N	50%	4.71	1.63	2.44	1.46	AG	TT
329	Control	67	F	N	N	N	N	N	4.16	0.79	2.36	1.33	AG	TT
330	Control	50	F	N	N	N	N	N	3.41	2.76	1.74	0.36	AG	TC
331	Patient	31	F	N	N	N	N	N	6.4	3.01	3.62	1.31	AG	TT
332	Patient	55	F	N	N	N	N	N	7.1	0.7	4.87	1.82	AG	TT
333	Control	90	F	N	Y	N	N	50%	5.56	1.64	3.41	1.33	AG	TC
334	Patient	71	M	Y	Y	N	N	70%	5.62	2.00	3.74	0.87	AG	TC
335	Patient	85	M	Y	Y	N	N	70%	7.28	2.98	4.21	0.9	AG	TC
336	Control	77	F	Y	N	N	N	50%	8.16	1.94	5.18	1.97	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
337	Patient	83	F	Y	N	N	N	50%	4.19	0.78	2.05	1.72	AA	TT
340	Control	57	M	Y	Y	N	N	N	4.52	0.55	2.64	1.56	AG	TT
341	Control	68	M	N	N	N	N	N	3.54	0.58	1.85	1.38	AG	TC
342	Patient	74	F	Y	Y	N	N	50%	7.41	3.11	4.44	1.46	AG	CC
343	Patient	47	M	Y	Y	N	N	N	4.55	1.45	2.44	1.38	AG	TT
344	Patient	43	M	N	N	Y	N	N	7.83	3.48	4.74	1.38	AG	TT
346	Patient	67	M	Y	Y	N	N	70%	5.51	0.98	3.87	1.13	AG	TT
347	Patient	69	F	N	N	N	N	70%	6.45	1.65	4.36	1.26	AG	TT
348	Control	77	M	Y	Y	Y	N	N	5.23	0.91	3.26	1.49	AG	TT
349	Control	60	M	Y	N	N	N	N	4.00	1.00	2.36	1.13	AG	TT
351	Patient	74	M	N	N	Y	N	70%	4.00	1.08	2.72	0.74	AG	TT
352	Patient	84	M	Y	Y	N	N	70%	5.51	1.01	3.97	1.08	AA	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
354	Patient	74	M	Y	N	N	N	50%	3.64	1.37	2.05	0.92	AG	TT
356	Control	44	M	N	N	N	Y	N	4.45	1.22	2.82	1.00	AG	TT
357	Patient	68	F	Y	Y	Y	N	N	3.04	1.22	1.41	1.03	AG	TT
359	Control	52	F	Y	N	N	N	50%	5.2	1.94	2.85	1.38	AG	TT
360	Patient	68	F	Y	N	N	N	70%	5.33	1.03	3.62	1.18	AG	TT
363	Control	58	F	N	N	N	N	N	4.08	1.67	2.31	0.95	AG	TT
364	Patient	57	M	Y	Y	Y	N	70%	6.16	2.84	3.90	0.87	AG	TT
365	Control	79	M	Y	Y	N	N	N	5.17	1.94	3.31	0.90	AG	TT
366	Control	73	F	N	N	N	N	N	5.28	1.25	3.62	1.03	AG	TC
367	Patient	34	F	N	N	N	N	N	5.80	1.96	2.64	0.62	AG	TT
368	Patient	61	M	Y	N	N	N	100%	4.58	1.48	2.92	0.92	AG	TT
369	Patient	62	M	N	N	N	N	100%	4.71	2.11	2.69	0.97	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
371	Patient	62	M	Y	N	N	Y	50%	5.69	1.52	3.79	1.13	GG	TT
372	Patient	63	F	Y	Y	N	N	50%	6.4	1.84	4.51	0.95	GG	TT
373	Patient	68	M	Y	N	Y	Y	50%	4.86	1.06	3.59	0.72	GG	TC
375	Patient	84	F	N	N	N	N	50%	6.50	0.84	4.62	1.41	GG	TT
377	Patient	69	F	Y	N	N	N	50%	5.36	0.52	3.82	1.23	AG	TC
378	Patient	71	M	Y	N	N	N	50%	5.67	1.38	4.08	0.87	GG	TT
381	Patient	38	M	Y	N	Y	N	50%	6.63	1.69	4.72	1.05	AG	TC
382	Patient	71	F	Y	N	N	Y	50%	5.25	1.42	3.21	1.33	AG	TC
383	Patient	73	M	Y	N	Y	Y	90%	6.37	1.28	4.54	1.15	AA	TC
385	Patient	63	M	N	N	Y	Y	50%	4.47	0.78	2.59	1.46	AG	TT
386	Patient	30	M	N	N	N	Y	N	4.03	1.33	2.49	0.87	GG	TT
388	Patient	61	F	Y	N	N	Y	N	4.94	1.29	2.56	1.64	GG	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
389	Patient	75	M	N	N	N	N	50%	3.64	0.69	2.26	1.03	AG	TC
390	Patient	42	M	N	N	N	N	N	5.98	0.88	4.28	1.21	AG	TT
391	Patient	59	F	Y	N	N	Y	50%	6.81	1.82	4.05	1.85	AG	TT
392	Patient	83	M	Y	N	N	N	50%	2.39	0.92	1.00	0.95	AG	CC
393	Patient	73	M	Y	N	Y	Y	50%	6.19	1.97	3.9	1.31	AG	TC
394	Patient	78	M	N	N	N	Y	N	4.71	1.33	3.1	0.92	AG	TT
395	Patient	71	M	Y	Y	Y	Y	50%	5.72	3.78	3.69	1.00	AG	TT
396	Patient	76	F	N	N	Y	Y	50%	5.12	2.07	3.33	0.77	AG	TC
397	Patient	74	F	Y	Y	N	Y	50%	5.62	3.34	3.41	0.62	AG	TC
398	Patient	79	M	Y	Y	N	Y	50%	5.07	1.08	3.77	0.74	AG	TT
399	Patient	59	M	Y	N	N	Y	50%	4.39	1.28	2.79	0.95	AG	TT
402	Patient	52	M	N	N	N	N	N	5.28	0.69	3.33	1.31	AG	TC

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
403	Patient	77	F	Y	Y	Y	Y	50%	6.79	1.64	4.74	1.21	AA	TT
404	Patient	77	M	Y	N	Y	N	50%	3.33	1.61	1.9	1.05	AG	TT
405	Patient	85	F	Y	N	N	Y	50%	2.11	0.75	1.05	0.69	AG	TT
407	Patient	79	F	Y	N	N	N	50%	4.55	0.81	3.05	1.08	AG	TC
409	Patient	68	M	Y	N	Y	Y	70%	6.34	1.18	4.64	1.08	AG	TC
410	Patient	85	M	N	Y	N	N	50%	5.46	0.93	3.82	1.13	AG	TT
411	Patient	79	F	Y	Y	N	Y	50%	6.42	1.71	4.62	0.95	AG	TT
413	Patient	46	M	Y	Y	N	Y	N	5.02	1.16	3.15	1.26	AG	TT
415	Patient	70	M	Y	Y	N	Y	70%	6.6	2.18	4.51	1.00	AG	TT
417	Patient	79	F	N	N	Y	Y	70%	6.63	1.03	3.97	2.10	AG	TC
419	Patient	81	F	N	N	N	N	70%	5.64	2.35	3.54	0.95	AA	TC
420	Patient	79	M	Y	Y	N	Y	70%	7.88	0.66	6.10	1.36	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
422	Patient	75	M	Y	N	Y	Y	70%	6.47	2.78	4.00	1.13	AG	TT
424	Patient	83	F	Y	Y	N	N	50%	5.12	2.01	3.23	0.9	AG	TT
425	Patient	64	M	Y	N	Y	Y	50%	2.81	0.87	1.77	0.62	AG	TC
426	Patient	81	M	Y	N	Y	Y	50%	2.52	0.83	1.46	0.64	AG	TT
428	Patient	72	F	Y	N	Y	N	90%	5.07	1.08	3.18	1.38	AA	TT
431	Patient	71	M	Y	Y	Y	Y	50%	4.32	1.16	3.03	0.69	AG	TT
433	Patient	51	M	Y	N	Y	Y	N	4.52	1.92	2.92	0.67	AG	TT
435	Patient	60	F	N	N	Y	N	N	5.88	1.20	4.00	1.26	AG	TT
438	Patient	77	F	Y	N	N	N	50%	5.07	1.25	3.28	1.15	AG	TT
440	Patient	71	F	Y	Y	N	Y	50%	3.51	1.08	2.03	0.95	AG	TT
441	Patient	75	M	Y	N	Y	Y	50%	4.97	1.11	2.92	0.92	AG	TC
442	Patient	65	M	Y	Y	Y	Y	50%	5.41	2.10	3.33	1.13	AG	TT

Table C.1 Cont'd

		Demographic Characteristics							Lipid Parameters				CYP1A1 Genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	A4889G	T6235C
443	Patient	72	F	Y	Y	N	N	N	4.26	1.58	2.38	1.10	AA	TT
444	Patient	70	F	Y	N	N	Y	50%	5.54	0.96	3.38	1.64	AA	TT
445	Patient	57	M	N	N	N	Y	N	6.66	1.17	4.62	1.41	AG	TT