ASSOCIATION ANALYSIS OF GLUTATHIONE S-TRANSFERASE OMEGA 1 AND OMEGA 2 GENETIC POLYMORPHISMS AND ISCHEMIC STROKE RISK IN TURKISH POPULATION

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ASSOCIATION ANALYSIS OF GLUTATHIONE S-TRANSFERASE OMEGA 1 AND OMEGA 2 GENETIC POLYMORPHISMS AND ISCHEMIC STROKE RISK IN TURKISH POPULATION

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ABSTRACT

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Stroke is defined as the acute neurological cerebrovascular disease based on interruptions to blood flow in the brain. These interruptions are caused by loss of blood supply due to vessel bursts or vessel blocked by clotting. Atherosclerosis, a main cause of stroke, is blockage of endothelium layer of arteries and losing the flexibility of tissue. The oxidative stress is known as a risk factor for atherosclerosis. The increased free radicals such as reactive oxygen species (ROS) and decreased antioxidant level cause the oxidative stress and this situation damages the tissue on brain and blood vessels. Oxidative stress is influenced by imbalance in production of phase II enzymes which are responsible for xenobiotic mechanism in terms of decreasing oxidative stress. Glutathione S-transferase omega 1 and omega 2 are members of phase II enzymes family which catalyze detoxification reactions. The polymorphisms, Ala140Asp (C→A) in GSTO1 gene and Asn142Asp (A→G) in GSTO2 gene may cause a decrease in enzyme activity and this situation promotes oxidative stress damage in blood vessels. The aim of this study is to investigate the possible association between GSTO1

Ala140Asp and GSTO2 Asn142Asp SNPs and ischemic stroke risk in Turkish population.

The study population includes 239 patients and 130 controls and both polymorphisms were determined by PCR/RFLP method. Conventional risk factors for ischemic stroke such as hypertension, diabetes mellitus, obesity and smoking were found significantly higher in patients compared to controls (Odds Ratios; OR=3.35, OR=2.614, OR=4.191 and OR=2.662, respectively). Mutant allele 'A' frequencies for Ala140Asp polymorphism of GSTO1 gene were calculated as 0.358 for patients and 0.342 for controls. For the Asn142Asp SNP of GSTO2 gene, the mutant allele 'G' frequencies were found 0.370 for patients and 0.404 for controls. There was no statistically significant difference between patients and controls in terms of allele frequencies.

Detailed analyses have shown that stroke risk can change depending on the genotypes of GSTO1 and GSTO2 genes within conventional risk factors. For Ala140Asp SNP, the risk of having stroke is significantly lower within hypertensive subgroup and obesity subgroup when the individual carrying the mutant allele (OR=2.24 for hypertensive and OR=2.75 for obesity subgroup). Besides, having mutant allele increased the stroke risk for diabetics and smokers (OR=3.873 for diabetics and OR=3.55 for smokers). As regard to Asn142Asp SNP, the mutant allele caused an increase in stroke risk for people with obesity while decrease in diabetics (OR=9.09 for obesity and OR=2.4 for diabetics). In addition, the wild type allele increased the stroke risk for smokers (OR=3.171).

Logistic regression analysis revealed that hypertension, smoking, obesity and HDL were significant predictors of stroke. Hypertension, smoking and obesity increased the stroke risk (OR= 3.043, OR=3.258 and OR=2.593, respectively) while HDL had protective role (OR=0.270).

In this study, the association between Ala140Asp of GSTO1 and Asn142Asp of GSTO2 genetic polymorphisms and ischemic stroke risk in Turkish population was investigated for the first time. No significant difference was found between patient and control groups in terms of C and A allele frequencies in GSTO1 Ala140Asp polymorphism and A and G allele frequencies in GSTO2 Asn142Asp polymorphism. However, different

genotypes of GSTO1 and GSTO2 considering the Ala140Asp and Asn142Asp SNPs have effect on stroke risk in conventional subgroups of hypertension, diabetes, smoking and obesity.

Keywords: GSTO1, GSTO2, genetic polymorphism, ischemic stroke, Ala140Asp, Asn142Asp, SNP, Turkish population

ÖZ

GLUTATYON S-TRANSFERAZ OMEGA 1 VE OMEGA 2 GEN POLİMORFİZİMLERİ İLE TÜRK POPULASYONUNDA İSKEMİK İNME RİSKİ İLİŞKİSİNİN İNCELENMESİ

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İnme beyne giden kan akışının yetersiz olduğu veya tamamıyla kesildiği durumlarda ortaya çıkan nörolojik bir hastalıktır. İnmenin iki temel nedeni vardır: birincisi beyin damarlarının çatlamasıyla oluşan kanın istenen dokuya ulaşamaması, ikincisi ise beyin damarlarında daralmalardan ve kan pıhtısından kaynaklanan tıkanmadır. İnme hastalığının büyük bir yüzdesi aterosklerotik plaktan kaynaklanan kan akışının tıkanması ve beynin belli bir bölümünün besinsiz ve oksijensiz kalması ile gerçekleşir. Yapılan çalışmalarda dokuda oksidatif stresin artması aterosklerozun oluşumunu tetikler. Bu durumda, normalden fazla serbest radikal üretimi ya da antioksidan mekanizmasında oluşan aksaklıklar inme riskini arttırır şeklinde düşünülebilir. Glutatyon S- transferaz (GST) enzimi faz II enzimlerine mensup olup, özellikle detoksifikasyon metabolizmasında etkin rol oynar. Glutatyon S- transferaz enzimi yedi alt gruba sahiptir. GST omega 1 ve omega 2 bu alt gruptandır ve serbest radikallerin inhibe edilmesinde aktif rol oynar. Bu çalışmanın amacı GSTO1 Ala140Asp ve GSTO2 Asn142Asp

polimorfizmleri ile iskemik inme riski arasındaki olası ilişkinin Türk popülasyonunda araştırılmasıdır.

Çalışma grubu 239 iskemik inme hastası ve 130 sağlıklı kontrol bireylerden oluşmaktadır. Gruptan toplanan kanlardan tam DNA izolasyonu yapılarak PZR/SEPUÇ yöntemi ile genotip tayini yapılmıştır. Hipertansiyon, diyabet, obezite ve sigara kullanımı gibi iskemik inmeye sebep olan risk faktörleri bu çalışma grubunda da istatistiksel anlamlı olarak hastalık için risk faktörü olduğu tespit edilmiştir. (eşitsizlik oranı sırasıyla; OR=3.35, OR=2.614, OR=4.191 ve OR=2.662). Ala140Asp SNP için mutant alel olan 'A' aleli frekansı iskemik inme hasta grubu için 0.358 ve kontrol grubu için 0.342 bulunmuştur. Asn142Asp SNP genotip frekansını incelediğimizde ise, mutant olan 'G' aleli hasta grupta 0.370 iken kontrol grupta 0.404'tür. Her iki tek nükleotid polimorfizminde de mutant alel frekansı istatistik olarak anlamlı bulunmamıştır.

Vasküler risk faktörlerle ilgili yapılan ayrıntılı analizlerde farklı GSTO1 ve GSTO2 genotiplerinin iskemik inme riskini değiştirdiği saptanmıştır. Ala140Asp tek nükteotid polimorfizmi ile ilgili olarak, mutant alele sahip hipertansiyonu olan veya obez bireylerde iskemik inme riskini azaltırken (eşitsizlik oranı hipertansiyon için 2.24 ve obezite için 2.75), diyabetik veya sigara kullanan bireylerde aynı riski arttırdığı tespit edilmiştir (eşitsizlik oranı diyabet için 3.87 ve sigara kullananlar için 3.55). Asn142Asp SNP için bakıldığında, obez bireylerde yabanıl tip 'A' aleli taşıdığında risk neredeyse 10 kat artarken (eşitsizlik oranı 9.09), diyabetik bireylerde yabanıl tip alelin iskemik inme riskini azalttığı tespit edilmiştir (eşitsizlik oranı bu grup için 2.4 iken tüm grup için 2.614'tür). Mutant 'A' aleli taşıyan ve sigara kullanan bireylerde ise iskemik inme riskinin yaklaşık 3 kat arttığı belirlenmiştir (eşitsizlik oranı 3.171).

Lojistik regresyon testi ile hipertansiyon, diyabet, obezite ve sigara gibi bilinen vasküler risk faktörler, total kolesterol, trigliserit, LDL ve HDL gibi lipit parametreleri ve GSTO1 ve GSTO2 genotiplerinin iskemik inme hastalığı üzerindeki etkisi analiz edilmiştir. Bu testin sonucunda, hipertansiyon, sigara kullanımı ve obezitenin iskemik inme için istatistiksel olarak anlamlı risk faktörü olduğu ortaya çıkmıştır. Aynı zamanda, yüksek

dansiteli lipoprotein kolesterolün (HDL) bu hastalığa karşı koruyucu etkisi olduğu saptanmıştır.

Sonuç olarak, bu çalışmada GSTO1 geni üzerindeki Ala140Asp ve GSTO2 geni üzerindeki Asn142Asp tek nükleotit polimorfizmleri ile iskemik inme riski ilişkisi Türk popülasyonunda ilk defa çalışılmıştır. GSTO1 geni üzerindeki mutant alel 'A' nın ve GSTO2 gen üzerindeki mutant alel 'G' nin iskemik inme için risk faktörü oluşturmadığına; fakat farklı vasküler risk faktörlere sahip bireylerde farklı genotiplerin risk durumunu etkilediğine karar verilmiştir.

Anahtar kelimeler: GSTO1, GSTO2, genetik polimorfizm, iskemik inme, Ala140Asp, Asn142Asp, Türk popülasyonu.

Dedicated to my bellowed family,

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CI	Confidence interval
CT	Computed tomography
CAD	Coronary artery disease
CAT	Choloramphenicol acetyl transferase
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
GSH	Glutathione
GST	Glutathione S Transferases
GSTO1	Glutathione S Transferases Omega 1
GSTO2	Glutathione S Transferases Omega 2
HDL	High density lipoprotein
LDL	Low density lipoprotein
NADPH	Nicotinamide adenine dinucleotide phosphate
OR	Odds ratio
OS	Oxidative stress
Ox-LDL	Oxidized Low Density Lipoprotein
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RE	Restriction endonuclease
ROS	Reactive Oxygen Species
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TIA	Transient ischemic attack

CHAPTER 1

INTRODUCTION

1.1. Stroke

Stroke is defined as the acute neurological and irreversible appearances of cerebrovascular disease based on interruptions to blood flow in the brain. These interruptions are caused by loss of blood supply due to vessel bursts or vessel blocked by clotting. As a result, the brain tissues are disrupted with lack of nutrient and oxygen which are supplied by blood circulation, and so the brain become unable to function and severe long-term disability.

Many symptoms and signs are pointed for stoke such as a numb or weak feeling in the face, arm or leg, loss of balance, difficulty in speaking and understanding, blurred or poor vision, severe headache and fainting (http://www.who.int). A term FAST (face, arm, speech and time) is proposed by Department of Health (United Kingdom) and the Stroke Association to understand the stroke attack.

According to World Health Organization reports, stroke is the third-leading cause of death behind coronary heart disease. Mostly, although there is stroke patient at any age, stroke is considered age-related because half of all stroke patients are over age of 75. The WHO is reported that new 15 million stroke event occur in annually and 33% of them die, then others become permanently disabled (http://www.who.int).

1.1.1. Classification of Stroke

The stroke is mainly divided two types in terms of pathologic mechanisms: ischemic stroke and hemorrhagic stroke. According to American Stroke Association, the 80% of all stroke accounts is ischemic and 15% is hemorrhagic. The remaining is subarachnoid hemorrhage. In Turkish population, according to a study, the prevalence of ischemic stroke is 77%, hemorrhagic stroke is 19% and the remaining is subarachnoid hemorrhage (Kumral et al., 1998).

1.1.1.1. Ischemic Stroke

An ischemic stroke is caused by an obstruction within a blood vessel supplying blood to the brain (Figure 1.1). The obstruction occurs by three ways:

- By stenosis; the development of fatty deposits lining the vessel walls which supply nutrient and oxygen to brain cells. This fatty deposits cause a plaque formation and vessel narrowing, condition called atherosclerosis.
- By cerebral thrombosis; formation of a thrombus, blood clot which develops at the clogged part of the vessel.
- By cerebral embolism; blood clot which is located in another region in body arrive the cerebral vessels in brain by circulation system (http://www.strokeassociation.org).

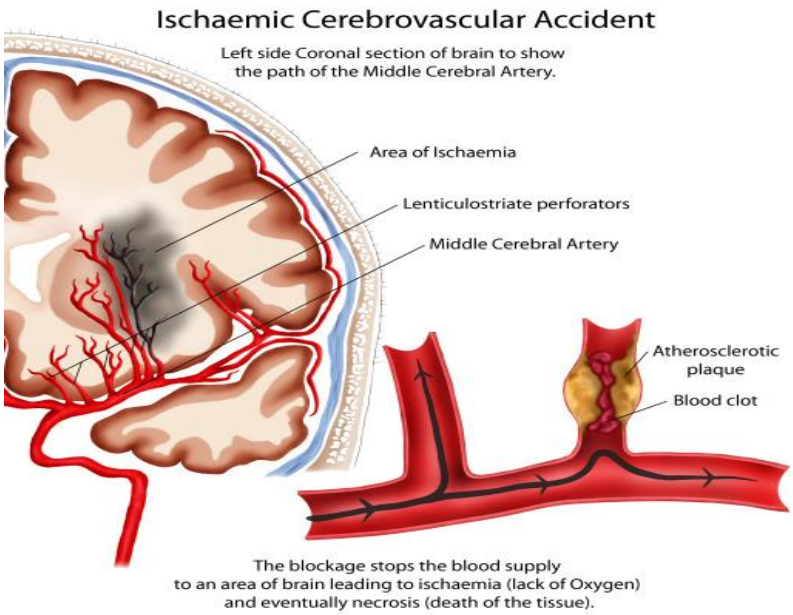

Figure 1.1 Ischemic stroke (taken from http://neuro4students.wordpress.com)

Transient ischemic attack (TIA) is defined as a sudden block of brain vessel by clotting and the symptoms are observed for a short time as less than 24 hours. TIA can be called as ‘mini stroke’. Moreover, it can be an indicator for an ischemic stroke attack because 90-day risks of stroke reported as high as 10.5% and the greatest stroke risk apparent in the first week (Johnston et al., 2000; Rothwell et al., 2005).

1.1.1.2. Hemorrhagic Stroke

Hemorrhagic Stroke is defined as bleeding in brain caused by weakened vessel which breaks and bleeds into the surrounding brain (Figure 1.2). 13% of all stroke events are hemorrhagic. It can be divided into two main types: intracerebral hemorrhagic stroke and subarachnoid hemorrhagic stroke. The causes of weakened blood vessel are aneurysms (ballooning of weakened region, then bursting) and arteriovenous

malformations (clustering of abnormal blood vessel formation) (http://www.strokeassociation.org).

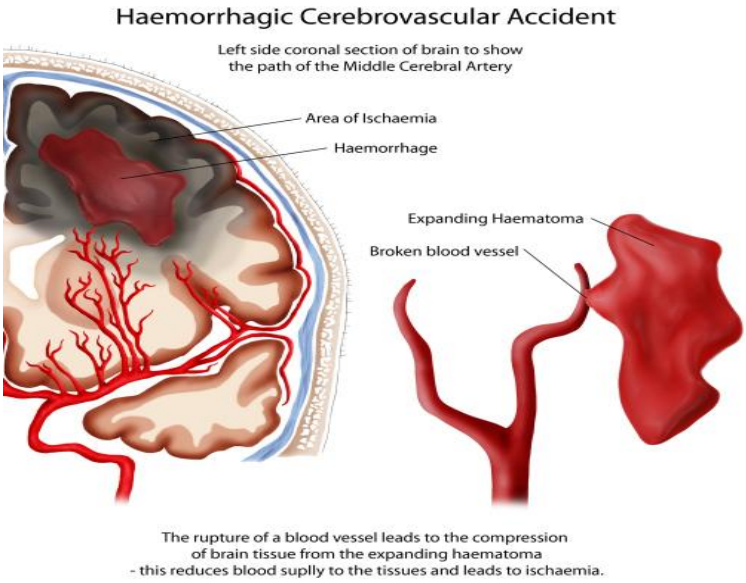

Figure 1.2 Hemorrhagic stroke (taken from http://neuro4students.wordpress.com)

1.1.2. Risk Factor of Stroke

Ischemic stroke is a complex neurological disorder and it has relation with many diseases that are related to heart and blood circulation. Therefore, many risk factors can influence the stroke attack. Some risk factors are unalterable, so they cannot be decreased or eliminated. The others are alterable by treatment and changing life style and therefore, the stroke risk can be reduced (http://medicalcenter.osu.edu, http://stroke.ahajournals.org). Unalterable and alterable as well as uncertain risk factors of stroke were given in Table 1.1.

Table 1.1 Risk factors of stroke

Unalterable Risk Factor	• Age • Gender • Heredity • Race • TIA
Alterable Risk Factor	• Hypertension • Diabetes mellitus • High blood cholesterol • Smoking • Obesity • Poor physical activity • Atrial fibrillation • Atherosclerosis and heart disease • Malnutrition
Uncertain Risk Factor	• Alcohol abuse • Drug abuse • Socioeconomic factor • Geographic factor (temperature, climate, location)

1.1.2.1. Unalterable Risk Factor

According to the previous investigations, age, heredity, gender, race and TIA are considered as unalterable risk factors for stroke. These factors cannot be modified and the risk for stroke cannot be decreased by changing life style or treatment.

Age is strongly related with stroke. The stroke attack risk increases more than two times after 55 years old and each 10 years after this age (Brown et al., 1996).Gender is another important risk factor in this disease. The stroke is seen more common in men than women and the death event in stroke is higher in men. This decreased risk in women is suggested to be related with usage of birth control pills (Brown et al., 1996; http://www.strokeassociation.org).

The risk of stroke increases when the parents or other close relatives are stroke patient. In a study, when both paternal and maternal have had stroke, the stroke risk in offspring has became higher (Kiely et al., 1993). Another study has revealed that some genetic disorders can trigger the stroke attack such as Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy (CADASIL). In this disease, a genetic mutation causes a defect in blood vessels in brain and occlusion in circulation (http://www.strokeassociation.org).

The studies on race have shown that black men are more prone to get a stroke attack (Howard et al., 1994). Unlikely, this relation might be associated with environmental factors like climate and geographic location (Sacco et al., 1997). In Japan and Chinese, stroke patients' death ratio is found to be very high (Jiang et al., 1995).

Transient ischemic attack (TIA) is considered as unalterable risk factor because TIA is followed by ischemic stroke (Johnston et al., 2000; Rothwell et al., 2005).

1.1.2.2. Alterable Risk Factor

Hypertension, diabetes mellitus, high blood cholesterol, smoking, obesity, poor physical activity, atrial fibrillation, atherosclerosis and malnutrition are most important alterable risk factors for stroke.

Hypertension can be considered most important controllable risk factor for ischemic stroke due to the damage of high blood pressure to brain vessels. According to the results of a study done by the Centers for Disease Control and Prevention (CDC), the risk of stroke decreased 37% by reducing the blood pressure (http://medicalcenter.osu.edu). Hypertension has been found a main risk factor in many studies (Demirdöğen et al., 2008; Demirdöğen et al., 2009;, Türkanoğlu et al., 2010; Türkanoğlu et al., 2013). Also, antihypertensive treatment was seen to decrease the risk of stroke by 38% to 40% (Collins et al., 1994).

Diabetes mellitus is an independent risk factor for stroke. Most diabetes patients are also has high blood pressure, high blood cholesterol, atherosclerosis and obesity problem, which have very close relationship with stroke (http://www.strokeassociation.org). In Japanese men, it is revealed that the thromboembolic stroke risk was two-fold higher in diabetes patients than control (Burchfiel et al., 1994). In Turkish population, the prevalence of diabetes was found significantly higher in stroke patient than nondiabetic groups (Türkanoğlu et al., 2013).

High blood cholesterol is a factor increasing the stroke risk. High cholesterol level in blood cause promoting the plaque formation in blood vessels by thickening of the arteries called atherosclerosis. This plaque formation is the most important factor in obstruction of blood circulation and ischemic stroke. There are two types of cholesterol in blood; LDL (low density lipoprotein) and HDL (high density lipoprotein). While HDL has protective role against the stroke, the LDL triggers the stroke attack. The total cholesterol level increase is also a risk factor for ischemic stroke, especially certain stroke subtypes and patient subgroups (http://www.strokeassociation.org).

The smoking is also considered as a risk factor for stroke because smoking can cause change in structure of plasma fibrinogen and this structure damage can triggers the blocking of vessels (Sacco, 2005). According to a study, the smoking creates doubling the risk for ischemic stroke and it is considered that the reason may be nicotine and carbon monoxide (Shinton et al., 1989).

Obesity and poor physical activity are related to many diseases such as high blood pressure, high blood cholesterol, diabetes and heart disease and therefore, they are strongly related with stroke. It is revealed that the body mass index has effect of increasing the risk of stroke in women (Kurth et al., 2007).

Atrial fibrillation, irregular heartbeat, triggers the stroke because defect in heart rhythm increases the risk of embolism which is a cause of ischemic stroke. According to Framingham Study, the atrial fibrillation was related with three times to five times

increased risk for stroke. Also in the same study, myocardial disease was found as risk factor for stroke which causes increased risk for two times (Wolf et al., 1991).

Malnutrition can be another risk factor for stroke. A diet including nutrition which are rich in sodium and iron due to effect on hypertension and formation of atherosclerosis increases the risk of having stroke (Larsson et al., 2011).

1.1.2.3. Uncertain Risk Factor

Alcohol abuse, drug abuse, socioeconomic factors and geographic factors (temperature, climate, and location) are defined as uncertain risk factors of stroke. In spite of this, there are some clues they affect the stroke prevalence but these studies have not enough results yet.

1.1.3. Atherosclerosis

Atherosclerosis is blockage of endothelium layer of arteries and losing the flexibility of tissue (Figure 1.3). This accumulation is composed of by debris of dead cells, fats and cholesterol and other substances in blood circulation like ions. Especially, white blood cells, macrophages, cause the plaque formation by triggering the low-density lipoprotein (LDL) when the high lipoprotein (HDL) level is low. The inner layer of arteries start to narrowing and the proper circulationis blocked. The tissues, especially heart and brain, are under risk due to lack of nutrients and oxygen.

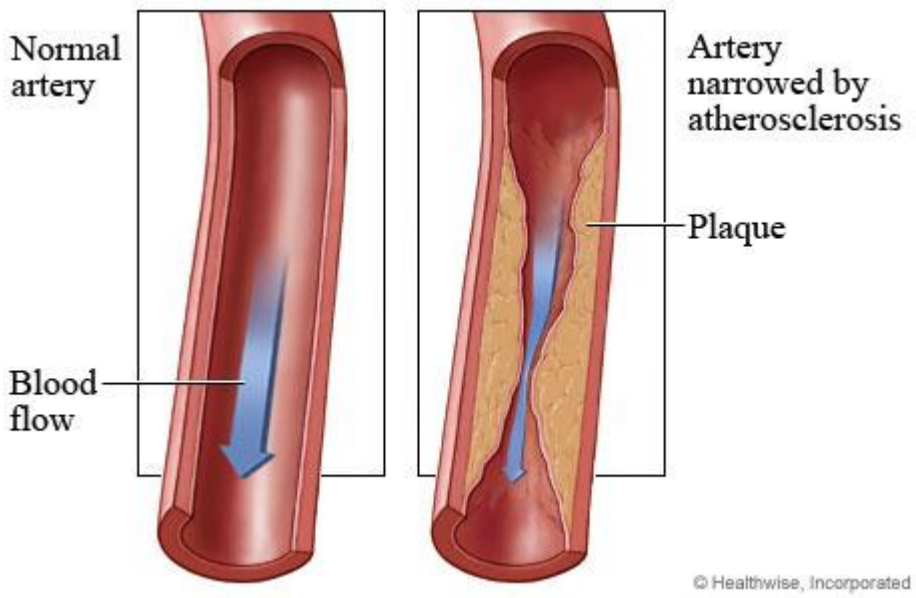

Figure 1.3 The normal artery and artery with plaque (taken from: http://www.webmd.com)

The atherosclerosis can be triggered by many factors like hypertension, diabetes, hyper cholesterol, age, genders and ethnic origin. Moreover, the oxidative stress (OS) is related with these risk factors. So, OS plays important role in formation of atherosclerosis. The increased free radicals, reactive oxygen species (ROS) and decreased antioxidant level cause the oxidative stress and this situation damages the tissue on brain and blood vessels. The ROS enhances the formation of plaque from macrophages by modifying low density lipoprotein (LDL), called oxidized LDL (ox-LDL) (Bonomini et al., 2008). Ox-LDL combines with the monocytes and foam cells are formed, which play role in formation of atherosclerotic plaque by linking smooth muscle cells and thrombus formation (Figure 1.4).

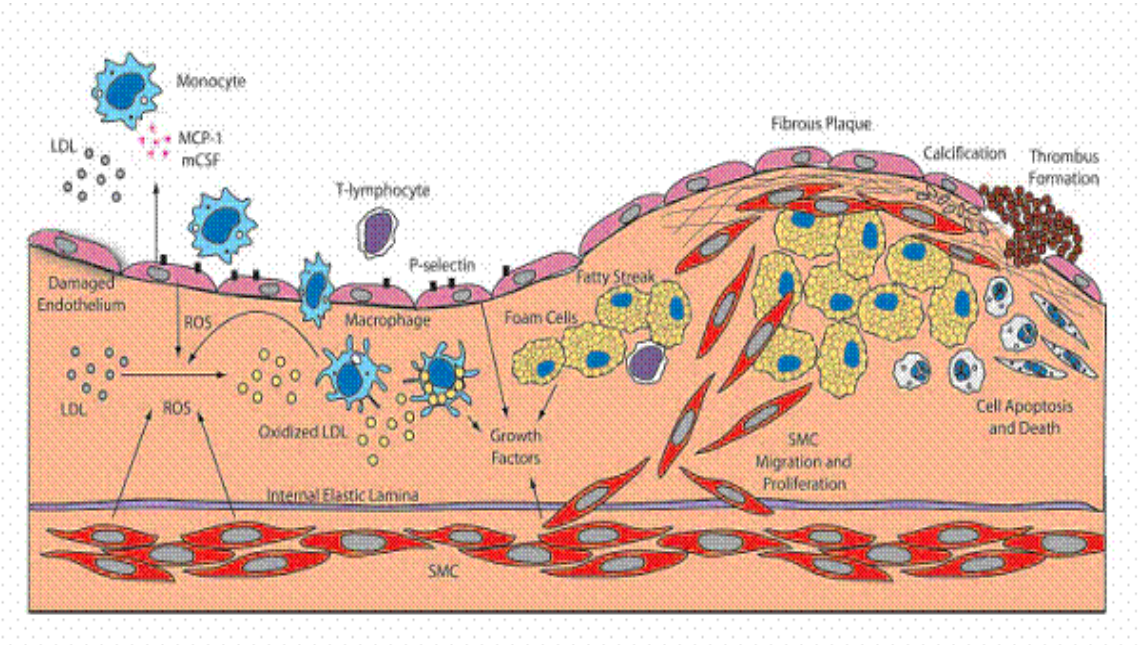

Figure 1.4 Formation of atherosclerotic lesions (taken from http:// sabanciuniv.edu)

1.1.4. Prevention of Stroke

The key factor to be protected from stroke is identification of risk factors of stroke. The unalterable risk factors cannot be changed and there is no way to decrease the risk from this way. However, by focusing alterable risk factors, the stroke risk can be decreased by 80% (http://www.stroke.org). Treatments of high blood pressure, high blood lipid, diabetes, atrial fibrillation, heart disease etc. are important for prevention of stroke.

Controlling high blood pressure is one of the most important prevention. Studies revealed that the decreasing of high blood pressure certainly prevented from stroke by using some drugs (MacMahon et al., 1990; MacMahon et al., 1986). Post-stroke Antihypertensive Treatment Study (PATS) and Perindopril protection against Recurrent Stroke Study (PROGRESS) were performed by using a drug, indapamide was given to patients and the risk of recurrence was reduced 29%. Also, angiotensin converting

enzyme (ACE) inhibitor is another protective drug from stroke (Progress Collaborative Group, 2001).

Heart disease treatments are much related with stroke prevention because the atherosclerosis causes both heart disease and stroke. Anticoagulant and antiplatelet reagents prevent from stroke due to reduced risk of thromboembolism. The European Stroke Prevention Study 2 has revealed that the treatment including both aspirin and dipyridamole causes a reduction stoke recurrence up to 37% (Diener et al., 1996). However, the doze of aspirin should be a certain level.

Decreasing the high blood cholesterol is a key factor from prevention of stroke, because LDL became a very important compound in formation of atherosclerosis. The statin treatment showed a decrease risk of stroke (Heart Protection Study Collaborative Group, 2002).

Losing weight, quitting smoking, increasing physical activity and a balanced diet are also important in prevention of stoke (http://www.stroke.org).

1.2. Free Radicals and Oxidative Stress

Free radicals are highly reactive atoms which are produced in normal biological reactions such as cellular respiration. In aerobic metabolism, oxygen participates in the reduction reactions. There is a balance in generation of free radicals and antioxidant defense mechanism which eliminates the damaging effects of free radicals by converting them less reactive forms (Chan, 2001).

There are two types' free radicals in biological mechanisms. Reactive nitrogen species come from reactions including nitrogen and cause nitrositive stress. Reactive oxygen species (ROS) are known as major free radicals. ROS are produced in normal reaction and they have role in many cellular metabolism and defense systems (Lu et al., 2007),

for example in neuronal communication, cell differentiation, proliferation and apoptosis (Bergendi et al., 1999; Klan et al., 1998 and Suzuki et al., 1997).

In Figure 1.5, the metabolism of reactive oxygen species is summarized. The first reaction for formation ROS is superoxide formation. They come from NADPH oxidase, xanthine oxidase, or the mitochondrial electron transport chain. Then, antioxidant defense system enzyme, superoxide dismutase, converts the superoxide less harmful molecules. Hydrogen peroxide, H_2O_2, less reactive molecule, is converted to water molecule by helping CAT, GSH-Px. However, when an imbalance occurs in ROS and antioxidants, the high level of ROS start to damage the organism. The high level of ROS, which is not coped with antioxidant system, causes oxidative stress. Hydrogen peroxide reacts with metal ions by Fenton reaction and causes tissue injury (Olmez et al., 2012). The ROS trigger the development of many diseases such as atherosclerosis.

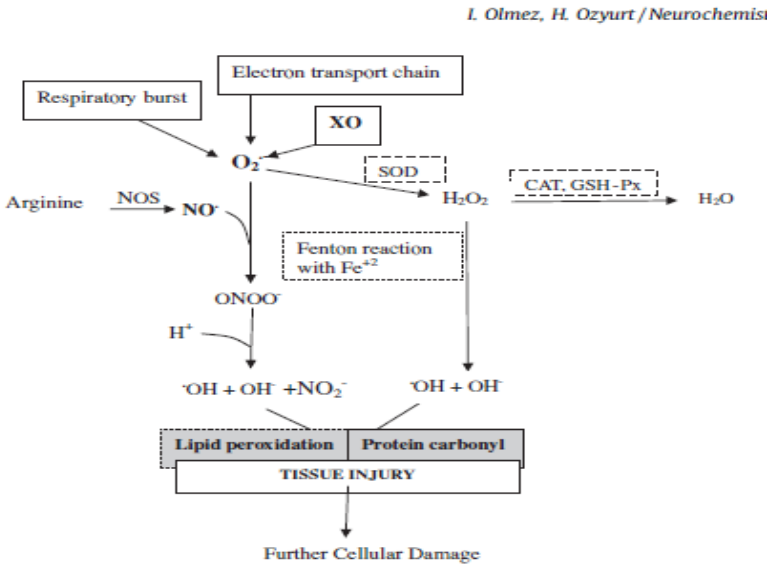

Figure 1.5 Reactive oxygen species mechanism (Olmez et al., 2012)

The brain is vulnerable to free radical attacks because the antioxidant level is low in brain and the brain includes much polyunsaturated fatty acids, which prone to free

radical attack (Wang et al., 2010). Moreover, relation between platelet formation and ROS was found in studies carried out by Krötz et al., 2004. High level ROS production cause an oxidation reaction with low-density lipoprotein and this molecule damages the endothelium of blood vessels. By combining macrophage cells, the oxidized LDL create foam cell which is a cause of atherosclerosis as given in Section 1.1.3. Therefore, increasing ROS level causes platelet aggregation due to formation of oxidized LDL (Kaul et al., 2001).

1.3. Xenobiotic Metabolism

Xenobiotic metabolism means a series of biochemical reactions which modify the molecules called xenobiotics including foreign substances for organisms like toxins, poisons, drugs and also some endogenous compounds. The xenobiotic compounds are general lipophilic, so their excretion from organism is difficult or impossible. By means of xenobiotic metabolism, these lipophilic compounds are converted to hydrophilic compounds and they become neutralized, and so they can be eliminated from the body more easily (Figure 1.6). This metabolic pathway has a crucial role in drug metabolism and organism defense mechanism.

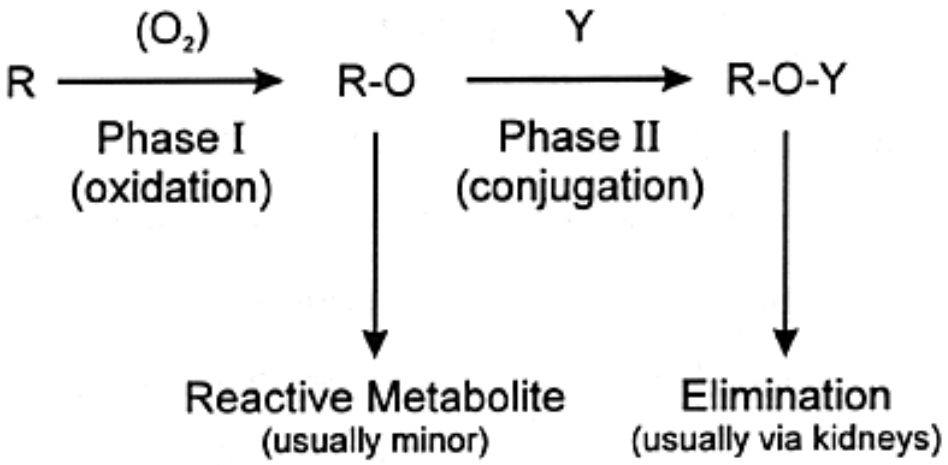

Figure 1.6 The summary of xeobiotic metabolism phases (taken from http://www.preskorn.com)

The xenobiotic metabolism occurs in two phases (Figure 1.6). In phase I, the xenobiotics enter the oxidation-reduction and hydrolysis reactions and polar or reactive groups are added to those by helping enzymes like flavin monooxygenases, cytochrome P450s, and monoamine oxidases. The metabolites produced by phase I reactions have reactive groups, so this reaction may cause production of ROS. Also, the metabolites are not fast eliminated by urine. For this process, the phase II enzymes are responsible. These enzymes have the ability of transformation of the metabolites to more water soluble form and therefore, prevent the toxicity. The ways to convert metabolites to more water soluble form are to change the molecular shape and make molecule larger and heavier to promote the elimination with bile salts (Coleman, 2005). The phase II reactions are conjugation reactions including glucuronidation, sulfation and acetylation. The compound is converted to more soluble form by enzymes, glutathione S-transferases (GSTs), UDP glucuronosyltransferases (UGTs), N-acetyltransferases (NATs), sulfotransferases (SULTs), etc. These phase II reaction enzymes are important in the elimination of the toxic effect of metabolites and they prevent the oxidative stress damage of metabolites produced from phase I reactions.

1.4. GST Enzymes

Glutathione S-transferase (GST) enzymes are known as important multifunctional antioxidant enzyme family playing role in phase II reactions. They are cytosolic enzymes which are responsible for detoxification of active xenobiotics and for drug metabolism (Mannervik et al., 1992). GSTs have small molecular weight; 25000 MW and general they have two subunit structures (Coleman, 2005) (Figure 1.7).

Figure 1.7 The crystallographic structure of glutathione S-transferase (taken from http://en.wikipedia.org/)

The GSTs catalyze conjugation reaction of glutathione (GSH, an antioxidant) and electrophilic agents, so they become more stable and non-toxic. The thiol group (-SH) in GSH is not stable enough to react with ROS and so the thiol molecule can be oxidized before function of conjugation. The GST creates hydrogen bonding from N-terminus of GST and prevents this oxidation by stabilizing the molecule (Figure 1.8).

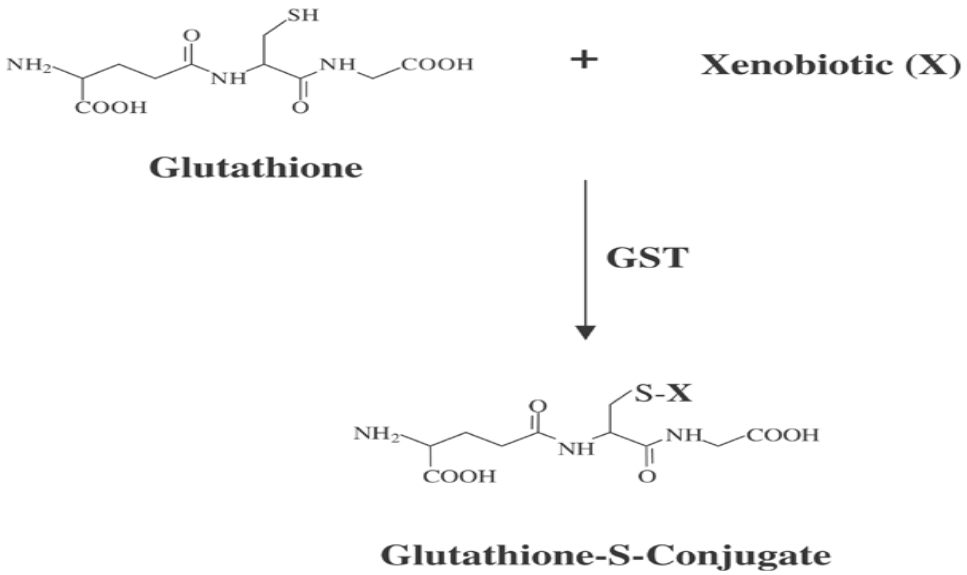

Figure 1.8 Glutathione and xenobiotic conjugation via GST (Townsend et al., 2003)

The glutathione S-transferase enzyme family has seven main class in human organism; Alpha, Mu, Pi, Theta, Zeta, Sigma and Omega (Hayes of Pulford, 1995). Some groups of GSTs are located in cytosol and the others are attached in endoplasmic reticulum. The lipophilic GSTs have different structure and they metabolize the prostaglandins and leukotriens. The cytosolic GSTs are responsible both detoxification of harmful toxic molecules and repairing of some damaged proteins (Coleman, 2005).

Moreover, the level of glutathione S-transferases can be an indicator for some disease such as the high level of GST is alarm for a viral infection or toxification in an organ (Loguercio et al., 1998 and Beckett et al., 1985). For example; the GSTP class enzyme is abundant in renal tubules, so the mean of high level GSTP can be a sign for nephrotoxicity (Sundberg et al., 1994 and Harrison et al., 1989).

The glutathione S-transferase omega class has two isoforms, GST omega 1 and GST omega 2 which are expressed by GSTO1 gene and GSTO2 gene located in chromosome 14q11.2 and 10q24.3, respectively (Whitbread et al., 2003). The structure of omega family has some characteristic feature like other class of GSTs and also this family

shows differences only in omega class. The crystal structure of glutathione S- transferase is shown in figure 1.9.

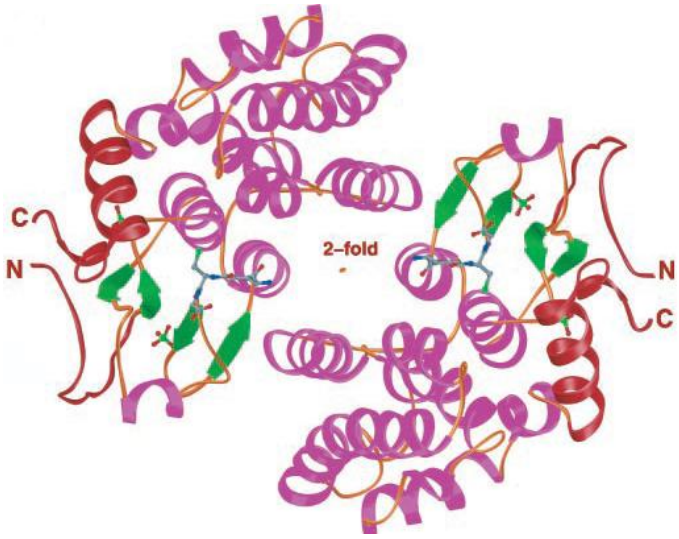

Figure 1.9 Crystal structure of glutathione S-transferase omega

There are two domains; N terminal and C- terminal. The N- terminal is thioredoxin-like domain and C- terminal includes α helical structures. Unlike other GSTs, omega class has a 19 residues N-terminal extension. This region is proline rich and they conjugate with C-terminal by hydrogen bonds. The function of this structure is no clear. Moreover, the active site residue is different in GST omega family which has cysteine while other GSTs have serine and tyrosine (Board et al., 2000). Due to this residue, the omega family creates disulfide bond between GSH molecule rather than hydrogen bonding (Board et al., 2000).

The omega family catalyzes glutathione-dependent detoxification reaction. The GST omega is found in the most of tissues, but this enzyme is abundant in liver, pancreas, skeletal muscle, prostate, spleen, colon and ovary. The expression in brain is low, mostly they are found in glial cells (Yin et al., 2001). The substrate of GST omega family is different from other GSTs. Unlike the other GST family members, these enzymes possesses substrates like 1-chloro 2, 4-dinitrochlorobenzene, 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole and p-nitrophenyl acetate (Board et al., 2000). The glutathione S-

transferase omega family also plays an antioxidant activity role such as thioltransferases and dehydroascorbate reductases with subsrates hydroxyethyl disulfide and dehydroascorbate, respectively (Board et al., 2000). Also, some forms of omega class have a big and open hydrophobic binding site, so they can contribute reaction in protein repairing (Coleman, 2005).

Moreover, the GSTO1 has a similar structure with intracellular chloride channel proteins (CLIC), so this enzyme can bind the receptors which are promoted by CLIC such as ryanodine receptors (Yin et al., 2001). These receptors are a type of Ca channel and located in endoplasmic reticulum. Therefore, the GSTO1 can modulate the process of apoptosis that are induced by Ca (Dulhunty et al., 2001). Also, the GST omega 1 class promotes the activation of inflammation process by affecting the cytokines and interleukin 1β via modifying in posttranslation (Laliberte et al., 2003). Although the mechanism is not completely clear, GSTO1 enzymes have also role in regulation of estrogen receptor negative breast cancer (Adam et al., 2002).

The glutathione S-transferase omega 1 and 2 play important role in arsenic metabolism. Arsenic is an environmental toxic element and is a source for oxidative stress (Sampayo-Reyes et al., 2010). The harmful effect of arsenic decreases with reaction catalyzed by GST omega family. Arsenic is converted to two types of metabolites, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), which are less toxic, via reaction promoted by GST omega family (Zakharyan et al., 2001). Therefore, it can be considered that the risk and severity of diseases, which can be contributed by inorganic arsenic like some types of cancer, neurotoxicity, hyperkeratosis, cerebral infarction etc, can be decreased by GST omega enzymes (Sampayo- Reyes et al., 2010 and Paiva et al., 2010).

1.5. Genetic Polymorphisms

The genetic polymorphism is existence of variance in DNA sequence among individuals of a population. This variance can be functional or non-functional. When the variance is functional, it affects the phenotypes, homeostasis, enzyme activity etc. There are two main types of polymorphism: length polymorphisms and single base changes. The former one is divided into three classes: inversions, insertions-deletions and simple sequence repeats (like microsatellites). The other type of polymorphism is separated two groups: single nucleotide polymorphisms and point mutations.

1.5.1. Single Nucleotide Polymorphism

The single nucleotide polymorphism means that only one nucleotide changes in DNA molecule with a frequency of more than 1% in at least one population. The SNP may be located at coding region in DNA or non-coding region. The SNP located in coding region have two affects: the expressed protein can change or not. If the amino acid change does not occur, it is called silent mutations. If the SNP cause a change in expression, it can be missense; means a different amino acid is expressed, and it can be nonsense; i.e., stop codon expression. These missense and nonsense polymorphisms can cause protein losing functionality or efficiency. Therefore, this situation affects the biochemical reaction in homeostasis.

1.5.2. Polymorphism of Glutathione S-Transferase Omega 1 and Omega 2

Ala140Asp and Asn142Asp polymorphisms are misssense mutations and they occur in GSTO1 and GSTO2 genes, respectively. GSTO1 Ala140Asp is found in exon 4. The 419^{th} nucleotide cytosine is converted to adenine, so the amino acid alanine is substituted to aspartic acid. The Asp genotype caused a reduced enzyme activity (Tanaka-Kagawa et al, 2003). GSTO2 Asn142Asp polymorphism is found in exon 4.

GSTO2 Asn142Asp polymorphism, a substitution adenine nucleotide to guanine at position 424 causes amino acid conversion asparagine to aspartic acid (Rafiee et al., 2010). According to a previous study, this polymorphism may be associated to lower enzyme activity (Whitbread et al., 2003).

In literature, there are some studies related with GSTO1 and GSTO2 gene polymorphisms by focusing the various appointments of the enzymes expressed by these genes. Due to the function of Glutathione S-Transferase omega family on arsenic metabolism, the effect of SNP in GST omega genes on cancer tissues were investigated. Environmental arsenic is a risk factor for cancer and the GST omega family is responsible in detoxification of arsenic in organism. The effects of GSTO1 Ala140Asp and GSTO2 Asn142Asp polymorphisms were studied on urothelial carcinoma patients (Chung et al., 2011).

In another study, it is suggested that there is a relationship between arsenic metabolism and atherosclerosis. Therefore, polymorphisms on the genes responsible for converting inorganic arsenic to less toxic molecules, like GST omega 2, were investigated. The results of this study have shown that, the increased risk for carotid atherosclerosis was observed when the arsenic metabolism was impaired (Hsieh et al., 2011).

The GSTO1 enzyme plays role in redox homeostasis, so this situation may affect the Alzheimer disease and therefore polymorphism of Ala140Asp was studied on sporadic Alzheimer disease patients (Capurso et al., 2010). Moreover, due to relation between smoking and pesticide metabolism with glutathione S-transferase enzymes, the effect of polymorphism in GST Omega genes on Parkinson disease was investigated. The smoking and pesticides decrease the Parkinson disease risk, so the polymorphisms Ala140Asp on GSTO1 gene and Asn142Asp on GSTO2 gene may affect the disease severity (Kiyohara et al., 2010 and Wahner et al., 2007).

1.6. Aim of the Study

Ischemic stroke is an interruption of blood flow in brain vessels due to formation of atherosclerosis. Therefore, lack of nutrients and oxygen damages the brain tissue. Despite of many risk factors, such as age, sex, some chronic diseases are reported in ischemic stroke, the genetic variations are also affect the formation of atherosclerosis and increased the risk of ischemic stroke. Because a genetic variation in DNA molecule can change the enzyme expression in organism, the biochemical reactions which are related to this change are also affected. This situation may increase or decrease the risk of a disease.

Glutathione S-transferase enzyme family is responsible for conjugation reaction with glutathione and xenobiotic molecules. Therefore, this enzyme family play important role in detoxification reactions and decreasing oxidative stress. The endogenous and exogenous toxic elements are converted to less harmful type by GST enzymes.

The oxidative stress (OS) is a risk factor for ischemic stroke because OS promotes the formation of atherosclerosis. When the reactive oxygen species (ROS) level is high and the antioxidant level is low, the oxidative stress increases. ROS contributes the oxidation reaction with low density lipoprotein (LDL). Combination of oxidized LDL and macrophage cells creates a foam cell and then atherosclerosis occurs (Section 1.1.3). Therefore, it can be suggested that due to role in decreasing oxidative stress, the Glutathione S-transferases may have close relationship with ischemic stroke.

The Glutathione S-transferase omega class enzymes were investigated for their disease association. Because the enzyme affects the oxidative stress in tissues, the alteration in enzyme function may cause formation a disease or increasing the severity of a disease. The GST omega 1 prevents organism from oxidative stress, so the diseases affected by oxidative damage are strongly related with GST omega 1 functionality. In a study reported by Kölsch et al., 2004, GSTO1 Ala140Asp single nucleotide polymorphism was studied in Alzheimer disease, vascular dementia and stroke. This SNP reduce the enzyme activity, so the oxidative stress reaches higher level in brain tissue and induces

the neuronal cell death in brain. It was concluded that the Asp genotype may be a risk factor for these cerebrovascular diseases (Kölsch et al., 2004). According to another research carried out by Peddareddy et al., 2009, high oxidative stress occurs when the GST omega 1 enzyme activity changes by SNP Ala140Asp. The researchers suggested that reduced enzyme activity may affect the stroke volume (Peddareddy et al., 2009).

This study was aimed to investigate the possible association between GSTO1 Ala140Asp and GSTO2 Asn142Asp SNPs and ischemic stroke risk in Turkish population. This study also determined the genotype and allele frequencies of GST Omega Class SNPs in Turkish population and compared them with other populations.

The steps to reach this aim of study were listed below:

- Collecting the whole blood samples from ischemic stroke patients and control group,
- Isolation of genomic DNA from whole blood samples,
- Amplification of interested regions in GSTO1 gene and GSTO2 gene by PCR method,
- Applying restriction endonuclease digestion to PCR products and determine the genotypes of patients and controls for interested SNPs,
- Detection of genotype and allele frequencies for GSTO1 and GSTO2 genes for ischemic stroke and control groups in Turkish population,
- Assessment of genotype and allele frequencies between patients and controls by categorizing subgroups like age, gender, conventional risk factors for stroke.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Population and Blood Sampling

Blood samples were collected from 239 acute hemispheric ischemic stroke patients and 130 symptom free controls with the collaboration of Gülhane Military Medical Academy, Department of Neurology, Ankara, from October 2005 to April 2011. Both patients and control group was unrelated Caucasian people from same geographic region, central Anatolia, Turkey. The cerebral infarction of stroke patients was diagnosed by using neurological examination such as brain computer tomography scan, transthoracic echocardiographic examination, Holter study and Transcranial Doppler emboli detection. According to results of these tests, the patients who included were selected: having anterior circulation stroke without other major disease including autoimmune disease, carcinoma, any failure in clotting, hepatic or renal failure, no embolic defeat, no having familial background for hematological, autoimmune or chronic inflammatory illnesses, no having heart attack in three weeks or transient ischemic attack. Control group was selected randomly from out of neurology department who has no stroke or transient stroke at any time. All criteria applied for patient group were also considered for controls and in addition to not having carotid stenosis > 50% or ulcerated carotid plaque.

All subjects underwent bilateral carotid Doppler ultrasound (CUSG) and echocardiographic studies. The detailed information about these canvasses can be found in research of former studies (Demirdöğen et al., 2008, Demirdöğen et al., 2009 and Türkanoğlu et al., 2010).

An overall history of conventional vascular risk factors and conditions from each participant was acquired from our collaborator Gülhane Military Medical Academy, Neurology Department. Hypertension was assessed as a systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg and/or use of antihypertensive drugs. Diabetes was defined as fasting glucose ≥6.99 mmol/L and/or use of pharmacological treatment. Obesity was defined by measuring body mass, which was higher or equal to 30. Smoking status of an individual was decided as "yes" if the individual was a current smoker or quitted smoking less than three months ago.

All participants were subjected to routine laboratory tests including electrocardiogram, chest-X ray, complete blood count, leukocyte differential, erythrocyte sedimentation rate, routine biochemical tests such as fasting glucose, lipid profile (triglycerides, total cholesterol, LDL, HDL, VLDL-C) creatinine, sodium, potassium, bilirubin, and liver function tests, routine urine tests and rheumatologic screening tests. These evaluations and all laboratory tests were performed blinded to medical conditions of the subjects.

All participating individuals were informed about the study and then consent forms were signed by them (see APPENDIX A and B). Also, the study was approved by the Ethical Committee of the Medical Academy and was carried out according to the principles of the Declaration of Helsinki (see APPENDIX C).

2.1.2. Chemicals and Enzymes

Some chemicals that are necessary for our experimental procedures such as agarose (A-9539), bromophenol blue (B-5525), ethidium bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), sodium chloride (NaCl; S-3014), sodium

dodecyl sulfate (SDS; L-4390), 2-amino-2(hydroxymethyl)-1,3- propandiol (Tris; T-1503), were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Borate (11607) and absolute ethanol (32221) were the products of Riedel de Haën, Seelze. Magnesium chloride (A4425) and potassium chloride (A2939) were purchased from AppliChem, Ottoweg, Darmstadt. Sucrose (7653) and Triton X -100 (11869) were the products of Merck and Co., Inc., Whitehouse Station, NJ, USA.

Taq DNA Polymerase (supplied together with $MgCl_2$ and amplification buffer, (#EP0407), dNTP mix (#R0191), 50 bp Gene Ruler^{TM} DNA Ladder (#SM0371) and restriction enzyme MboI (#ER0811) which includes its buffer were purchased from Fermentas, USA. Moreover, the restriction enzyme Cac8I (R0597L) was obtained from New England Biolabs.

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

2.1.3. Primers

Two primer pairs were purchased from Iontek (Iontek, Istanbul, Turkey) for amplifying our desired region. The commercial primer stocks had 100 pmol/µL and they are preserved in -20 °C. The primers were aliquoted to 10 pmol/µL in order to use in PCR.

In this study, the primer pairs used for polymorphisms Ala140Asp of GSTO1 gene and Asn142Asp of GSTO2 gene were selected by literature searches (Chung et al., 2010).

The sequences of forward and reverse primers used for analysis of Ala140Asp polymorphism of GSTO1 gene were:

5′ GAA CTT GAT GCA CCC TTG GT 3′
5′ TGA TAG CTA GGA GAA ATA ATT AC 3′

The sequences of forward and reverse primers used for analysis of Asn142Asp polymorphism of GSTO2 gene were:

5′ AGG CAG AAC AGG AAC TGG AA 3′
5′ GAG GGA CCC CTT TTT GTA CC 3′

2.2. Methods

Human genomic DNA was isolated from whole blood samples. Buffers used for this process include Tris-HCl buffer containing EDTA, pH 8.0, TKME buffer saturated with NaCl, pH 7.6 and TE buffer, pH 8.0. In agarose gel electrophoresis of PCR and restriction digestion products, TBE buffer, pH 8.3, ethidium bromide, gel loading dye solutions were used. Reagents and chemical used for DNA isolation from human whole blood samples, PCR, restriction endonuclease digestion and agarose gel electrophoresis were attached to Appendix D.

2.2.1. Preparation of Human Genomic DNA Sample for PCR

2.2.1.1. Isolation of Genomic DNA from Human Whole Blood Samples

Isolation of genomic DNA from human whole blood was carried out using the method of Lahiri and Shnabel, 1993, with slight modifications. The whole blood samples were collected by Gülhane Military Medical Academy into EDTA containing tubes to eliminate blood clotting. The reagents used in this procedure were given in Appendix D.

DNA Isolation Procedure:

750 µL of whole blood was transferred into eppendorf tube and was treated with the same volume of low-salt TKM Buffer, pH 7.6. 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. The centrifugation process was repeated at 14000 g for 10 minutes at 4°C and supernatant fraction containing the DNA was taken to a separate eppendorf tube. Then, 2x volume ice-cold ethanol was added 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. Finally, supernatant was removed and DNA containing pellet was solubilized with 100 µL of TE Buffer, pH 8.0. Tubes were incubated at 37°C for more than 2 hours to completely dissolve the DNA.

2.2.1.2. Quantification of Genomic DNA Samples 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 wavelength 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:

Concentration (µg/mL) = A_{260nm} x 50 (µg/mL) x Dilution Factor

2.2.1.3. Qualification of Genomic DNA Samples by Spectrophotometry

The purity of nucleic acids was determined by measurements of the absorbance at 280 nm and 260 nm. They give maximum absorbance for proteins and for DNA, respectively. The ratio of these absorbances was used to assess protein and RNA contamination in DNA sample. The ratio for pure DNA is 1.8 while higher and lower values represent RNA and protein contamination, respectively.

2.2.1.4. Qualification of Genomic DNA Samples by Agarose Gel Electrophoresis

Determination of the intactness of DNA samples was performed by 0.5% agarose gel electrophoresis using Biogen horizontal gel electrophoresis system which gave a gel tray in 8 cm x 9 cm dimensions. Reagents used for qualification of human genomic DNA samples by agarose gel electrophoresis were given in Appendix D.

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. This mix was heated in microwave oven till all of the grains of the agarose dissolved.

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 throughly.

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 allowed to solidify 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 and the gel in the plastic tray was mounted in the electrophoresis tank so that the slots of the gel faced towards the negative pole-cathode. 0.5x TBE buffer was added to the tank until it covered the gel to a depth of 1 mm. Any air bubbles in the wells were removed with the help of a pipette tip.

5 μL (0.25-0.5 ng) of DNA sample was mixed with 1 μL of gel loading buffer by sucking in and out of a micropipette. The mixture was slowly loaded into the slots 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 so that the voltage of 5 V/cm (measured as the distance between the electrodes) was applied. 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.

Single band in agarose gel electrophoresis shows pure DNA preparations, while RNA contaminated preparations yield two bands. A smear shows that the DNA is degraded.

2.2.2. Genotyping of Ala140Asp Polymorphism in GSTO1 Gene and Asn142Asp Polymorphism in GSTO2 Gene

In this study, genetic polymorphisms of GSTO1 gene (Ala140Asp) and GSTO2 gene (Asn142Asp) were examined. The restriction fragment length polymorphisms detection method was used to determine the polymorphisms. In PCR-RFLP technique, polymerase chain reaction was done for desired gene region by using specific primers and then was followed by restriction enzyme digestion. Techne Progene (Cambridge, UK) and Eppendorf Mastercycler (Hamburg, Germany) thermocyclers were used for PCR; also TDB-120 Heat block (Biosan Ltd., Latvia) was used to incubate samples during digestion. The procedure details for PCR and digestion were given in following sections.

In Table 2.1, SNP involving genes, polymorphisms, region of amplification, PCR product size, restriction endonucleases and fragment size were given.

Table 2.1 Genes of interest, polymorphisms, regions of amplification, PCR product size, restriction endonucleases and fragment size for GSTO1 and GSTO2 genes.

Gene	Polymorphism	Region of Amplification	PCR Product Size	Restriction Endonuclease	Fragment Size
GSTO1	Ala140Asp (C→A)	Coding Region	254bp	Cac8I	CC:186-68 CA:254-186-68 AA:254
GSTO2	Asn142Asp (A→G)	Coding Region	185bp	MboI	AA: 185 AG:185-122-63 GG: 122-63

2.2.2.1. Genotyping of Ala140Asp Single Nucleotide Polymorphisms on GSTO1 Gene

2.2.2.1.1 Polymerase Chain Reaction for Ala140Asp SNP

Ala140Asp single nucleotide polymorphism is involved in GSTO1 gene, exon4. The reagents for PCR protocol (details were given in Appendix D):

- Taq DNA polymerase
- PCR amplification buffer with KCl
- dNTP mixture
- $MgCl_2$ solution
- Forward and reverse primers (section 2.1.3)

Initially, polymerase chain reaction protocol was optimized to gather single specific product for desired SNP region. To obtain best concentration of components for PCR, twenty four different conditions by using three concentrations of Taq polymerase (1.5 U, 2 U and 2.5U), five concentrations of $MgCl_2$ (2 nm, 2.5 nm, 3 nm, 3.5 nm and 4 nm) and three concentrations of primer (30 pmole, 40 pmole and 50 pmole) were tested. The concentrations of compounds used for optimized PCR mixture were given in Table 2.2.

Table 2.2 The components of PCR mixture for Ala140Asp polymorphism.

Constituent	Stock Concentration	Volume Added	Final Concentration in 50 µL Reaction Mixture
Amplification Buffer	10X	5 µL	1X
$MgCl_2$	25 mM	6 µL	3 mM
dNTP Mixture	10 mM	1 µL	0.2 mM
Forward Primer	10 pmole/µL	5 µL	50 pmole
Reverse Primer	10 pmole/µL	5 µL	50 pmole
Taq DNA Polymerase	5U/µL	0.5 µL	2.5 U
Template DNA	Varies	Varies	~200 ng
Sterile Apyrogen H_2O		Up to 50 µL	

The thermal cycling program to amplify Ala140Asp SNP was given Table 2.3.

Table 2.3 Thermal cycling conditions for Ala140Asp polymorphism.

	Temperature	Time	
Initial Denaturation	94 °C	5 min	
Denaturation	94 °C	1 min	30 cycles
Annealing	60 °C	1min	
Extension	72 °C	5 min	

Amplified region of GSTO1 gene including Ala140Asp single nucleotide polymorphism was illustrated in Figure 2.1 with highlighted forward and reverse primers, recognition site of endonuclease and single nucleotide substitution.

The amplified PCR products were loaded on 2% agarose gel and gel electrophoresis method was used as described in section 2.2.4. 10 μL of PCR product and 3 μL of bromophenol blue dye were loaded each well. 5 μL of DNA ladder was loaded in a well. The gel was run for 45 min at 120 V until dye reached the bottom of the gel. Then, the gel was analyzed under UV light and photographed.

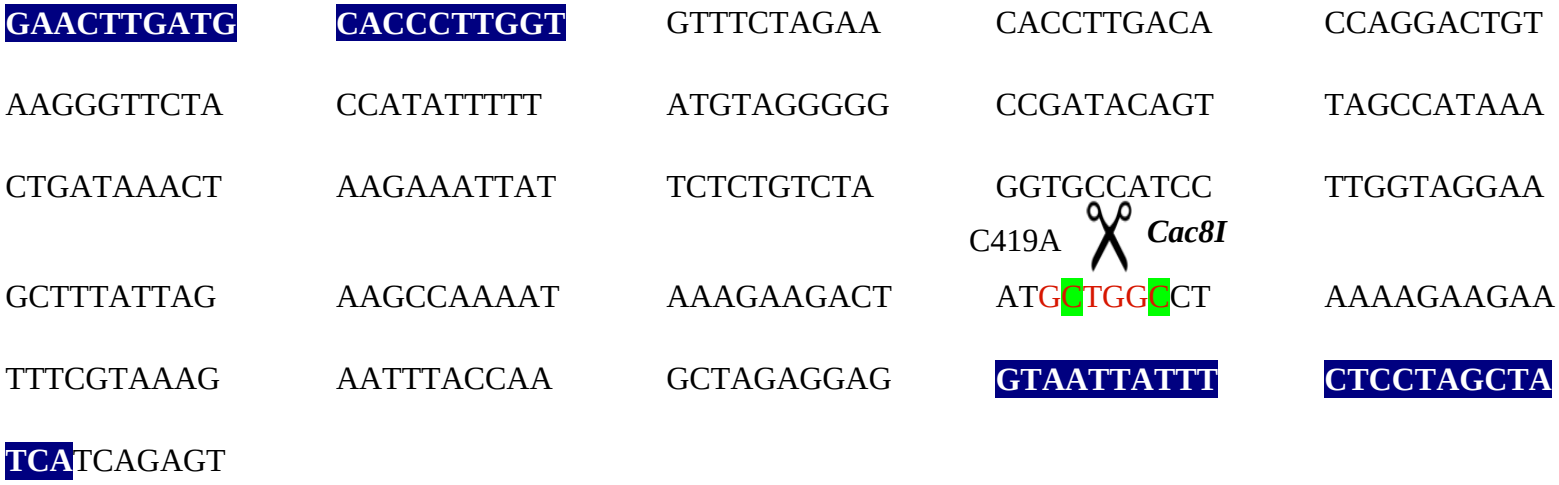

Figure 2.1 Schematic representation of the glutathione S-transferase omega 1 gene that includes C419A (Ala140Asp) single nucleotide polymorphism. The blue highlighted sequences are the forward and reverse primers used for PCR procedure. The red region is enzyme recognition site; when the sequence is GCNNGC, the enzyme can cut. In wild type, the enzyme is cut between 'T' and 'G'. In the mutant, 419th allele is 'A' and the enzyme could not cut. (The nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov)

2.2.2.1.2 Restriction Endonuclease Digestion of GSTO1 Ala140Asp Polymorphism

Determination of genotypes in terms of Ala140Asp polymorphism in GSTO1 gene was carried out using the restriction fragment length polymorphism technique. Cac8I restriction enzyme was used to digest PCR products of GSTO1 Ala140Asp SNP. This enzyme recognizes the wild type individuals (C), so when base substitution occurs (C/A), the enzyme could not cut the DNA and no fragment was observed. The enzyme Cac8I yields 186 bp and 68 bp long bands indicating wild type allele containing individuals. 254 bp long band means the individuals is mutant whereas 254 bp, 186 bp and 68 bp bands indicates heterogeneous individuals for this SNP (Figure 2.2).

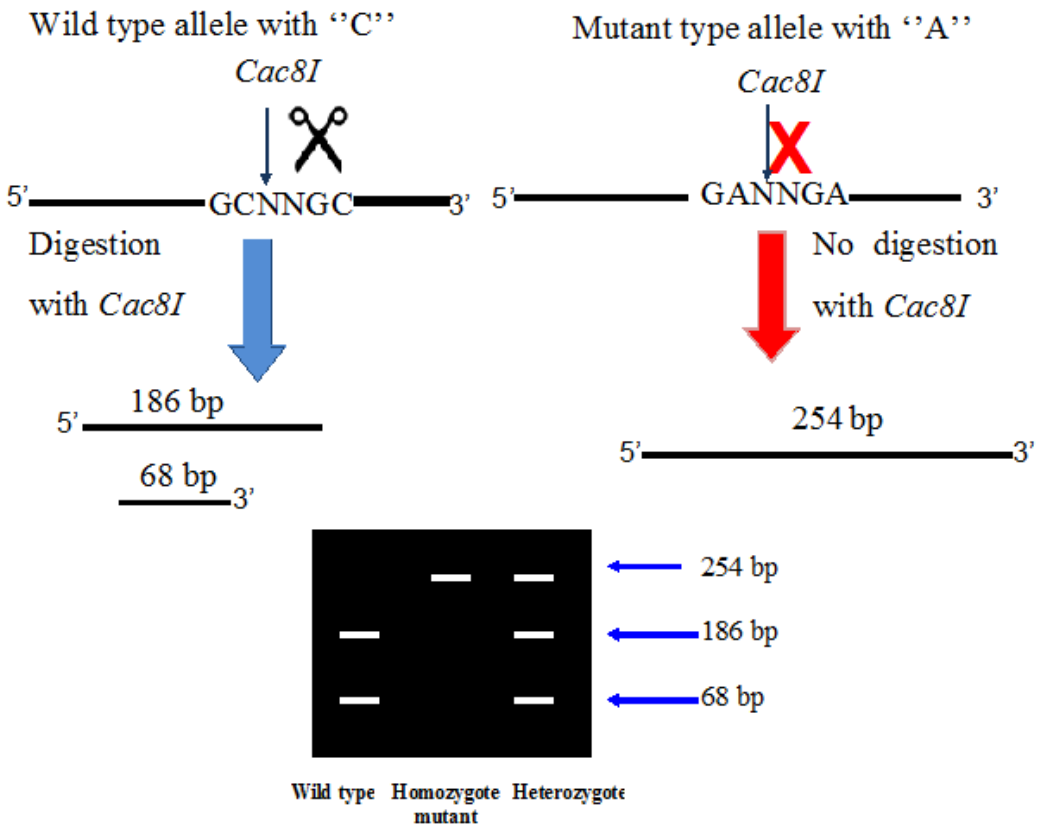

Figure 2.2 Schematic presentation of the determination of genotypes of *GSTO1* C419A (Ala140Asp) single nucleotide polymorphism. In the left panel, the wild type allele 'C' can be seen. *Cac8I* recognize the site and the enzyme cuts the 254 bp PCR product and cause two fragments of 186 bp and 68 bp. In the right panel, the mutant type allele 'A' can be seen. *Cac8I* does not recognize the site, thus the unfragmented 254 bp band represents the mutant type 'A' allele. At the bottom, a representative agarose gel photograph of wild type, heterozygous and mutant genotypes are given. The reagents used for determination of genotypes of *GSTO1* C419A (Ala140Asp) single nucleotide polymorphism were given in Appendix D.

To obtain the best digestion products image in agarose gel electrophoresis, restriction enzyme was tested by using different concentrations. The optimized condition used for restriction endonuclease digestion was given in Table 2.4.

Table 2.4 Restriction endonuclease digestion mixture constituents for Ala140Asp polymorphism

Constituent	Stock Concentration	Volume to be Added	Final Concentration in 30µL Reaction Mixture
PCR Product		10µL	
NE Buffer 4	1X	3µL	0.1X
Cac8I	5U/µL	0.4µL	2U
Sterile apyrogen H_2O		16.6µL	

10 µL of PCR product was incubated in 2U Cac8I restriction enzyme at 37°C for 18 to 24 hours. After the incubation, the digestion product was mixed with 6 µL loading dye and it was loaded to 3% agarose gel to analyze the length of digestion products. 5 µL of 50 bp ladder was also loaded to determine the length of fragments. The gel was run for 45 min at 120 V.

2.2.2.2. Genotyping of Asn142Asp Single Nucleotide Polymorphism on GSTO2 Gene

2.2.2.2.1 Polymerase Chain Reaction for Asn142Asp SNP

Asn142Asp single nucleotide polymorphism is involved in GSTO2 gene, exon4. The reagents for PCR protocol (details were given in Appendix D):

- Taq DNA polymerase
- PCR amplification buffer with $NaSO_4$
- dNTP mixture
- $MgCl_2$ solution
- Forward and reverse primers (section 2.1.3)

Initially, polymerase chain reaction protocol was optimized to gather single specific product for desired SNP region. To obtain best concentration of components for PCR, twenty four different conditions by using three concentrations of Taq polymerase (1.5 U, 2 U and 2.5U), five concentrations of $MgCl_2$ (2 nm, 2,5 nm, 3 nm, 3.5 nm and 4 nm) and three concentration of primer (30 pmole, 40 pmole and 50 pmole) were tested. The concentrations of compounds used for optimized PCR mixture were given in Table 2.5.

Table 2.5 The components of PCR mixture for Asn142Asp polymorphism.

Constituent	Stock Concentration	Volume Added	Final Concentration in 50 µL Reaction Mixture
Amplification Buffer	10X	5 µL	1X
$MgCl_2$	25 mM	3 µL	1.5 mM
dNTP Mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmole/µL	4 µL	40 pmole
Reverse Primer	10 pmole/µL	4 µL	40 pmole
Taq DNA Polymerase	5U/µL	0.5 µL	2.5 U
Template DNA	Varies	Varies	~200 ng
Sterile Apyrogen H_2O		Up to 50 µL	

The thermal cycling program to amplify Asn142Asp SNP was given Table 2.6.

Table 2.6 Thermal cycling conditions for Asn142Asp polymorphism.

	Temperature	Time	
Initial Denaturation	94 °C	5 min	
Denaturation	94 °C	1 min	30 cycles
Annealing	60 °C	1min	
Extension	72 °C	5 min	

Amplified region of GSTO2 gene including Asn142Asp single nucleotide polymorphism was illustrated in Figure 2.1 with highlighted forward and reverse primers, recognition site of endonucleases and single nucleotide substitution.

The amplified PCR products were loaded on 2% agarose gel and gel electrophoresis method was used as described in section 2.2.4. 10 µL of PCR product and 3 µL of bromophenol blue dye were loaded each well. 5 µL of DNA ladder was loaded in a well. The gel was run for 45 min at 120 V until dye reached the bottom of the gel. Then, the gel was analyzed under UV light and photographed.

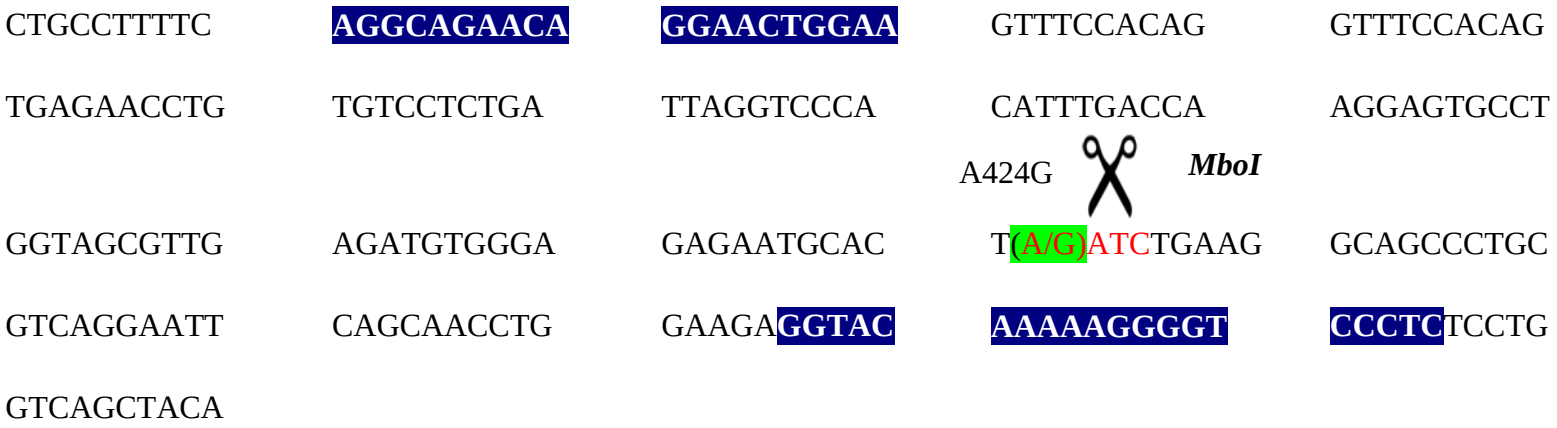

Figure 2.3 Schematic representation of the Glutathione S-Transferase Omega 2 Gene that includes A424G (Asn142Asp) single nucleotide polymorphism. The blue highlighted sequences are the forward and reverse primers used for PCR procedure. The red region is enzyme recognition site; when the sequence is GATC (AATC is wild type), the enzyme can cut. In mutant type, the enzyme is cut between 'G' and 'A'. In the wild type, 424th allele is 'A' and the enzyme could not cut. (The nucleotide sequence is taken from http://www.esembl.org).

2.2.2.2.2 Restriction Endonuclease Digestion of GSTO2 Asn142Asp Polymorphism

Determination of genotypes in terms of Asn142Asp polymorphisms in GSTO2 gene was carried out using the restriction fragment length polymorphism technique. MboI restriction enzyme was used to digest PCR products of GSTO2 Asn142Asp SNP. Wild type individuals for Asn142Asp have no recognition site for MboI enzyme while base substitution (A/G) causes formation of recognition site. So when base substitution occurs (A/G), the enzyme could cut the DNA and fragments were observed. The enzyme MboI yields 122 bp and 63 bp long bands indicating mutant allele containing individuals, 185 bp long band means the individual is wild type whereas 185 bp, 122 bp and 63 bp bands indicates heterogeneous individuals for this SNP (Figure 2.6).

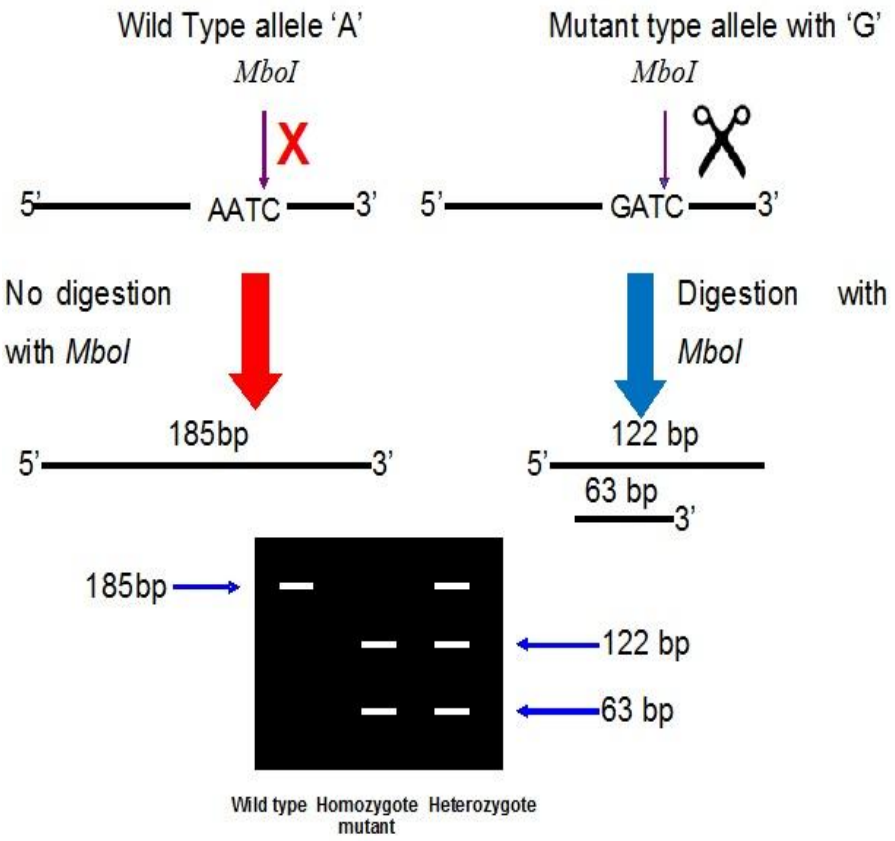

Figure 2.4 Schematic presentation of the determination of genotypes of GSTO2 A424G (Asn142Asp) single nucleotide polymorphism. In the left panel, the mutant type allele 'G' can be seen. *MboI* recognize the site and the enzyme cuts the 185 bp PCR product and cause two fragments of 122 bp and 63 bp. In the right panel, the wild type allele 'A' can be seen. *MboI* does not recognize the site, thus the unfragmented 185 bp band represents the wild type 'A' allele. At the bottom, a representative agarose gel photograph of wild type, heterozygous and mutant genotypes are given. The reagents used for determination of genotypes of GSTO2 A424G (Asn142Asp) single nucleotide polymorphism were given in Appendix D.

To obtain the best digestion products image in agarose gel electrophoresis, restriction enzyme was tested by using different concentrations. The optimized condition used for restriction endonuclease digestion was given in Table 2.7.

Table 2.7 Restriction endonuclease digestion mixture constituents for Asn142Asp polymorphism

Constituent	Stock Concentration	Volume to be Added	Final Concentration in 30µL Reaction Mixture
PCR Product		10 µL	
Buffer R	10X	3 µL	1X
MboI	10U/ µL	0.2 µL	2U
Sterile apyrogen H_2O		16.8 µL	

10 µL of PCR product was incubated in 2U MboI restriction enzyme at 37°C for 18 to 24 hours. After the incubation, the digestion product was mixed with 6 µL loading dye and it was loaded to 3% agarose gel to analyze the length of digestion products. 5 µL of 50 bp ladder was also loaded to determine the length of fragments. The gel was run for 45 min at 120 V.

2.2.3. Statistical Analysis

Statistical analysis was done by using the software program SPSS 20.0 (developed by SPSS, Chicago, IL, USA). In these analyses, continuous variables were expressed as mean ± standard deviation. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov-Smirnov test. Moreover, the Independent T-Test and Mann-Whitney U test were used to evaluate differences of continuous variables depending on the shape of distribution curves. Categorical variables such as sex, statin usage, hypertension, smoking status, diabetes and obesity were expressed as proportions and compared using Chi-square test. The gene counting method was used to determine

allele frequency and Chi-square test was used to evaluate departure from the Hardy-Weinberg equilibrium and to compare of genotype distribution and allele frequency.

A logistic regression analysis with backward selection method was used to determine the effects of vascular risk factors, lipid parameters and genotypes in the prediction of ischemic stroke. 2-tailed probability values with 95% confidence intervals were used for each odds ratio. Calibration of the logistic regression analysis was done with Hosmer-Lemeshow goodness of fit test.

Significance of the result was determined with P value. If the value was less than 0.05, this result was accepted as significant, on the other hand, ones higher than 0.05 accepted as insignificant.

CHAPTER 3

RESULTS

3.1. Study Population

The study population consisted of 239 ischemic stroke patients and 130 control subjects without any symptoms related to ischemic stroke. The polymorphisms in GSTO1 gene and GSTO2 gene were determined by using PCR/RFLP and the genotypes and allele frequencies of these genes were calculated. The blood samples used for isolation of whole human DNA were collected from Central Anatolia, Turkey, by Gülhane Military Medical Academy, Department of Neurology. The clinical parameters and risk factors including lipid level, hypertension, diabetes, obesity and cigarette smoking were recorded and added to Appendix E. The details about preparing study population were given in section 2.1.1.

The demographic features, clinical laboratory test result and the prevalence of conventional risk factors for the patients and the controls in the study population were given in Table 3.1.

The age of study population varied between 38 to 90 for control group and 20 to 83 for patients. The mean of ages were 65.57±13.54 for patients and 64.16±12.17 for controls. Age is a risk factor for stroke. Due to this reason, we should eliminate the significant differences of ages between patients and controls (P=0.067). The patient group included 133 male and 106 females, and the control group included 63 males and 67 females in our study population.

The gender is a risk factor for ischemic stroke; males are more susceptible than females. For this reason the number of males and females should be close within patients and controls, in order to eliminate the effect of susceptibility. There was approximately similar percentage of male and female in the study population (55.6% of patients were males and 51.5% of controls were males, P=0.186).

The prevalence of conventional risk factors for stroke such as hypertension, diabetes, cigarette use and obesity were higher in patients than control group (Table 3.1). Also, these risk factors were found significant risk factors for ischemic stroke (P values are lower than 0.05).

Table 3.1 Clinical characteristic and conventional risk factor of ischemic stroke patients and controls.

Parameter	Patients (n=239)	Control (n=130)	*P*
Age (years)[a]	65.57±13.54	64.16±12.17	0.067
Male, n (%)[b]	133 (55.6)	63 (51.5)	0.186
Hypertension, n (%)[b]	160 (66.9)	49 (37.7)	0.000
Diabetes mellitus, n(%)[b]	77 (32.2)	20 (15.4)	0.000
Smokers, n (%)[b]	65 (27.2)	16 (12.3)	0.001
Obesity, n (%)[b]	53 (22.2)	8 (6.2)	0.000
Total cholesterol (mmol/L)[c]	4.76 ±1.34	4.70±1.28	0.679
Triglycerides(mmol/L)[c]	1.54 ±0.75	1.44±0.60	0.195
HDL(mmol/L)[c]	1.08±0.31	1.20±0.31	0.001
LDL(mmol/L)[c]	2.85±1.17	2.76±0.04	0.499

Values are either number of subjects, percentage or mean ± SD

[a]Mann Whithey U test is applied

[b]Chi-square test is applied

[c]Independent Samples T-test is applied

Hypertension was observed in 66.9% of the patients, while 37.7% of the control group was hypertensive (P=0.000). In patient group, the frequency of diabetic individuals (32.3%) was higher than control group (15.4%) (P=0.000). Similarly, the frequency of smoking people was significantly higher in patient than control group (P=0.001). The obesity was observed in 22.2% of patient group, while 6.2% in control group (P=0.000).

According to the clinical laboratory tests given in Table 3.1, HDL level was significantly lower in patient (1.08±0.31 mmol/L) than controls (1.20±0.31 mmol/L) (P=0.001). Also, LDL level is insignificantly higher in patients (2.85±1.17 mmol/L) than controls(2.76±0.04 mmol/L, P=0.499). The total cholesterol levels of patients (4.76±1.34 mmol/L) and controls (4.70±1.28 mmol/L, P=0.679) were close each other. The level of triglycerides in patients (1.54±0.75 mmol/L) is slightly higher than in controls (1.44±0.60 mmol/L, P=0. 195).

Table 3.2 represents the effects of those conventional risk factors on ischemic stroke. Except gender (P=0.186), all conventional risk factors; hypertension, diabetes mellitus, smoking and obesity, have significant risk effect in stroke. The hypertension caused approximately 3-fold risk for stroke (OR=3.35, P=0.000). Diabetes and smoking have approx. 2.5-fold relative risk in patients when compared to controls. (OR= 2.614, P=0.000 for diabetes and OR=2.662, P=0.001 for smoking). Obesity had approx. 4 fold risk for stroke (OR=4.191, P=0.000).

Table 3.2 Effects of conventional risk factor on ischemic stroke

Parameter	OR (%95 CI)	*P*
Male, n (%)[a]	1.334 (0.870-2.048)	0.186
Hypertension, n (%)[a]	3.35 (2.144-5.227)	0.000
Diabetes mellitus, n (%)[a]	2.614(1.511-4.523)	0.000
Smokers, n (%)[a]	2.662(1.467-4.829)	0.001
Obesity, n (%)[a]	4.191(1928-9.113)	0.000

Values are either number of subjects, percentage or mean ± SD

[a]Chi-square test is applied

3.2. Genotyping for Single Nucleotide Polymorphisms in GSTO1 and GSTO2 Gene

The PCR/RFPL method was used for determination of GSTO1 and GSTO2 genotype frequencies. In this procedure, the amplified region by PCR and the products from RFLP was visualized by agarose gel electrophoresis.

3.2.1. Genotyping for Ala140Asp Single Nucleotide Polymorphism of GSTO1 gene

Determination of Ala140Asp single nucleotide polymorphism of GSTO1 gene was achieved by PCR/RFLP method. The polymerase chain reaction was followed by restriction enzyme digestion. The specific enzyme for this procedure is Cac8I.

3.2.1.1. Polymerase Chain Reaction Results for Ala140Asp Single Nucleotide Polymorphism

Ala140Asp Single Nucleotide Polymorphism is found in the exon4. The 'C' nucleotide is converted to 'A' nucleotide, so the gene encodes 'aspartic acid' instead of 'alanine'. The polymerase chain reaction was optimized for best results like desired base pairs and elimination of non-specific band. The optimized PCR mixture for the amplification of Ala140Asp SNP was given in section 2.2.2.1.1. Approximately 200 ng DNA, 0.2 mM dNTPs, 50 pmol of each primer, 3 mM $MgCl_2$ and 2.5 Unit of Taq Polymerase were included in the PCR reaction. PCR products were analysed on 2% agarose gel prepared by adding 4 g agarose to 200 mL of 0.5X 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.

The amplified region of DNA fragments was 254 bp due to selected primers. Figure 3.1 shows the gel electrophoresis photograph including result of PCR product for Ala140Asp SNP. The bands were located approximately at 254 bp as expected.

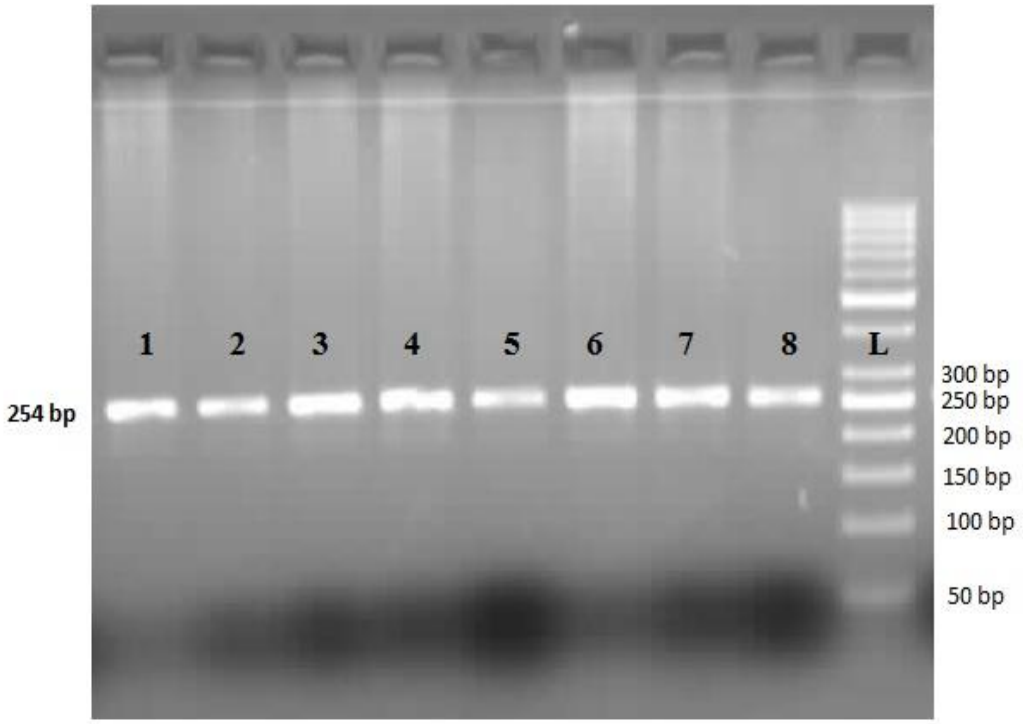

Figure 3.1 Agarose gel electrophoresis image for polymerase chain reaction product of GSTO1 gene. The lane labeled with L shows the DNA ladder (50 bp-1000 bp). Other lanes are PCR products which are located 254 bp, as expected.

3.2.1.2. Restriction Endonuclease Digestion Results for Ala140Asp Single Nucleotide Polymorphism

The genotype determination was done by restriction fragment length polymorphism method. A specific endonuclease enzyme, Cac8I, was used. This enzyme can cut from wild type, C nucleotide. When the product is wild type, the enzyme gives two fragments: 186 bp and 68 bp length. However, when the product is mutant, the enzyme cannot recognize the 'A' nucleotide, so the yield is 254 bp. The heterozygous mutant product yields three fragments, 254 bp, 186 bp and 68 bp. The procedure details and optimized condition were given in section 2.2.2.1.2. The result of restriction fragments for Ala140Asp SNP was given in Figure 3.2.

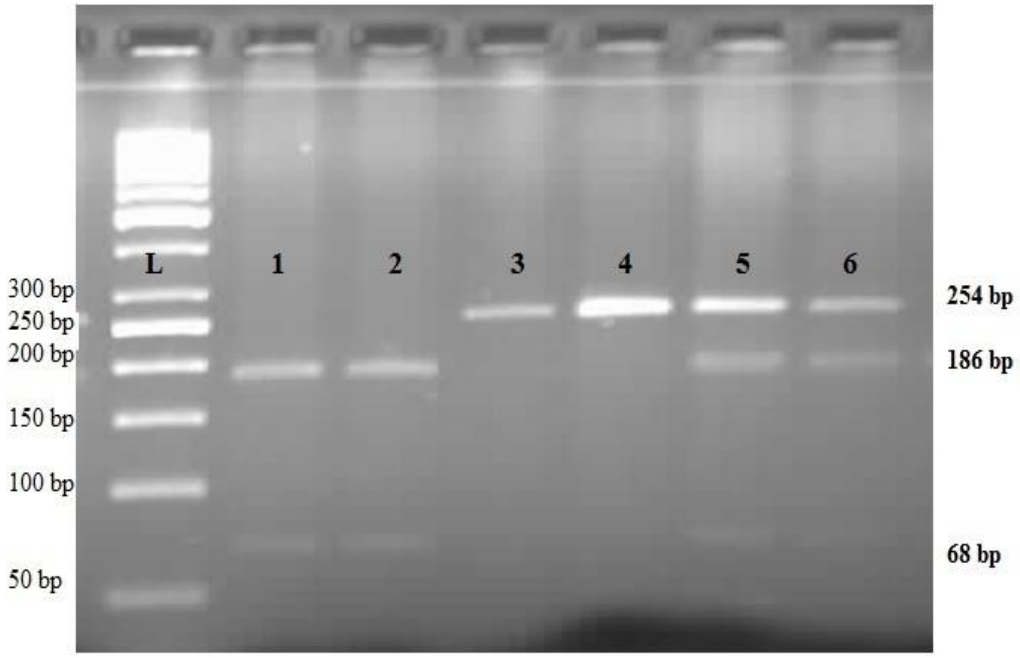

Figure 3.2 Agarose gel electrophoresis image for restriction endonuclease digestion with Cac8I of amplified GSTO1 gene. The lane labeled with L shows the DNA ladder (50 bp-1000 bp). The other lanes are digestion products. Lane 1 and 2 show the banding patterns of wild type homozygous individuals due to including two bands at 186 bp and 68 bp. Lane 3 and 4 are homozygous mutant individuals with single band at 254 bp. Lane 5 and 6 have three bands at 254 bp, 186 bp and 68 bp, so these are heterozygous mutant individuals.

3.2.2. Genotyping for Asn142Asp Single Nucleotide Polymorphism GSTO2 Gene

Determination of Asn142Asp single nucleotide polymorphism of GSTO2 gene was achieved by PCR/RFLP method. The polymerase chain reaction was followed by restriction enzyme digestion. The specific enzyme for this procedure is MboI.

3.2.2.1. Polymerase Chain Reaction Results for Asn142Asp Single Nucleotide Polymorphism

Asn142Asp Single Nucleotide Polymorphism is found in the exon 4. The 'A' nucleotide is converted to 'G' nucleotide, so the gene encodes 'aspartic acid' instead of 'asparagine'. The polymerase chain reaction was optimized for best results like desired base pairs and elimination of non-specific band. The optimized PCR mixture for the amplification of An142Asp SNP was given in section 2.2.2.2.1. Approximately 200 ng DNA, 0.2 mM dNTPs, 40 pmol of each primer, 1.5 mM $MgCl_2$ and 2.5 Unit of Taq Polymerase were included in the PCR reaction. PCR products were analysed on 2% agarose gel prepared by adding 4 g agarose to 200 mL of 0.5X 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.

The amplified region of DNA fragments was 185 bp due to selected primers. Figure 3.3 shows the gel electrophoresis photograph including result of PCR product for Asn142Asp. The bands were located approximately at 185 bp as expected.

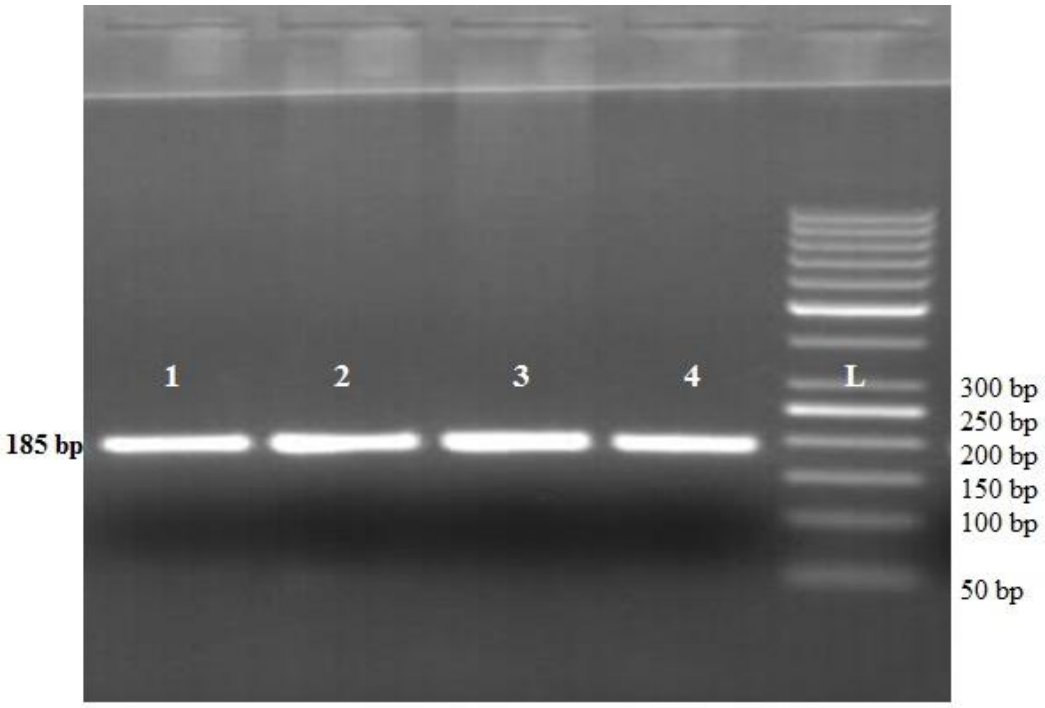

Figure 3.3 Agarose gel electrophoresis image for polymerase chain reaction product of GSTO2 gene. The lane labeled with L shows the DNA ladder (50 bp-1000 bp). Other lanes are PCR products which are located 185 bp, as expected.

3.2.2.2. Restriction Endonuclease Digestion Results for Asn142Asp Single Nucleotide Polymorphism

The genotype determination was done by restriction length fragment polymorphism method. A specific endonuclease enzyme, MboI, was used. This enzyme can cut from mutant allele, G nucleotide. When the product is homozygous mutant, the enzyme gives two fragment, 122 bp and 63 bp length. However, when the product is wild type, the enzyme cannot recognize the 'A' nucleotide, so the fragment is 185 bp. The heterozygous mutant product yields three fragments, 185 bp, 122 bp and 63 bp. The procedure details and optimized condition was given in Section 2.2.2.2.2. The result of restriction fragments for Asn142Asp SNP was given in Figure 3.4.

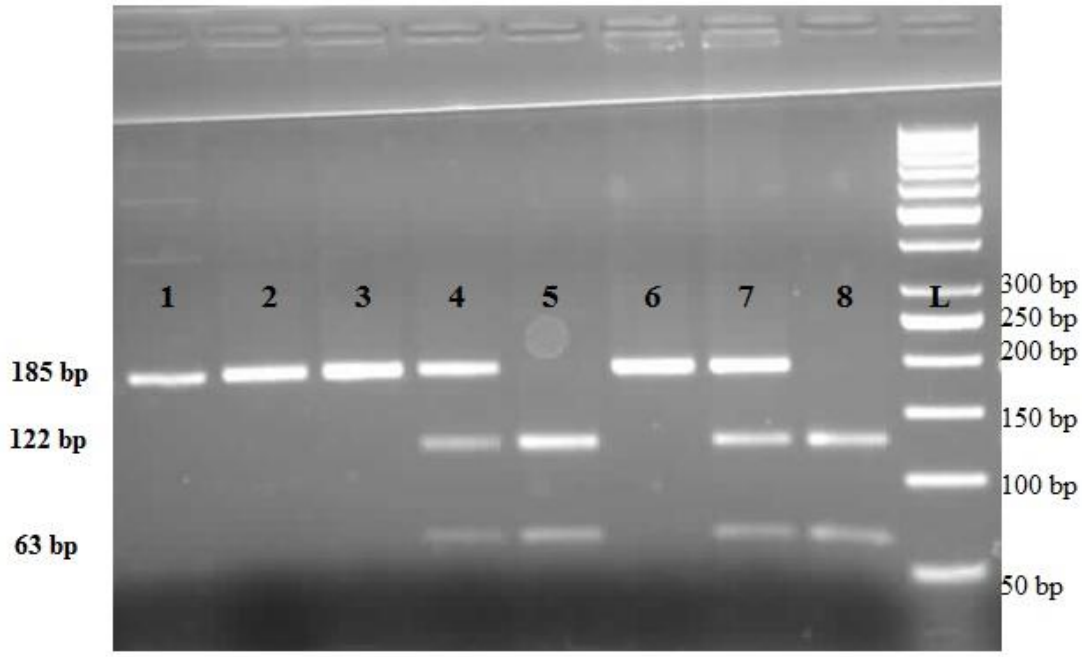

Figure 3.4 Agarose gel electrophoresis image for restriction endonuclease digestion with MboI of GSTO2 gene. The lane labeled with L shows the DNA ladder (50 bp-1000 bp). The other lanes are digestion products. Lane 1, 2, 3 and 6 show the banding patterns of wild type homozygous individuals due to including one band at 185 bp. Lane 5 and 8 are homozygous mutant individuals with two band at 122 bp and 63 bp. Lane 4 and 7 have three bands at 185 bp, 122 bp and 63 bp, so these are heterozygous mutant individuals.

3.3. Genotypes and Allele Frequencies of Ala140Asp Single Nucleotide Polymorphism of GSTO1 gene and Asn142Asp Single Nucleotide Polymorphism of GSTO2 Gene

In this study, Ala140Asp SNP of GSTO1 gene and Asn142Asp SNP of GSTO2 gene were analyzed in 130 controls and 239 patients in terms of genotype and allele frequency.

The analysis was done to evaluate whether SNPs have effect on risk of stroke. The odds ratio was calculated to detect risk assessment in terms of risk group and non-risk group. The individuals carrying one or two mutant allele (homozygous mutant or heterozygous mutant) was considered as risk group and the homozygous wild type individuals were counted as non-risk group because the polymorphisms cause reduction in enzyme activity

The formula for calculation of odds ratio is:

$$OR= \frac{\text{\# of patients with risky allele /\# of patients without risky allele}}{\text{\# of controls with risky allele /\# of controls without risky allele}}$$

3.3.1. Genotypes and Allele Frequencies of Ala140Asp Single Nucleotide Polymorphism of GSTO1 gene

The distribution of genotypes and allele frequencies for Ala140Asp single nucleotide polymorphism of GSTO1 gene were shown in Table 3.3. In this study, 239 patient and 130 control individuals were studied in terms of conversion of ‘C’ allele to ‘A’ allele. Among study population, 101 (42.3%) patients and 62 (47.7%) controls were homozygous wild type (CC), 105 (43.9%) patients and 47 (36.2%) controls were heterozygote (CA), 33 (13.8%) patients and 21 (16.2%) controls were homozygous mutant genotype (AA). The heterozygous and homozygous mutant types were considered risky and the odds ratio was calculated. As a result, there is no significant difference in the genotype frequencies of Ala140Asp single nucleotide polymorphism between stroke patients and controls (OR=1.245, CI: 0.811-1.914, and P= 0.315).

The distributions of wild type allele frequencies were found 0.642 for patients and 0.658 for controls. On the other hand, the mutant type allele frequencies were found 0.358 for patients and 0.342 for controls. The odds ratio was calculated according to proportion of risky allele (A) and no-risk allele (C), and no significant difference was found (OR=0.934, CI: 0.68-1.28, and P= 0.675).

Table 3.3 Genotypes and allele frequency distribution of patients and controls for Ala140Asp single nucleotide polymorphism.

	Patients (n=239)	**Controls (n=130)**	**OR (95%CI)**	***P***
Ala140Asp polymorphism Genotypes, n(%)				
CC	101 (42.3)	62 (47.7)		
CA	105(43.9)	47(36.2)	1.245[a] (0.811-1.914)	0.315
AA	33(13.8)	21(16.2)		
Allele frequency				
C	0.642	0.658	0.934[b] (0.68-1.283)	0.675
A	0.358	0.342		

[a]CA+AA vs. CC, [b]C vs. A

3.3.2. Genotypes and Allele Frequencies of Asn142Asp Single Nucleotide Polymorphism of GSTO2 gene

The distribution of genotypes and allele frequencies for Asn142Asp single nucleotide polymorphism of GSTO2 gene were shown in Table 3.4. In this study, 239 patient and 130 control individuals were studied in terms of conversion of 'A' allele to 'G' allele. Among study population, 97 (40.6%) patients and 50 (38.5%) controls were homozygous wild type (AA), 107 (44.8 %) patients and 55 (42.3%) controls were heterozygous (AG), 35 (14.6%) patients and 25 (19.2%) controls were homozygous mutant genotype (GG). The heterozygous and homozygous mutant types were considered risky and the odds ratio was calculated. As a result, there is no significant difference in the genotype frequencies of Ala140Asp single nucleotide polymorphism between stroke patients and controls (OR=0.941, CI: 0.813-1.146, and P= 0.691).

The distributions of wild type allele frequencies were found 0.630 for patients and 0.596 for controls. On the other hand, the mutant type allele frequencies were found 0.370 for patients and 0.404 for controls. The odds ratio was calculated according to proportion of risky allele (G) and no-risk allele (A), and no significant differences was found (OR=1.152, CI: 0.845-1.57, and P= 0.370).

Table 3.4 Genotypes and allele frequency distribution of patients and controls for Asn142Asp single nucleotide polymorphism

	Patients (n=239)	**Control (n=130)**	**OR (95%CI)**	***P***
Asn142Asp polymorphism Genotypes, n(%)				
AA	97 (40.6)	50 (38.5)		
AG	107(44.8)	55 (42.3)	0.941[a] (0.813-1.146)	0.691
GG	35(14.6)	25 (19.2)		
Allele frequency				
A	0.630	0.596	1.152[b] (0.845-1.57)	0.370
G	0.370	0.404		

[a]AG+GG vs. AA, [b]A vs. G

3.4. GSTO1 and GSTO2 Genotypes in Different Subgroups of Stroke Patient and Control Group

Ala140Asp SNP and Asn142Asp SNP genotypes were analyzed in four different subgroups which are conventional risk factors for ischemic stroke. The distribution of genotypes were counted for patients and control groups in subgroups of hypertensive vs. normotensive, diabetic vs. non-diabetic, obesity vs. non-obesity and smoker vs. non-

smoker. The odds ratio (OR) was calculated for investigation of risk between groups. While calculating the OR, the heterozygous and homozygous mutant genotypes were considered as one group against homozygous wild type. Therefore, CA and AA genotypes were grouped against CC; similarly, AG and GG against AA.

3.4.1. GSTO1 and GSTO2 Genotypes in Hypertensive vs. Normotensive Subgroup of Stroke Patients and Controls

The frequency distribution of GSTO1 and GSTO2 genotypes between patients and controls in terms of hypertensive and normotensive were represented in Table 3.5. The relation between hypertension and stroke risk was investigated by calculating OR of hypertensive stroke patient group and hypertensive control group. Similarly, same calculation was done for normotensive group. 209 hypertensive subjects and 160 normotensive subjects were analyzed for this investigation.

According to Table 3.5, for Ala140Asp polymorphism, there were 70 wild type (CC) stroke patients and 18 wild type (CC) controls within hypertensive group. Moreover, the number of heterozygous (CA) individuals was 70 for hypertensive patients, 18 for hypertensive controls and the number of homozygous mutant type (AA) individuals number was 20 for hypertensive patients and 13 for hypertensive controls. The risky genotype frequency (CA+AA) was 56.3% in hypertensive patients while it is 63.2% in hypertensive control groups. CC genotype frequency was 43.8% in hypertensive patients and 36.7% in hypertensive controls. There was no significant difference between stroke patients and controls in hypertensive subgroup (OR=0.747, CI: 0.386-1.444, and P=0.384). As regards normotensive group for Ala140Asp SNP, there were 31 CC, 35 Ca and 13 AA individuals in patients. On the other hand, these numbers changed to 44, 2 and 8, respectively for controls. The non-risky ‘CC’ genotype frequency was 39.2% in patient and 54.3% in control group. The ‘A’ allele carrier genotypes (CA+AA) frequency was 60.8% in patients and 45.7% in control group. Like hypertensive

subgroup, there was no significant difference between them (OR=1.841, CI: 0.982-3.452, and P=0.056).

For Asn142Asp polymorphism given in Table 3.5, there were 64 wild type (AA) individuals, 76 heterozygous (AG) individuals and 20 homozygous mutant (AA) individuals in hypertensive patients. Moreover, the number of AA individuals was 14, AG is 14 and GG is 11 for hypertensive controls. Wild type genotype, AA, frequency was 40.0% and other genotypes (AG+GG) frequency was 60% in hypertensive patients while they were 28.6% and 51% in hypertensive controls, respectively. There was no significant difference (OR=0.84, CI: 0.406-1.737, and P=0.638). In respect to normotensive group for this polymorphism, there were 33 wild type (AA) individuals, 31 heterozygous (AG) and 15 homozygous mutant (GG) individuals in patients, and 36 AA, 31 AG and 14 GG in controls. The frequencies of AA and AG+GG genotypes were almost the same in the stroke and control groups. AA genotypes frequencies were 41.8% in patients and 44.4% in controls. The genotype including risky ‘G’ allele frequency was 58.2% in patient and 55.6% in controls. The difference between them was not significant (OR=1.115, CI: 0.596-2.086, and P=0.732).

Table 3.5 Genotype distributions of GSTO1 Ala140Asp and GSTO2 Asn142Asp single nucleotide polymorphisms in hypertensive vs. normotensive subgroup of stroke patients and controls

	Hypertensive (n=209)				**Normotensive (n=160)**			
	Patient (n=160)	**Control (n=49)**	**OR(95% CI)**	***P***	**Patient (n=79)**	**Control (n=81)**	**OR(95% CI)**	***P***
Ala140Asp								
CC	70 (43.8%)	18 (36.7%)			31 (39.2%)	44(54.3%)		
CA	70 (43.8%)	18 (36.7%)	0.747[a]	0.384	35 (44.3%)	29 (35.8%)	1.841[a]	0.056
AA	20 (12.5%)	13 (26.5%)	(0.386-1.444)		13 (16.5%)	8 (9.9%)	(0.982-3.452)	
Asn142Asp								
AA	64 (40.0%)	14 (28.6%)			33 (41.8%)	36 (44.4%)		
AG	76 (47.5%)	24 (49%)	0.84[b]	0.638	31 (39.2%)	31 (38.3%)	1.115[b]	0.732
GG	20 (12.2%)	11 (22.4%)	(0.406-1.737)		15 (19.0%)	14 (17.3%)	(0.596-2.086)	

[a]CA+AA vs. CC

[b]AG+GG vs. AA

3.4.2. GSTO1 and GSTO2 Genotypes in Diabetic vs. Non-diabetic Subgroup of Stroke Patients and Controls

The frequency distribution of GSTO1 and GSTO2 genotypes between patients and controls in terms of diabetic and non-diabetic were represented in Table 3.6. The relation between diabetes mellitus and stroke risk was investigated by calculating OR of diabetic stroke patient group and diabetic control group. Similarly, same calculation was done for non-diabetic group. 97 diabetic subjects and 272 non-diabetic subjects were analyzed for this investigation.

For Ala140Asp polymorphism in Table 3.6, there were 30 wild type (CC) individuals, 36 heterozygous (CA) and 11 homozygous mutants (AA) in diabetic patients, and 12 wild type (CC) individuals, 5 heterozygous (CA) and 3 homozygous mutants (AA) in diabetic controls. The wild type, CC, genotype frequency was 39% in patient and 60% in control group in diabetic subgroup. The frequency of genotype including risky allele, CA+AA, was 61.1% in patients and 35% in controls within diabetic subgroup. There was no significant difference between patients and control in diabetic subgroup (OR=2.35, CI: 0.86-6.42, and P=0.090). In non-diabetic subgroup, the number of individuals was 71 for CC, 69 for CA and 22 for AA in patients, and 50 for CC, 42 for CA and 18 for AA in controls. The CC genotype frequency was 43.8% in patients and 45.5% in controls, and CA+AA genotype frequency was 56.2% in patients and 54.5% in controls for non-diabetic subgroup. There was no significant difference between them (OR=1.068, CI: 0.656-1.738, and P=0.790).

In respect of Asn142Asp polymorphism genotypes, there were 31 wild type (AA) individuals, 34 heterozygous (AG) individuals and 12 homozygous (AA) individuals in diabetic patients. Moreover, the number of AA individuals was 8, AG is 10 and GG is 2 for diabetic controls. The frequencies of patients and controls were approximately same in diabetic subgroups. The wild type genotype, AA, frequency was 40.2% in patients and 40% in controls. The risky genotype group, AG+GG, frequency was 59.8% in patients and 60% in controls. Analysis of patients and controls in diabetic subgroup

showed that there was no significant relation between stroke risk and Asn140Asp polymorphism (OR=0.989, CI: 0.363-2.699, and P=1). As regards non-diabetic group, there were 66 AA, 73 AG and 23 GG in patients, and 42 AA, 45 AG and 23 GG in controls. The frequency of AA genotype in patients is 40.7% and in controls 38.2%. The other genotypes had 59.3% for patients and 61.8% for controls. Similar to diabetic subgroup, there was no significant differences between patients and controls in different genotypes (OR=0.898, CI: 0.547-1.476, and P=0.672).

Table 3.6 Genotype distributions of GSTO1 Ala140Asp and GSTO2 Asn142Asp single nucleotide polymorphisms in diabetic vs. non-diabetic subgroup of stroke patients and controls

	Diabetic (n=97)				**Non-Diabetic (n=272)**			
	Patient (n=77)	**Control (n=20)**	**OR (95% CI)**	***P***	**Patient (n=162)**	**Control (n=110)**	**OR (95% CI)**	***P***
Ala140Asp								
CC	30 (39.0%)	12 (60.0%)			71 (43.8%)	50(45.5%)		
CA	36 (46.8%)	5 (25.0%)	2.35[a]	0.090	69 (42.6%)	42 (38.2%)	1.068[a]	0.790
AA	11 (14.2%)	3 (10.0%)	(0.86-6.42)		22 (13.6%)	18 (16.4%)	(0.656-1.738)	
Asn142Asp								
AA	31 (40.2 %)	8 (40.0%)			66 (40.7%)	42 (38.2%)		
AG	34 (44.2%)	10 (50.0%)	0.989[b]	1	73 (45.1%)	45 (40.9%)	0.898[b]	0.672
GG	12 (15.6%)	2 (10.0%)	(0.36-2.7)		23 (14.2%)	23 (20.9%)	(0.547-1.476)	

[a]CA+AA vs. CC

[b]AG+GG vs. AA

3.4.3. GSTO1 and GSTO2 Genotypes in Obesity vs. Non-obesity Subgroup of Stroke Patients and Controls

The frequency distribution of GSTO1 and GSTO2 genotypes between patients and controls in terms of obesity and non- obesity were represented in Table 3.7. The relation between obesity and stroke risk was investigated by calculation OR of obesity stroke patient group and obesity control group. Similarly, same calculation was done for non-obesity group. 61 subjects with obesity and 308 subjects without obesity are analyzed for this investigation.

According to Table 3.7, for Ala140Asp SNP, there were 24 CC, 22 CA and 7 AA in patient with obesity, and 2 CC, 3 CA and 3 AA in control with obesity. The genotypes frequencies in obesity subgroup were 45.3% for CC genotype, 54.7% for CA+AA genotypes and in non- obesity subgroup; these frequencies were 25.0% for CC genotype and 75% for CA+AA genotypes. There was no significant difference between control and patient group in obesity subgroup in terms of different genotypes (OR=0.402, CI: 0.074-2.181, and P=0.447 In non- obesity subgroup analysis, the number of individuals was 77 for CC, 83 for CA and 26 for AA in non- obesity patients and also, they were 60 CC, 44 CA and 18 AA. The frequency of CC genotype was 41.4% in patients and 49.2% in controls. On the other hand, the risky allele including genotype, CA+AA, frequency was 58.6% in patients and 50.9% in controls. It was found that there was not any significant difference between patients and controls in different genotypes (OR=1.370, CI: 0.865-2.169, and P=0.179).

As regards Asn142Asp SNP in Table 3.7, there are 25 AA, 25 AG and 3 GG in patients with obesity and 2 AA, 3 AG and 3 GG in controls with obesity. The genotype distribution of stroke and people with obesity is found that AA genotype is 47.2% for patients and 25.0% for controls in Asn142Asp polymorphism. AG+GG genotype frequency is 52.8% for patients and 75% for controls. The differences is not significant (OR=0.373, CI: 0.069-2.121, and P=0.283). Also, 66 AA, 73 AG and 23 GG were found in non-obesity patients and 48 AA 52 AG and 22 GG were found in non-obesity

controls. The AA frequencies for non-obesity subgroup were 40.7% in patients and 39.3% in controls. The frequencies for other genotype group were almost same; 59.3% for patients and 60.7% for controls. There was no significant differences between them (OR=0.943, CI: 0.584-1.524, and P=0.812).

Table 3.7 Genotype distributions of GSTO1 Ala140Asp and GSTO2 Asn142Asp single nucleotide polymorphisms in obesity vs. non- obesity subgroup of stroke patients and controls.

	Obesity (n=61)				**Non-Obesity (n=308)**			
	Patient (n=53)	**Control (n=8)**	**OR (95% CI)**	***P***	**Patient (n=186)**	**Control (n=122)**	**OR (95% CI)**	***P***
Ala140Asp								
CC	24 (45.3%)	2 (25.0%)			77 (41.4%)	60(49.2%)		
CA	22 (41.5%)	3 (37.5%)	0.402[a]	0.447[c]	83 (44.6%)	44 (36.1%)	1.370[a]	0.179
AA	7 (13.2%)	3 (37.5%)	(0.136-2.675)		26 (14.0%)	18 (14.8%)	(0.865-2.169)	
Asn142Asp								
AA	25 (47.2%)	2 (25.0%)			66 (40.7%)	48 (39.3%)		
AG	25 (47.2%)	3 (37.5%)	0.373[b]	0.283[c]	73 (45.1%)	52 (42.6%)	0.943[b]	0.812
GG	3 (5.7%)	3 (37.5%)	(0.069-2.021)		23 (14.2%)	22 (18.1%)	(0.584-1.524)	

[a]CA+AA vs. CC

[b]AG+GG vs. AA

[c] Fisher Exact Test is applied

3.4.4. GSTO1 and GSTO2 Genotypes in Smoker vs. Non-smoker Subgroup of Stroke Patients and Controls

The frequency distribution of GSTO1 and GSTO2 genotypes between patients and controls in terms of smoker and non-smoker were represented in Table 3.8. The relation between smoke use and stroke risk was investigated by calculation OR ratio of stroke patient using smoke group and control group using smoke. Similarly, same calculation was done for non-smoker group. 81 smoker subjects and 288 non-smoker subjects were analyzed for this investigation.

In Ala140Asp polymorphism in Table 3.8, there were 25 CC, 29 CA and 11 AA in smoker patient, and 9 CC, 6 CA and 1 AA in smoker control. The genotype frequencies in smoker patient subgroup were 38.5% for CC and 61.5% for CA+AA, and also, in smoker control group 56.3% for CC genotypes and 43.7% for CA+AA genotypes. There was no significant difference between control and patient group in smoker individuals (OR=2.057, CI: 0.68-6.223, and P=0.196). Moreover, in non-smoker group, 76 CC, 76 CA and 22 AA were found in non-smoker patients and 53 CC, 41 CA and 20 AA was found in non-smoker controls. The CC genotype frequencies were 43.7% for patients and 46.5% for controls and CA+AA genotypes frequencies were 56.3% for patients and 53.5% for controls. Similarly to smoker group, there was no significant difference between controls and patients (OR=1.074, CI: 0.667-1.73, and P=0.767).

When looking at the Asn142Asp SNP in Table 3.8, there were 28 AA, 27 AG and 10 GG in smoker patients group, and also, 8 AA, 6 AG and 2 GG in smoker controls. The genotype distribution for Asn142Asp SNP was found that AA genotypes were 43.1% for smoker patients and 50.0% for smoker controls and AG+GG genotypes were 56.9% for smoker patients and 50.0% for smoker controls. Any significant difference could not be found statistically (OR=0.964, CI: 0.317-2.936, and P=1). As regard non-smoker group, the AA, AG and GG numbers of individuals were 69, 80 and 25 for patients, respectively. Also, they were 42, 49 and 23 for control group. The distributions were 39.6% for AA genotype in patients, 60.4% for AG+GG genotype in patients and 36.8%

for AA genotypes in controls, 63.2% for AG+GG genotypes in controls. There was no statistical significance between them (OR=0.887, CI: 0.546-1.444, and P=0.631).

Table 3.8 Genotype distributions of GSTO1 Ala140Asp and GSTO2 Asn142Asp single nucleotide polymorphisms in smoker vs. non-smoker subgroup of stroke patients and controls.

	Smoker (n=81)				**Non-Smoker (n=288)**			
	Patient (n=65)	**Control (n=16)**	**OR (95% CI)**	***P***	**Patient (n=174)**	**Control (n=114)**	**OR (95% CI)**	***P***
Ala140Asp								
CC	25 (38.5%)	9 (56.3%)			76 (43.7%)	53(46.5%)		
CA	29 (44.6%)	6 (37.5%)	2.057[a]	0.196	76 (43.7%)	41 (36.0%)	1.074[a]	0.767
AA	11 (16.9%)	1 (6.2%)	(0.68-6.223)		22 (12.6%)	20 (17.5%)	(0.667-1.73)	
Asn142Asp								
AA	28 (43.1%)	8 (50.0%)			69 (39.6%)	42 (36.8%)		
AG	27 (41.5%)	6 (37.5%)	0.964[b]	1	80 (46.0%)	49 (43.0%)	0.887	0.631
GG	10 (15.4%)	2 (12.5%)	(0.317-2.936)		25 (14.4%)	23 (20.2%)	(0.546-1.444)	

[a]CA+AA vs. CC

[b]AG+GG vs. AA

3.5. Effects of Conventional Vascular Risk Factors in Different GSTO1 and GSTO2 Genotypes of Ischemic Stroke Patients and Controls

In this analysis, the conventional vascular risk factors like hypertension, diabetes, obesity and smoking were examined in terms of proportion of ischemic stroke patients to control group according to risky (heterozygous or homozygous mutant type) and non-risky group (wild type). The heterozygous genotypes (CA for Ala140Asp SNP and AG for Asn142Asp SNP) and homozygous mutant genotypes (AA for Ala140Asp SNP and GG for Asn142Asp SNP) were counted one group and the comparison analysis was done versus wild type (CC for Ala140Asp SNP and AA for Asn142Asp SNP). The analysis was evaluated significantly meaningful when P value was lower than 0.05 and the odds ratio were determined.

3.5.1. Effects Conventional Vascular Risk Factors in Different GSTO1 Ala140Asp Single Nucleotide Polymorphism Genotypes of Ischemic Stroke Patients and Control Group

In Table 3.9, the odds ratio of patients to controls in hypertensive, diabetic, obesity and smoker groups were shown in terms of different genotypes of GSTO1 Ala140Asp single nucleotide polymorphism.

When considering hypertensive-normotensive group, in subgroup analysis, subjects having CA+AA genotypes have 2.24 times higher risk for stroke in hypertensive group when compared to normotensive (OR=2.24, P=0.007). The risk of having stroke is insignificantly higher in hypertensive CC genotype group compared to normotensive (OR=5.52, P=1).

As regard diabetic group, in CA+AA genotype group, the risk was 3.873 times higher risk for stroke in diabetic group than non-diabetic group (OR=3.873, P=0.000). In CC genotypes, the risk was insignificantly higher (OR=1.76, P=0.142).

When focusing obesity and non-obesity analysis, having CC genotypes had 9.35 times higher stroke risk in obesity group than non-obesity group (OR=9.35, P=0.000). However, the risk decreased 2.75 in CA+AA group (OR= 2.75, P=0.028).

According to analysis, there was no significant difference between smoker and non-smoker patients to control groups in terms of CC (P=0.118) but the risk of stroke was 3.55 fold higher in smoker group than non-smokers when having CA+AA genotypes (OR= 3.55, P=0.002).

Table 3.9 Stratification of hypertensive/normotensive, diabetic/non-diabetic, smoker/non-smoker, obesity /non-obesity individuals according to GSTO1 Ala140Asp genotypes

	CC Genotype(n=163)	**OR (95%)**	***P***	**CA+AA Genotype(n=206)**	**OR (95%)**	***P***
	Stroke/Control			**Stroke/Control**		
Hypertensive (n=209)	70/18	5.52[a]	1	90/31	2.24[a]	0.007
Normotensive(n=160)	31/44	(2.762-11.032)		48/37	(1.238-4.045)	
Diabetic (n=97)	30/12	1.76[b]	0.142	47/8	3.873[b]	0.000
Non-diabetic (n=272)	71/50	(0.823-3.768)		91/60	(1.71-8.772)	
Obesity (n=61)	24/2	9.35[c]	0.000[e]	29/6	2.75[c]	0.028
Non- obesity (n=308)	77/60	(2.125-41.137)		109/62	(1.08-6.987)	
Smoker (n=81)	25/9	1.937[d]	0.118	40/7	3.55[d]	0.002
Non-smoker (n=288)	76/53	(0.837-4.482)		98/61	(1.499 -8.442)	

[a]OR calculated against normotensive

[b] OR calculated against non-diabetic

[c] OR calculated against non- obesity

[d] OR calculated against non-smoker

[e] Fisher Exact Test is applied

3.5.2. Effects Conventional Vascular Risk Factors in Different GSTO2 Asn142Asp Single Nucleotide Polymorphism Genotypes of Ischemic Stroke Patients and Control Group

In Table 3.10, the odds ratio of patients to controls in hypertensive, diabetic, obesity and smoker groups were shown in terms of different genotypes of GSTO2 Asn142Asp single nucleotide polymorphism.

When hypertensive is considered, according to analysis in terms of heterozygous and homozygous mutant group (AG+GG) to wild type (AA), even though the risk was higher, there was no significant increased risk between hypertensive and normotensive groups (OR=4.98, P=1 and OR=3.75, P=1).

When looking the diabetic and non-diabetic group analysis, the risks were 2.465 times for AA genotype and 2.71 times for AG+GG genotypes (OR= 2.465, P=0.037 and OR= 2.71, P=0.004).

For obesity and non-obesity groups, the wild type 'AA' had 9.09 fold higher risk of stroke in diabetes than non-diabetes (OR= 9.09, P=0.000); however, the risk decreased to 3.59 in heterozygous and homozygous mutant group (OR= 3.59, P=0.004).

The risk of stroke increased to 3.171 times in smoker when having AG+GG genotype (P=0.004), while any significant differences were get for AA genotype (P=0.085).

Table 3.10 Stratification of hypertensive/normotensive, diabetic/non-diabetic, smoker/non-smoker, obesity/non-obesity individuals according to GSTO2 Asn142Asp genotypes

	AA			**AG+GG**		
	Genotype(n=147)	**OR (95%)**	***P***	**Genotype(n=222)**	**OR (95%)**	***P***
	Stroke/Control			**Stroke/Control**		
Hypertensive (n=209)	64/14	4.98[a]	1	96/25	3.75[a]	1
Normotensive(n=160)	33/36	(2.364-10.523)		46/45	(2.057 -6.859)	
Diabetic (n=97)	31/8	2.465[b]	0.037	46/12	2.71[b]	0.004
Non-diabetic (n=272)	66/42	(1.035-5.875)		96/68	(1.339-5.507)	
Obesity (n=61)	25/2	9.09[c]	0.000[e]	28/6	3.59[c]	0.004
Non-obesity (n=308)	66/48	(2.054-40.23)		96/74	(1.416-9.139)	
Smoker (n=81)	28/8	2.13[d]	0.085	37/8	3.171[d]	0.004
Non-smoker (n=288)	69/42	(0.889-5.107)		105/72	(1.395 -7.208)	

[a]OR calculated against normotensive

[b] OR calculated against non-diabetic

[c] OR calculated against non- obesity

[d] OR calculated against non-smoker

[e] Fisher Exact Test is applied

3.6. Logistic Regression

The logistic regression test was applied with backward selection method to evaluate the effects of conventional risk factors such as age, sex, hypertension, diabetes, obesity, smoking, lipid parameter etc. and GSTO1 and GSTO2 genotypes on the ischemic stroke risk.

Model 1:

The first model contained by using all subjects in study population. The conventional risk factors such as sage, sex, hypertension, diabetes mellitus, obesity, smoking, lipid parameters (total cholesterol, triglyceride, LDL and HDL), GSTO1, GSTO2 genotypes were added as covariates. The result for logistic regression test was shown in Table 3.11.

Table 3.11 Model 1; Logistic regression analysis of conventional risk factors (age, sex, hypertension, smoking status, diabetes, obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and GSTO1, GSTO2 genotypes

Parameters	OR	95%CI	*P*
Hypertension	3.043	1.840-5.031	0.000
Smoking	3.258	1.658-6.50	0.001
Obesity	2.593	1.129-5.956	0.025
HDL	0.270	0.111-0.654	0.004

According to the result given in Table 3.11, the hypertension (OR=3.043, 95%CI: 1.840-5.031, P=0.000), smoking (OR=3.258, 95%CI: 1.658-6.50, P=0.001) and obesity (OR=2.593, 95%CI: 1.129-5.956, P=0.025) were revealed as significant risk factor for ischemic stroke. On the other hand, HDL played protective role in stroke by decreasing the risk (OR=0.270, 95%CI: 0.111-0.654, P=0.004).

The model predicted 70.4% of cases correctly and also, Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=5.093; 8 degrees of freedom; P=0.658).

Model 2:

The second model was done by using only male subjects. The conventional risk factors such as age, hypertension, diabetes mellitus, obesity, smoking, lipid parameters (total cholesterol, triglyceride, LDL and HDL), GSTO1, GSTO2 genotypes were added as covariates. The result for logistic regression test was shown in Table 3.12.

Table 3.12 Model 2, Logistic regression analysis of conventional risk factors (age, hypertension, smoking status, diabetes, obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and GSTO1, GSTO2 genotypes in males.

Parameters	OR	95%CI	*P*
Hypertension	3.907	2.078-7.346	0.000
Smoking	2.227	1.009-4.914	0.047
Obesity	5.105	1.125-23.175	0.035

The logistic regression test revealed that the hypertension (OR=3.907, 95%CI: 2.078 - 7.346, P=0.000), smoking (OR=2.227, 95%CI: 1.009-4.914, P=0.047) and obesity (OR=5.105, 95%CI: 1.125-23.175, P=0.035) were significant risk factor for ischemic stroke in male subjects.

The model predicted 68.9% of cases correctly and also, Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=7.880; 8 degrees of freedom; P=0.445).

Model 3:

The third model was done by using only female subjects. The conventional risk factors such as age, sex, hypertension, diabetes mellitus, obesity, smoking, lipid parameters

(total cholesterol, triglyceride, LDL and HDL), GSTO1, GSTO2 genotypes were added as covariates. The result for logistic regression test was shown in Table 3.13.

Table 3.13 Model 3, Logistic regression analysis of conventional risk factors (age, hypertension, smoking status, diabetes, obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and GSTO1, GSTO2 genotypes in females.

Parameters	OR	95%CI	*P*
Hypertension	4.997	2.312-10.798	0.000
Diabetes	3.217	1.260-8.213	0.015
Smoking	9.818	2.231-43.204	0.003
Cholesterol	1.776	1.228-2.567	0.025
HDL	0.127	0.032-0.052	0.004

According to results of logistic regression analysis model 3, the hypertension (OR=4.997, 95%CI: 2.312-10.798, P=0.000), diabetes (OR=3.217, 95%CI: 1.260 - 8.213, P=0.015) smoking (OR=9.818, 95%CI: 2.231-43.204, P=0.003) and cholesterol (OR=1.776, 95%CI: 1.228-2.567, P=0.025) were revealed as significant risk factor for ischemic stroke. On the other hand, HDL (OR=0.127, 95%CI: 0.032-0.152, P=0.004) was founded as protective effect for ischemic stroke in female subjects.

The model predicted 72.8% of cases correctly and also, Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=4.462; 8 degrees of freedom; P=0.813).

CHAPTER 4

DISCUSSION

Stroke, third-leading cause of death, occurs with lacking blood supply to brain by damaging the blood vessels. There are two main types of stroke: ischemic stroke and hemorrhagic stroke. Ischemic stroke is blockage of brain vessel due to pathological effect like atherosclerosis. There can be many reasons for existing ischemic stroke, so there are variable risk factors. These risk factors are divided into three classes; alterable, unalterable and uncertain risk factor. The alterable ones are generally conventional risk factors like hypertension, diabetes mellitus, high blood cholesterol, smoking, obesity, atrial fibrillation etc. The unalterable risk factors include age, gender, heredity, race and TIA (transient ischemic attack). The uncertain risk factors are drug use, socioeconomic factors and geographic conditions such as climate. The investigations with stroke focus on the risk factors and the reasons of risk factors, so beneficial treatments can be created by eliminating or decreasing the risk factors.

Free radicals are produced by normal biochemical reactions in organism. Even though free radicals are responsible some necessary reactions like cell signaling, the high level of free radicals is harmful for organism. Xenobiotic metabolism is a protective system against harmful effects of free radicals, endotoxins and exotoxins. The xenobiotic metabolism includes enzymes which catalyze the detoxification reactions. The high level free radical production and low level antioxidant system functionality like impaired enzyme efficiency cause increased oxidative stress. The oxidative stress can induce many negative situations such as lipid peroxidation, DNA modifications and protein denaturation. These damages result in tissue injury. Moreover, free radicals affect the

vascular tone in brain blood flow. In addition to these effects, the oxidative stress can promote the atherosclerosis formation in vessels by foam cells. Therefore, an impaired antioxidant system can trigger the ischemic stroke. The investigations about the oxidative stress and ischemic stroke risk were carried out by focusing the enzymes responsible for antioxidant metabolism.

Glutathione S-transferase enzymes (GSTs) are enzyme families which are responsible in antioxidant metabolism. GST enzymes catalyze the reaction of conjugation of glutathione with xenobiotics, thereby, the harmful effects of molecules decrease and they become less toxic elements. The Glutathione S-Transferase enzyme family has seven main classes in human organism; Alpha, Mu, Pi, Theta, Zeta, Sigma and Omega. Most of these classes are cytosolic, others are lipophilic.

The glutathione S-transferase omega class, our interested enzyme, has two isoforms GST omega 1 and GST omega 2. The GST omega enzymes have been found in most tissues in human, contribute the detoxification reaction. Different from other GST families, the GST omega enzyme play role in apoptosis, inflammation and arsenic metabolism (Section 1.4).

The aim of this study is to investigate the possible association between single nucleotide polymorphisms Ala140Asp in GSTO1 gene and Asn142Asp in GSTO2 gene and ischemic stroke risk in Turkish population. Moreover, the subgroup analyses were done between conventional risk factors, different genotypes and stroke.

The study population is composed of 239 ischemic stroke patients and 130 controls without any symptoms related to stroke. The age is one of important unalterable risk factor for ischemic stroke. When the age mean in a group increased, the probability of stroke also increased. Because of this reason, to eliminate the effect of age in our results, the mean differences between patients and control groups were tried to minimum level. In our study population, the age means are 65.57 for patients and 64.16 for controls. The age differs from 20 to 83 in patients and from 38 to 90 in controls. There is

no significant age difference statistically between patients and controls (P=0.067) (Table 3.1).

The gender is another important risk factor for ischemic stroke. In study population, there are 133 male in 239 patients (55.6%) and 63 male in 130 controls (51.5%). According to many previous studies, the male are more prone to stroke than female (Sacco et al., 1997 and Demirdöğen et al., 2008). As regard to this study, male has 1.33 times higher risk for stroke (OR=1.33, CI: 0.870-2.048) and any significant difference could not be found (P=0.186) (Table 3.1).

The conventional risk factors such as hypertension, diabetes mellitus, obesity and smoking were counted alterable risk factors for stroke in the previous studies. In this study, these conventional risk factors were also found to be effective on development of stroke. There is statically significant difference between patient and control group as expected (P<0.05) (Table 3.1 and Table 3.2).

In the study population, 160 individual of 230 patients (66.9%) and 49 individual of 130 controls (37.7%) were hypertensive. According to the results of analyses, hypertensives have 3.35 times higher risk for stroke (OR=3.35, P=0.000). This result is found to be parallel with previous studies (Türkanoğlu et al., 2013 and Demirdöğen et al., 2009).Another important conventional risk factor for stroke is diabetes. In fact, effect of diabetes on stroke is due to the other results of diabetes such as high blood cholesterol which can cause development of atherosclerosis. According to our results, the number of diabetics is 77 in patients (32.3%) and 20 in controls (15.4%). Moreover, diabetes has 2.614 times higher risk for stroke than non-diabetics (OR=2.614, P=0.000). All these are in concordance with previous studies (Türkanoğlu et al., 2010 and Demirdöğen et al., 2012). Smoking has negative effect on cardiovascular diseases due to deformation in blood vessels. In our study population, there are 65 smokers in patients (27.2%) and 16 smokers in controls (12.3%). As expected, the risk of having stroke in smoker group 2.662 times higher than non-smoker (OR=2.662, P=0.001). The smoking was also revealed as risk factor in other studies (Demirdöğen et al., 2012). Obesity is known as

another conventional risk factor for stroke. In the present study, 53 patient with obesity (22.2%) and 8 controls with obesity (6.2%) were counted. Statistically significant difference was found between patients and controls (P=0.000) and the obesity has approximately 4 fold higher risk for stroke (OR=4.191). The previous studies reported are also similar to our results (Türkanoğlu et al., 2013).

It is well known from previous studies that the lipid level of blood has a close relationship with ischemic stroke (Sacco et al., 2001, Psaty et al., 2004 and Kurth et al., 2007). Increased LDL, triglyceride and total cholesterol level could be seen in stroke patients. Similarly, the patients have generally decreased HDL level. In our study population, the total cholesterol level in patients and controls are close to each other (4.76 mmol/L for patients, 4.70 mmol/L for controls). Triglyceride level is higher in patients (1.54 mmol/L) than controls (1.44 mmol/L). However, there is no significant difference between them (P=0.679 for total cholesterol and P=0.195 for triglyceride). The higher LDL level and lower HDL level is very important in increased risk of ischemic stroke. LDL is a lipoprotein which plays role in distribution of cholesterol in tissues like blood vessels. HDL, another lipoprotein, is responsible for return the excess cholesterol from vessels. Therefore, it can be said LDL can trigger the development of atherosclerosis in blood vessel by transport cholesterol while HDL has a protective role. As expected, in our population, the level of LDL is higher in patients (2.85 mmol/L in patients and 2.76 mmol/L in controls) whereas the HDL level is higher in control group (1.08 mmol/L in patients and 1.20 mmol/L in controls). The HDL level is significantly lower in patients (P=0.001), but for LDL level, there is no significant difference between patient and control groups (P=0.499) (Table 3.1).

According to the results of previous studies, the GSTO1 and GSTO2 genotypes and allele frequencies can vary in different population (previous studies given in Table 4.1 and Table 4.2). To understand the distribution of genotype and allele frequencies of GSTO1 Ala140Asp and GSTO2 Asn142Asp polymorphisms of populations, investigation and comparison of healthy control groups are important.

The genotypes of GSTO1 Ala140Asp polymorphism and the frequencies of wild ‘C’ and mutant ‘A’ allele in healthy control groups from different populations are given in Table 4.1. In this study, the genotype frequencies of Ala140Asp polymorphism in GSTO1 gene for control group (n=130) are 47.7%, 36.1% and 16.2% for CC, CA and AA genotypes, respectively (Table 3.3). According to another study carried out in Turkish population, these frequencies are 48%, 41% and 11% for CC, CA and AA genotypes in healthy control group, respectively (Ada et al., 2013). The frequencies in control group for wild type allele, C and mutant allele, A in our study are 0.658 and 0.342, respectively. In the report by Ada et al., 2012, they are 0.689 and 0.311 for ‘C’ and ‘A’ allele (Table 4.1). These results are in concordance to each other. However, as regard to another study for Turkish population, the frequencies of these alleles are considerably different compared to our results (Takeshita et al., 2009). The wild type allele frequency is 0.915 and the minor one is 0.085 (Takeshita et al., 2009). This frequency closer to Taiwan, Chinese and Italian population, is higher than our results. The reason of this may result from the nature of study population. In our study, the blood sampling area is Ankara, which is a cosmopolitan city, so, the study population includes individuals from Anatolia and Turkey. However, in Takeshita study, the blood sampling was done only in Adana, which is not considered as a cosmopolitan city. Therefore, we suggested that our results were more reliable. Also, our population results for GSTO1 Ala140Asp polymorphism are similar to German, American and Serbian populations (C allele: 0.680, 0.655 and 0.637, respectively) (Table 4.1).

Table 4.1 Comparison of wild type and variant allele frequencies of GSTO1 gene in different populations for control groups

Population	Number of sample	Wild allele (C)	Variant allele (A)	Reference
Turkish	**130**	**0.658**	**0.342**	**This Study**
Turkish	214	0.689	0.311	Ada et al., 2013
	194	0.915	0.085	Takeshita et al., 2009
Taiwan	251	0.817	0.183	Chung et al., 2011
	764	0.834	0.166	Hsu et al., 2011
	184	0.818	0.182	Chung et al., 2009
German	280	0.680	0.320	Kölsch et al., 2004
American	727	0.655	0.345	Ozturk et al., 2005
Serbia	130	0.637	0.363	Stamenkovic et al., 2013
Chinese	215	0.853	0.137	Fu et al., 2008
Italian	157	0.876	0.124	Capurso et al., 2010

As regards the genotypes of GSTO2 Asn142Asp polymorphism, the percentages for AA, AG and GG genotypes are 38.5%, 42.3% and 19.2%, respectively (n=130). According to another study by Takeshita et al., 2009 which was focused on Turkish population, the percentage for wild type allele is higher and the heterozygous mutant and homozygous mutant allele are lower than our results (67% for AA, 22% for AG and 11% for GG). These results are considerably different from our results. The reason of that can be again the nature of study population. The sampling for their study were done from only Adana, this may be not represent the Turkish population. Our results are closer to Brazil and Iranian population; the wild type allele frequencies are 0.626 and 0.630, respectively. The American, Japanese, Taiwan, Serbian and Thailand populations show higher wild type allele frequency for this polymorphism (Table 4.2).

Table 4.2 Comparison of wild type and variant allele frequencies of GSTO2 gene in different populations for control groups

Population	Number of sample	Wild allele (A)	Variant allele (G)	Reference
Turkish	**130**	**0.596**	**0.404**	**This Study**
Turkish	194	0.781	0.219	Takeshita et al., 2009
Taiwan	251	0.741	0.259	Chung et al., 2010
	764	0.753	0.247	Hsu et al., 2011
Brazil	222	0.626	0.374	Morari et al., 2006
American	732	0.695	0.305	Ozturk et al., 2005
Serbia	124	0.669	0.331	Stamenkovic et al., 2013
Japanese	369	0.726	0.274	Kiyohara et al., 2010
	102	0.784	0.216	Takeshita et al., 2009
Thailand	151	0.770	0.230	Chariyalertsak et al., 2009
Iranian	134	0.630	0.370	Masoudi et al., 2009

In the present study, genotype and allele frequency analysis of Ala140Asp polymorphism in GSTO1 gene showed no significant difference between patients and controls (P= 0.315 for genotypes and P=0.675 for allele frequency, Table 3.3). In patients, the genotype frequencies of this polymorphism have been found as 42.3% for CC, 43.9% for CA and 13.8% for AA genotypes, while the frequencies of control group are 47.7% for CC, 36.1% for CA and 16.2% for AA genotypes. The allele frequencies are 0.642 for wild type allele ‘C’ and 0.358 for mutant allele ‘A’ in patients, and 0.658 and 0.342 in controls, respectively. These results suggest that there is no direct relation between Ala140Asp SNP and ischemic stroke risk.

As regard to GSTO2 Asn142Asp genotype analyses, in patients, the wild type 'AA', heterozygous 'GA' and homozygous mutant 'GG' genotypes are 40.6%, 44.8% and 14.6%, respectively. The same genotype frequencies are 38.5%, 42.3% and 19.2% for controls. The allele frequencies were also calculate and compared between groups. The wild type 'A' allele frequency is 0.630 in patients and 0.596 in controls, also; the mutant allele frequency is 0.370 in patients and 0.404 in controls. Both genotypes and allele frequencies have no significant differences between patients and controls (P=0.691 for genotypes and P=0.370 for allele, Table 3.4). Like the Ala140Asp SNP, there is no direct relation between Asn142Asp polymorphism and ischemic stroke risk.

The oxidative stress promotes the formation of atherosclerosis. The Glutathione S-Transferase omega enzyme family plays important role in detoxification metabolism and decreases the oxidative stress. Therefore, an impaired enzyme activity may change the risk ratio. Therefore, single nucleotide polymorphisms, Ala140Asp in GSTO1 gene and Asn142Asn in GSTO2 gene may result in a decrease of enzyme activity. In this study, we expect to observe a change in risk ratio for ischemic stroke, and the mutant genotypes and allele frequencies may be seen higher in patient group than controls. However, no significant difference was found between two groups, directly.

Previous studies (Kölsch et. al., 2004 and Peddareddy et al., 2009), have also considered the relation of oxidative stress and stroke, so the polymorphisms in genes expressing GST omega enzymes were focused. Kölsch and co-researchers, 2004, investigated the effect of Ala140Asp polymorphisms in GSTO1 gene on Alzheimer disease, vascular dementia (VaD) and stroke in German population. They have found that Asp/Asp genotypes or Asp/Ala genotypes may be a genetic risk factor for cerebrovascular disease. Also, the logistic regression analyses showed that the presence of at least one Asp allele was associated with increased risk of stroke (Kölsch et al., 2004). Moreover, another study which reports the association of GSTO1 Ala140Asp polymorphism and stroke, a major affect could not be found, but the researchers concluded that if the study population were selected in larger size, the results would be more meaningful (Peddareddy et al., 2009). In the present study, Ala140Asp and Asn142Asp genotypes

of GSTO1 and GSTO2 were analyzed to show whether risk factors for ischemic stroke or not in different subgroups. The genotypes were compared between ischemic stroke and controls with respect to hypertension (Table 3.5), diabetes (Table 3.6), obesity (Table 3.7) and smoking (Table 3.8). These four groups were found as the risk factors for ischemic stroke for all study population (Table 3.1); however, any significant difference between stroke patients and controls in terms of GSTO1 and GSTO2 genotype frequencies in any of the subgroups (all P values are higher than 0.05).

Population stratification was done to analyze the effects of the conventional risk factors in detail (Table 3.9 and Table 3.10). Risk analyses were calculated by comparing stroke vs control cases with respect to being wild type or carrying a mutant allele.

In Table 3.9, stroke risk ratio was evaluated for the individuals having CC genotype and CA or AA genotype in terms of hypertensive or normotensive, diabetic or non-diabetic, smoker or non-smoker and obesity or non-obesity. The risk of having stroke in wild type individuals (CC) is 5.5 fold higher in hypertensive than normotensive (OR=5.52, P=1). The stroke risk ratio decreases the 2.24 in heterozygous or homozygous mutant individuals in hypertensive than normotensive (OR=2.24, P=0.007). Risk of having stroke in hypertensive individuals is 3.35 for all study population (Table 3.2, OR=3.5 and P= 0.000). The risk decreases to 2.24 from 3.35. Therefore, we can conclude that, the mutant genotypes have a protective effect for stroke in hypertensive group although it could not completely eliminate. This conclusion is unexpected because, base exchange in GSTO1 gene cause decreasing the enzyme activity and we expect the SNP increase the stroke risk. According to the result for diabetics and non-diabetics (Table 3.9), the wild type individuals have 1.76 fold higher risks in diabetics than non-diabetics despite of being insignificant (OR=1.76, P=0.142). However, the risk increases to approximately 4 fold when the individual having CC or CA genotypes in diabetics (OR=3.873, P=0.000). The risk of having stroke in diabetic individuals is 2.64 for all study population (Table 3.2). Therefore, when the individual has both diabetes and mutant genotype, the stroke risk becomes higher. The risk of having stroke in obesity group is 9.35 fold higher than non-obesity group in wild type individuals (OR=9.35,

P=0.000). On the other hand, this risk assessment decreases 2.75 for CA and AA individuals (OR=2.75, P=0.028). For all study population risk assessment result, stroke risk is approximately 4 fold for obesity people in all study population (OR=4.19, P=0.000, Table 3.2). As a result, it can be said that having mutant genotype is a protective factor for ischemic stroke in individuals with obesity. The risk of having stroke is approx. 2 fold insignificantly higher in smoker than non-smoker within wild type individuals (OR=1.937, P=0.118) while this risk increases to 3.55 for heterozygous and homozygous mutant individuals (OR=3.55, P=0.002). , The risk of having stroke in smokers is 2.662 in all study population (Table 3.2). Therefore, the risk increases with having mutant allele within smokers.

In Table 3.10, the wild type individuals, AA genotypes, has approx.5 fold higher risk for stroke in hypertensive than normotensive and this risk decreases to 3.75 for heterozygous and homozygous mutant type individuals, AG and GG genotypes. However, the difference is not significant (OR=4.98, P=1 and OR=3.75, P=1). The risk of having stroke in diabetic group is 2.465 fold higher than non-diabetics in wild type individuals (OR=2.465, P=0.037). The risk increases to 2.71 in heterozygous and homozygous mutant individuals (OR=2.71, P=0.004)., so having a mutant allele cause increasing in ischemic stroke risk. The wild type obesity individuals have approx. 9 fold higher risks for ischemic stroke than non-obesity; while heterozygous or homozygous mutant obesity individuals have approx. 3.6 fold higher risk than non-obesity (OR=9.09, P=0.002 and OR=3.59, P=0.004). To conclude, if the individual is both obesity and wild type, the risk of having stroke is higher than non- obesity and individuals caring mutant allele. Therefore, the mutant allele has protective role in obesity individuals. The risk of having stroke in smoker group is 2.13 fold insignificantly higher than non-smokers in wild type individuals of Asn142Asp polymorphism (OR=2.13, P=0.085). The risk for other group, heterozygous and homozygous mutant type (AA+AG), increase to 3.171 (P=0.004). As a result, if a heterozygous or homozygous mutant individual is a smoker, the risk of stroke become higher than non-smoker and wild type individuals. For both SNP, the risk of having stroke increases with carrying mutant allele (homozygous or

heterozygous) in diabetics and smokers, and also, carrying wild type allele in obesity individuals.

Logistic regression analyses with backward selection method were applied to identify the effects of conventional risk factors (hypertension, diabetes, smoking, obesity, sex, and age), lipid parameters (total cholesterol, low density lipoprotein, high density lipoprotein and triglyceride) and different genotypes for Ala140Asp and Asn142Asp polymorphisms. Three models were established by grouping different parameters to investigate the effect of risk factors on ischemic stroke.

The first model was created with all parameters and different genotypes in whole study population. According to first model (Table 3.11), hypertension, smoking, and obesity were found significant risk factors for ischemic stroke. The risk of stroke in hypertensive group is approx. 3 fold higher risk (OR=3.043, P=0.000). The same risk is 3.26 for smokers and 2.6 for obesity group in all study population (OR=3.258, P=0.001 for smoking and OR=2.593, P=0.004 for obesity). Moreover, the high density lipoprotein was revealed as significant protective factor for ischemic stroke (OR=0.270, P=0.004).

The second model was established with all parameters and different genotypes in male subjects of study population. According to second model (Table 3.12), hypertension, smoking, and obesity were found significant risk factors for ischemic stroke. Hypertension group has approx. 4 fold higher risk (OR=3.907, P=0.000). The same risk is 2.2 for smokers and 5 for obesity group in male population (OR=2.227, P=0.047 for smokers and OR=5.05, P=0.035 for obesity).

The third model was created with all parameters which were used in other models and different genotypes in female subject of study population. According to third model (Table 3.13), hypertension, diabetes, smoking, and cholesterol were found significant risk factors for ischemic stroke. There is app. 5 fold higher risk for hypertension group (OR=4.997, P=0.000). This risk is approximately 10 fold higher for smokers, 3.2 higher for diabetic and 1.78 higher for elevated cholesterol in female population (OR=9.818, P=0.003, OR=3.217, P=0.015 and OR=1.777, P=0.025, respectively). Moreover, the

high density lipoprotein was revealed as significant protective factor for ischemic stroke (OR=0.127, P=0.004).

CHAPTER 5

CONCLUSION

Ischemic stroke, main death causes in world, is a brain vessel disease in which the blood supply is blocked due to plaque formation in vessels feeding the brain tissues. The ischemic stroke is a multiple cause's disease, so there are many reasons to occur stroke attack. Age, gender, hypertension, diabetes, smoking, obesity, blood lipid levels are known as conventional risk factors for stroke. In recent studies, the researchers have been focused on oxidative stress as a risk factor for stroke, because high level reactive oxygen species and impaired antioxidant mechanisms in body triggers the formation of atherosclerosis which is a main cause for stroke.

Glutathione S-transferase (GST) enzymes family known as phase II enzymes, play an important role in decreasing the oxidative stress. Therefore, decrease in expression of these enzymes can cause formation of atherosclerosis and ischemic stroke. The polymorphisms, Ala140Asp in GSTO1 gene and Asn142Asp in GSTO2 gene result in decreased enzyme activity, so we expect increased stroke risk due to impaired antioxidant mechanism.

The whole blood samples were collected from voluntary patients and non-patients and then DNA isolation was done. The desired gene region was amplified by PCR method and the genotyping was achieved by RFPL. Appropriate primers and restriction enzymes were selected to detect the single nucleotide polymorphisms of Ala149Asp in GSTO1 gene and Asn142Asp in GSTO2 gene.

The study population includes 239 patients and 130 controls. For age mean and gender, there is no significant difference between patients and controls (P=0.067, P=0.186).The conventional risk factors such as hypertension, diabetes mellitus, obesity and smoking are found significantly higher for stroke patients (P values are lower than 0.05). Moreover, the mean of total cholesterol, triglyceride and LDL level were higher in patients. HDL level was higher in controls and the HDL was found protective factor significantly for ischemic stroke (Table 3.1).

For Ala140Asp SNP, the frequency of wild type allele 'C' is 0.642 in patients and 0.658 in controls. It is 0.358 in patients and 0.342 in controls for mutant and risky allele 'A'. Any significant risk factor is not found for different genotypes and alleles for ischemic stroke (P=0.315, P=0.375).

As regard to Asn142Asp SNP, the frequency of wild type allele 'A' is 0.630 in patients and 0.596 in controls. It is 0.370 in patients and 0.404 in controls for mutant and risky allele 'G'. Similar to the other SNP, there is no significant risk for stroke in mutant allele (P=0.691, P=0.370).

The subgroup analyses, with conventional risk factors hypertensive/normotensive, diabetic/non-diabetic, obesity/non-obesity and smoker/non-smoker, revealed that even though these factors are risk factors for stroke, there is no significant risk due to different genotypes (Table 3.5, Table 3.6, Table 3.7 and Table 3.8).

According to population stratification analysis results (Table 3.9), while the stroke risk in hypertensive individuals are 3.35 fold higher than normotensive (Table 3.1), this risk decreases to 2.24 when individual having mutant allele in GSTO1 gene (CA+AA). Moreover, the risk of stroke for diabetics is 2.61 fold higher than non-diabetics (Table 3.1); however, it increases 3.873 in mutant genotypes. Within obesity group, the risk of stroke is 2.75 for mutant individuals and 9.35 for wild types. This risk is 4.19 without any grouping according to different genotypes (Table 3.1). Finally, the risk of stroke within mutant genotype individuals is 3.55 fold higher than non-smoker while this risk is 2.66 for smoker in all study population (Table 3.1). Consequently, the single nucleotide

polymorphism increases the risk of stroke within diabetics and smokers. Moreover, carrying mutant allele has protective effect for hypertensive and obesity individuals. If we summarize the situation that increases the stroke risk for this SNP;

- being wild type and hypertensive
- being mutant type and diabetic
- being wild type and obesity
- being mutant type and smoker

By focusing the population stratification of Asn142Asp polymorphism (Table 3.10), the risk of being stroke within wild type, AA, individuals 2.4 fold higher in diabetics than non-diabetics and this risk increases to 2.71 for mutant type, AG+GG genotype individuals. The stroke risk in diabetic individuals for all study population is 2.61 (Table 3.1). So, mutant allele increases the risk of stroke for diabetic individuals. Moreover, for AA individuals, the people with obesity have 9 fold higher risks for stroke than non-obesity and same risk decreases to 3.39 fold for AG+GG individuals. The people with obesity have 4.19 fold higher risks for stroke (Table 3.1). Therefore, it can be concluded that mutant allele has protective role for stroke in people with obesity. As regards smoking use, while AA genotypes have 2.13 fold higher risk for stroke when smoker, AG+GG genotypes have 3.171 fold higher risks. For all study population, the risk of stroke is 2.66 fold for smoker. As a result, mutant type allele causes increasing risk of stroke for smoker. If we summarize the situation that increases the stroke risk for his SNP;

- being mutant type and diabetic
- being wild type and obesity
- being mutant type and smoker

Logistic regression analyses was done to confirm the effects of conventional risk factors; such as hypertension, diabetes vs., blood lipid level; total cholesterol, LDL vs., and

different genotypes of Ala140Asp and Asn142Asp in stroke risk assessment. For model using all study population with all parameters investigated as risk factor, hypertension, smoking and obesity was revealed as risk factor for ischemic stroke (OR=3.258, P=0.000 for hypertension, OR=3.258, P=0.001 for smoking and OR=2.593, P=0.004 for obesity). The HDL was also found as protective factor (OR=0.270, P=0.004). Moreover, the second model constructed by using male subjects, hypertension, smoking and obesity was revealed as risk factor for stroke (OR=3.907, P=0.000 for hypertension, OR=2.227, P=0.047 for smoking and OR=5.05, P=0.035 for obesity). Finally, the third model with female subjects, hypertension, diabetes, smoking, and cholesterol were found significant risk factor for ischemic stroke (OR=4.997, P=0.000 for hypertension, OR=9.818, P=0.003 smoking, OR=3.217, P=0.015 diabetes and OR=1.777, P=0.025 cholesterol). Also, the high density lipoprotein was revealed as significant protective factor for ischemic stroke (OR=0.127, P=0.004).

In conclusion, any direct association could not be revealed between different genotypes and alleles of GSTO1 and GSTO2 genes and ischemic stroke risk; on the other hand, some risk assessment and relation was found in subgroup analyses. There were situations of increased or decreased risk of stroke when combining a conventional risk factor and different genotypes.

REFERENCES

Ada T G, Ada A O, Kunak S C, Alpar S, Gulhan M, and Iscan M. (2013). Association between Glutathione S-Transferase Omega 1 A140D Polymorphism in the Turkish Population and Susceptibility to Non-Small Cell Lung Cancer. Arhiv za higijenu rada i toksikologiju, 64(2), 247-252.

Adam G C, Sorensen E J, and Cravatt B F. (2002). Proteomic profiling of mechanistically distinct enzyme classes using a common chemotype. nature biotechnology, 20(8), 805-809.

Bergendi L, Benes L, Durackova Z, and Ferencik M. (1999). Chemistry, physiology and pathology of free radicals. Life Sci, 65:1865–74.

Beckett G, Chapman B, Dyson E, and Hayes J. (1985). Plasma glutathione S-transferase measurements after paracetamol overdose: evidence for early hepatocellular damage. Gut, 26(1), 26-31.

Board P G, Coggan M, Chelvanayagam G, Easteal S, Jermiin L S, Schulte G K, Danley D E, Hoth L R, Griffor M C, and Kamath A V. (2000). Identification, characterization, and crystal structure of the Omega class glutathione transferases. Journal of Biological Chemistry, 275(32), 24798-24806.

Bonomini F, Tengattini S, Fabiano A, Bianchi R, Rezzani R.(2008). Atherosclerosis and oxidative stress. Histol Histopathol,23: 381-390.

Brown R, Whisnant J P, Sicks J, O'fallon W M, and Wiebers D O. (1996). Stroke incidence, prevalence, and survival: secular trends in Rochester, Minnesota, through 1989. Stroke; a journal of cerebral circulation, 27(3), 373-380.

Burchfiel C M, Curb J D, Rodriguez B L, Abbott R D, Chiu D, and Yano K. (1994). Glucose intolerance and 22-year stroke incidence. The Honolulu Heart Program. Stroke, 25(5), 951-957.

Capurso C, Panza F, Seripa D, Frisardi V, Imbimbo B P, Verdile G, Vendemiale G, Pilotto A, and Solfrizzi V. (2010). Polymorphisms in glutathione S-transferase omega-1 gene and increased risk of sporadic Alzheimer disease. Rejuvenation research, 13(6), 645-652.

Chan P H. (2001). Reactive oxygen radicals in signaling and damage in the ischemic brain. Journal of Cerebral Blood Flow and Metabolism, 21(1), 2-14.

Chariyalertsak S, Purisa W, and Sangrajrang S. (2009). Role of glutathione S-transferase omega gene polymorphisms in breast-cancer risk. Tumori, 95(6), 739-743.

Chung C-J, Hsueh Y-M, Bai C-H, Huang Y-K, Huang Y-L, Yang M-H, and Chen C-J. (2009). Polymorphisms in arsenic metabolism genes, urinary arsenic methylation profile and cancer. Cancer Causes and Control, 20(9), 1653-1661.

Chung C-J, Pu Y-S, Su C-T, Huang C-Y, and Hsueh Y-M. (2011). Gene polymorphisms of glutathione S-transferase omega 1 and 2, urinary arsenic methylation profile and urothelial carcinoma. Science of the Total Environment, 409(3), 465-470.

Coleman M D. (2005). Front Matter: Wiley Online Library.

Collins R, and Macmahon S. (1994). Blood pressure, antihypertensive drug treatment and the risks of stroke and of coronary heart disease. British medical bulletin, 50(2), 272-298.

Demirdöğen B C, Demirkaya Ş, Türkanoğlu A, Bek S, Arınç E, and Adalı O. (2009). Analysis of paraoxonase 1 (PON1) genetic polymorphisms and activities as risk factors for ischemic stroke in Turkish population. Cell biochemistry and function, 27(8), 558-567.

Demirdöğen B C, Türkanoğlu A, Bek S, Sanisoğlu Y, Demirkaya Ş, Vural O, Arınç E, and Adalı O. (2008). Paraoxonase/arylesterase ratio, PON1 192Q/R polymorphism and PON1 status are associated with increased risk of ischemic stroke. Clinical biochemistry, 41(1), 1-9.

Demirdöğen B C, Şahin E, Özçelik A T, Bek S, Demirkaya Ş, and Adali O. (2012). Apolipoprotein A5 polymorphisms in Turkish population: association with serum lipid profile and risk of ischemic stroke. Molecular biology reports, 39(12), 10459-10468.

Diener H, Cunha L, Forbes C E, Sivenius J, Smets P, and Lowenthal A. (1996). European Stroke Prevention Study 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. Journal of the neurological sciences, 143(1), 1-13.

Dulhunty A, Gage P, Curtis S, Chelvanayagam G, and Board P. (2001). The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. Journal of Biological Chemistry, 276(5), 3319-3323.

Fu S, Wu J, Chen F, Sun D, and Fu S. (2008). Polymorphisms of Glutathione S-transferases Omega-1 among ethnic populations in China. BMC genetics, 9(1), 29.

Harrison D, Kharbanda R, Cunningham D S, Mclellan L, and Hayes J. (1989). Distribution of glutathione S-transferase isoenzymes in human kidney: basis for possible markers of renal injury. Journal of clinical pathology, 42(6), 624-628.

Hayes J D, and Pulford D J. (1995). The Glut athione S-Transferase Supergene Family: Regulation of GST and the Contribution of the lsoenzymes to Cancer Chemoprotection and Drug Resistance Part I. Critical reviews in biochemistry and molecular biology, 30(6), 445-520.

Howard G, Anderson R, Sorlie P, Andrews V, Backlund E, and Burke G L. (1994). Ethnic differences in stroke mortality between non-Hispanic whites, Hispanic whites, and blacks. The National Longitudinal Mortality Study. Stroke, 25(11), 2120-2125.

Hsieh Y-C, Lien L.-M, Chung W.-T, Hsieh F-I, Hsieh, P-F, Wu M-M (2011). Significantly increased risk of carotid atherosclerosis with arsenic exposure and polymorphisms in arsenic metabolism genes. Environmental research, 111(6), 804-810.

Hsu L-I, Chen W-P, Yang T-Y, Chen Y-H, Lo W-C, Wang Y-H, Liao Y-T, Hsueh Y-M, Chiou H-Y, and Wu M-M. (2011). Genetic polymorphisms in glutathione S-transferase (GST) superfamily and risk of arsenic-induced urothelial carcinoma in residents of southwestern Taiwan. J. Biomed. Sci, 18, 51.

Johnston S C, Gress D R, Browner W S, and Sidney S. (2000). Short-term prognosis after emergency department diagnosis of TIA. JAMA: the journal of the American Medical Association, 284(22), 2901-2906.

Kaul N, Devaraj S, and Jialal I. (2001). α-Tocopherol and atherosclerosis. Experimental Biology and Medicine, 226(1), 5-12.

Kiely D K, Wolf P A, Cupples L A, Beiser A, and Myers R. (1993). Familial aggregation of stroke. The Framingham Study. Stroke, 24(9), 1366-1371.

Kiyohara C, Miyake Y, Koyanagi M, Fujimoto T, Shirasawa S, Tanaka K, Fukushima W, Sasaki S, Tsuboi Y, and Yamada T. (2010). < i> GST</i> polymorphisms, interaction with smoking and pesticide use, and risk for Parkinson's disease in a Japanese population. Parkinsonism and Related Disorders, 16(7), 447-452.

Kölsch H, Linnebank M, Lütjohann D, Jessen F, Wüllner U, Harbrecht U, Thelen K, Kreis M, Hentschel F, and Schulz A. (2004). Polymorphisms in glutathione S-transferase omega-1 and AD, vascular dementia, and stroke. Neurology, 63(12), 2255-2260.

Krötz F, Sohn H-Y, and Pohl U. (2004). Reactive oxygen species Players in the platelet game. Arteriosclerosis, thrombosis, and vascular biology, 24(11), 1988-1996.

Kumral E, Özkaya B, Sagduyu A, Şirin H, Vardarli E, and Pehlivan M. (1998). The Ege Stroke Registry: a hospital-based study in the Aegean region, Izmir, Turkey. Cerebrovascular diseases, 8(5), 278-288.

Kurth T, Everett B M, Buring J E, Kase C S, Ridker P M, and Gaziano J M. (2007). Lipid levels and the risk of ischemic stroke in women. Neurology, 68(8), 556-562.

Laliberte R E, Perregaux D G, Hoth L R, Rosner P J, Jordan C K, Peese K M, Eggler J F, Dombroski M A, Geoghegan K F, and Gabel C A. (2003). Glutathione S-transferase omega 1-1 is a target of cytokine release inhibitory drugs and may be responsible for their effect on interleukin-1β posttranslational processing. Journal of Biological Chemistry, 278(19), 16567-16578.

Loguercio C, Caporaso N, Tuccillo C, Morisco F, Del Vecchio Blanco G, and Del Vecchio Blanco C. (1998). Alpha-glutathione transferases in HCV-related chronic hepatitis: a new predictive index of response to interferon therapy? Journal of hepatology, 28(3), 390-395.

Lu S-P, Lin Feng M-H, Huang H-L, Huang Y-C, Tsou W-I, and Lai M-Z. (2007). Reactive oxygen species promote raft formation in T lymphocytes. Free Radical Biology and Medicine, 42(7), 936-944.

Macmahon S, Peto R, Collins R, Godwin J, Cutler J, Sorlie P, Abbott R, Neaton J, Dyer A, and Stamler J. (1990). Blood pressure, stroke, and coronary heart disease: part 1, prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias. The Lancet, 335(8692), 765-774.

Macmahon S W, Cutler J A, Furberg C D, and Payne G H. (1986). The effects of drug treatment for hypertension on morbidity and mortality from cardiovascular disease: a review of randomized controlled trials. Progress in cardiovascular diseases, 29(3), 99-118.

Mannervik B, Awasthi Y, Board P, Hayes J, Di Ilio C, Ketterer B, Listowsky I, Morgenstern R, Muramatsu M, and Pearson W. (1992). Nomenclature for human glutathione transferases. Biochemical Journal, 282(Pt 1), 305.

Masoudi M, Saadat I, Omidvari S, and Saadat M. (2009). Genetic polymorphisms of GSTO2, GSTM1, and GSTT1 and risk of gastric cancer. Molecular biology reports, 36(4), 781-784.

Morari E C, Lima A B C, Bufalo N E, Leite J L, Granja F, and Ward L S. (2006). Role of glutathione-S-transferase and codon 72 of P53 genotypes in epithelial ovarian cancer patients. Journal of cancer research and clinical oncology, 132(8), 521-528.

Olmez I, and Ozyurt H. (2012). Reactive oxygen species and ischemic cerebrovascular disease. Neurochemistry international, 60(2), 208-212.

Ozturk A, Desai P P, Minster R L, Dekosky S T, and Kamboh M I. (2005). Three SNPs in the GSTO1, GSTO2 and PRSS11 genes on chromosome 10 are not associated with age-at-onset of Alzheimer's disease. Neurobiology of aging, 26(8), 1161-1165.

Paiva L, Hernández A, Martínez V, Creus A, Quinteros D, and Marcos R. (2010). Association between< i> GSTO2</i> polymorphism and the urinary arsenic profile in copper industry workers. Environmental research, 110(5), 463-468.

Peddareddygari L R, Dutra A V, Levenstien M A, Sen S, and Grewal R P. (2009). An analysis of Methylenetetrahydrofolate reductase and Glutathione S-transferase omega-1 genes as modifiers of the cerebral response to ischemia. BMC neurology, 9(1), 37.

Psaty B M, Anderson M, Kronmal R A, Tracy R P, Orchard T, Fried L P. (2004). The association between lipid levels and the risks of incident myocardial infarction, stroke, and total mortality: The Cardiovascular Health Study. Journal of the American Geriatrics Society, 52(10), 1639-1647.

Rafiee L, Saadat I, and Saadat M. (2010). Glutathione S-transferase genetic polymorphisms (GSTM1, GSTT1 and GSTO2) in three Iranian populations. Molecular biology reports, 37(1), 155-158.

Rothwell P M, and Warlow C P. (2005). Timing of TIAs preceding stroke time window for prevention is very short. Neurology, 64(5), 817-820.

Sacco R L. (2005). Vascular disease In: Rowland LP, Ed. Merritt's Neurology, 11th edition. Lippicotti, Williams & Wilkins, Philadelphia, PA; 275-290.

Sacco R L, Benson R T, Kargman, D E, Boden-Albala B, Tuck C, Lin I.-F. (2001). High-density lipoprotein cholesterol and ischemic stroke in the elderly: the Northern Manhattan Stroke Study. Jama, 2*85*(21), 2729-2735.

Sacco R L, Benjamin E J, Broderick J P, Dyken M, Easton J D, Feinberg W M, Goldstein L B, Gorelick P B, Howard G, Kittner S J, Manolio T A, Whisnant J P, Wolf P A. (1997). Stroke risk factors. Stroke, 28: 1507-1517.

Sampayo-Reyes A, Hernández A, El-Yamani N, López-Campos C, Mayet-Machado E, Rincón-Castañeda C B. (2010). Arsenic induces DNA damage in environmentally exposed Mexican children and adults. Influence of GSTO1 and AS3MT polymorphisms. Toxicological Sciences, 117(1), 63-71.

Shinton R, and Beevers G. (1989). Meta-analysis of relation between cigarette smoking and stroke. BMJ: British Medical Journal, 298(6676), 789.

Stamenkovic M, Radic T, Stefanovic I, Coric V, Sencanic I, Pljesa-Ercegovac M, Matic M, Jaksic V, Simic T, and Savic-Radojevic A. (2013). Glutathione S-transferase omega-2 polymorphism Asn142Asp modifies the risk of age-related cataract in

smokers and subjects exposed to ultraviolet irradiation. Clinical and experimental ophthalmology.

Sundberg A, Appelkvist E-L, Bäckman L, and Dallner G. (1994). Urinary π-class glutathione transferase as an indicator of tubular damage in the human kidney. Nephron, 67(3), 308-316.

Suzuki YJ, Forman HJ, and Sevanian A. (1997). Oxidants as stimulators of signal transduction. Free Radic Biol Med, 22:269–85.

Takeshita H, Fujihara J, Takastuka H, Agusa T, Yasuda T, and Kunito T. (2009). Diversity of glutathione s-transferase omega 1 (a140d) and 2 (n142d) gene polymorphisms in worldwide populations. Clinical and Experimental Pharmacology and Physiology, 36(3), 283-286.

Tanaka-Kagawa T, Jinno H, Hasegawa T, Makino Y, Seko Y, Hanioka N, and Ando M. (2003). Functional characterization of two variant human GSTO 1-1s (Ala140Asp and Thr217Asn). Biochemical and biophysical research communications, 301(2), 516-520.

Türkanoğlu A , Demirdöğen B C, Demirkaya Ş, and Adalı O. (2013). Flavin containing monooxygenase 3 genetic polymorphisms Glu158Lys and Glu308Gly and their relation to ischemic stroke. Gene.

Türkanoğlu A, Demirdöğen B C, Demirkaya Ş, Bek S, and Adalı O. (2010). Association analysis of GSTT1, GSTM1 genotype polymorphisms and serum total GST activity with ischemic stroke risk. Neurological sciences, 31(6), 727-734.

Wahner A D, Glatt C E, Bronstein J M, and Ritz B. (2007). Glutathione< i> S</i>-transferase mu, omega, pi, and theta class variants and smoking in Parkinson's disease. Neuroscience letters, 413(3), 274-278.

Wang X, and Michaelis E K. (2010). Selective neuronal vulnerability to oxidative stress in the brain. Frontiers in aging neuroscience, 2.

Whitbread A K, Tetlow N, Eyre H J, Sutherland G R, and Board P G. (2003). Characterization of the human Omega class glutathione transferase genes and associated polymorphisms. Pharmacogenetics and Genomics, 13(3), 131-144.

Wolf P A, Abbott R D, and Kannel W B. (1991). Atrial fibrillation as an independent risk factor for stroke: the Framingham Study. Stroke, 22(8), 983-988.

Yin Z-L, Dahlstrom J E, Le Couteur D G, and Board P G. (2001). Immunohistochemistry of omega class glutathione S-transferase in human tissues. Journal of Histochemistry and Cytochemistry, 49(8), 983-987.

Zakharyan R A, Sampayo-Reyes A, Healy S M, Tsaprailis G, Board P G, Liebler D C, and Aposhian H V. (2001). Human monomethylarsonic acid (MMAV) reductase is a member of the glutathione-S-transferase superfamily. Chemical research in toxicology, 14(8), 1051-1057.

APPENDIX A

INFORMED CONSENT FOR PATIENTS

İnme-felç hastalığı için risk oluşturan faktörleri bulmak üzere yeni bir araştırma yapmaktayız. Araştırmanın ismi "Paraoksonaz 1'in gen ve aktivite polimorfizmlerinin iskemik inme riski ile ilişkisinin araştırılması" dır. 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 yada 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

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 - 567 - 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**	
Ergun TOZKOPARAN Doç.Tbp. Alb.	Nalan AKBAYRAK Prof. Dr. Sağ. Yb.	Muharrem UÇAR Yrd.Doç.J.Tbp.Yb.	

APPENDIX D

REAGENTS

Reagents which used in human genomic DNA isolation from whole blood samples:

1. TKME Buffer

10 mM Tris-HCl (pH 7.6), 10 mM KCl, 4 mM $MgCl_2$, and 2 mM EDTA are used for solution. After preparation, the solution is autoclaved for sterilization and stored at 4°C.

2. TE Buffer

10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) are used for solution. After preparation, the solution is autoclaved for sterilization and stored at 4°C.

3. Tris-HCl (pH 8.0, 100 mM, 100 mL)

12.1 g Tris is weighed and dissolved in 70 mL of dH_2O. pH is adjusted to 8.0 with concentrated HCl and volume is completed to 100 mL. Solution is autoclaved for sterilization and stored at 4°C.

4. 10% SDS Solution

10 g SDS detergent in molecular weight is weighed and dissolved in 100 mL of distilled water. The solution is not autoclaved.

5. **Saturated NaCl (6M)**

35.06 g NaCl is weighed and dissolved in 100 mL of distilled water. Solution is autoclaved for sterilization and stored at 4°C.

6. **EDTA, pH 8.0 (500 mM)**

18.61 g $Na_2EDTA.2H_2O$ is weighed and dissolved in 70 mL distilled water. pH is adjusted to 8.0 by using NaOH, then volume is completed to 100 mL. The solution is autoclaved for sterilization and stored at 4°C.

Reagents which used in PCR:

1. **PCR Amplification Buffer (10x)**

The buffer is obtained commercially (Fermentas) and includes 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% Nonidet P40. The solutions are stored at -20°C.

2. **dNTP Mixture**

The solution is obtained commercially (Fermentas) and includes 10 mM of each dATP, dCTP, dGTP and dTTP in aqueous solution. The solution is stored at -20°C.

3. **25 mM $MgCl_2$**

The solution is obtained commercially (Fermentas) and is stored at -20°C.

Reagents which used in agarose gel electrophoresis:

1. **5X TBE Buffer (Tris-Borate-EDTA, pH 8.3, 1000 mL)**

54 g Trizma-Base, 27.5 g boric acid and 20 µL of 500 mM EDTA are mixed in a beaker and dissolved appropriate amount of distilled water. pH is adjusted to 8.3 and volume is completed to 1000 mL. After preparation, the solution is autoclaved.

2. **Ethidium Bromide**

0.1 g ethidium bromide is dissolved in 10 mL distilled water. The solution is stirred on magnetic stirrer for several hours to ensure that dye has completely dissolved. Because the solution is light sensitive, the bottle is covered with aluminum foil and stored at room temperature.

3. **Gel Loading Dye**

25 mg bromophenol blue and 4 g sucrose is mixed and it is completed to 10 mL distilled water. The solution is stored at 4°C

Reagents which used in agarose restriction endonuclease digestion:

1. **1X NE Buffer 4**

The buffer is obtained commercial with restriction enzyme Cac8I. The buffer includes 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate and 1mM Dithiothreitol at pH 7.9. The solution is stored at -20°C.

2. **1X Buffer R**

The buffer is obtained commercial with restriction enzyme MboI. The buffer includes 10 mMTris-HCl, 10 mM $MgCl_2$, 100 mM KCl and 0.1 mg/mL BSA at pH 8.5. The solution is stored at -20°C.

APPENDIX E

LIST OF STUDY POPULATION

Table E.1 List of study population composed of 239 stroke patients and 130 controls including demographic characteristics, lipid parameters, GSTO1 Ala140Asp and GSTO2 Asn142Asp the abbreviations means:

P: Patient; C: Control; M: Male; F: Female; Y: Yes; N: No; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; CC: Wild Type Homozygous for Ala14Asp polymorphism; CA: Heterozygous for Ala14Asp polymorphism; AA: Homozygous mutant for Ala14Asp polymorphism; AA: Wild Type Homozygous for Asn142Asp polymorphism; AG: Heterozygous for Asn142Asp polymorphism; GG: Homozygous mutant for Asn142Asp polymorphism

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
1.	P	75	M	Y	Y	Y	N	3,094	1,281	1,846	0,615	CC	GG
2.	P	57	F	Y	Y	N	N	5,460	1,528	3,385	1,308	CA	AG
3.	P	41	M	N	N	Y	N	4,316	1,573	2,667	0,872	CC	GG
4.	P	73	M	Y	N	N	N	3,718	0,719	1,897	1,462	CC	GG
5.	P	53	M	Y	Y	N	N	10,42	2,596	7,590	1,513	CC	GG
6.	P	66	M	Y	Y	Y	N	3,380	1,416	2,179	0,512	CA	GG
7.	P	56	F	Y	Y	N	N	3,588	2,382	1,795	0,974	CA	AA

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
8.	P	67	F	Y	N	N	N	5,434	1,191	3,564	1,256	CC	GG
9.	P	76	M	Y	N	Y	N	4,030	0,764	2,333	1,282	CA	GG
10.	P	75	F	Y	N	N	N	4,732	1,169	3,256	0,872	CA	AG
11.	P	74	F	Y	Y	N	N	4,342	0,697	2,744	1,231	CC	AA
12.	P	68	F	Y	N	Y	N	3,640	2,270	1,923	0,641	CC	AG
13.	P	72	F	N	N	N	N	6,656	2,843	4,231	1,026	CC	GG
14.	P	81	F	Y	N	N	N	4,888	1,753	2,949	1,077	CA	AG
15.	P	73	F	Y	Y	N	N	5,382	2,270	3,205	1,077	CC	AG
16.	P	73	F	Y	N	N	Y	4,680	0,989	2,744	1,410	CA	AG
17.	P	67	F	Y	N	N	N	5,408	1,393	3,667	1,026	CA	AG
18.	C	71	M	Y	N	N	N	3,848	1,337	2,103	1,077	CC	GG
19.	P	61	M	Y	Y	N	N	4,394	1,371	2,769	0,949	CA	AG
20.	P	40	M	N	N	Y	N	3,770	1,169	2,000	1,205	CA	AG
21.	C	61	F	Y	N	N	N	3,380	0,933	1,179	1,744	CC	AG
22.	P	60	F	Y	Y	N	Y	4,992	1,685	3,051	1,103	CA	AG
23.	P	75	M	N	N	N	N	4,550	1,079	1,821	2,179	CA	AG
24.	P	76	F	Y	N	N	N	3,224	1,517	1,282	1,179	CC	AA
25.	C	76	M	Y	Y	N	N	3,432	1,045	1,897	1,000	CA	AG
26.	C	51	M	N	N	Y	N	3,692	1,292	2,128	0,897	CC	AG
27.	C	50	F	N	N	N	N	3,614	2,022	1,872	0,769	CA	AG
28.	C	42	F	N	N	N	N	2,704	1,270	1,462	0,615	CA	AA
29.	C	45	F	N	N	Y	N	3,666	0,596	1,872	1,462	CA	AA
30.	P	70	M	Y	N	N	N	3,016	0,820	1,513	1,077	CC	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
31.	C	63	M	Y	Y	Y	N	3,042	1,124	1,538	0,949	CC	GG
32.	P	83	F	N	N	N	N	7,254	2,360	4,667	1,410	CC	GG
33.	C	63	F	N	N	N	N	5,200	2,483	3,128	0,872	CC	AA
34.	C	75	M	Y	Y	N	N	4,732	1,719	2,564	1,308	CC	AA
35.	C	58	F	Y	N	N	N	2,704	1,090	1,103	1,077	CA	AG
36.	C	78	F	Y	N	N	N	3,614	1,067	1,821	1,256	CA	AG
37.	C	74	M	N	N	N	N	4,342	0,921	2,564	1,308	CC	GG
38.	P	71	M	Y	N	N	N	5,382	2,607	3,231	0,897	CC	GG
39.	C	61	M	N	N	N	N	5,928	1,865	3,974	1,026	CC	GG
40.	C	85	M	Y	N	N	N	3,094	1,798	0,615	1,615	CC	AA
41.	C	65	F	N	Y	N	N	6,110	1,416	3,231	2,154	CC	AG
42.	C	65	M	N	N	N	N	4,966	0,640	3,282	1,333	CA	AG
43.	C	58	M	N	Y	Y	N	5,954	1,202	4,333	1,000	CC	GG
44.	C	61	F	N	N	N	Y	6,812	1,831	4,359	1,513	CC	AA
45.	C	80	F	N	N	N	N	3,354	0,708	1,179	1,821	CC	GG
46.	C	76	M	N	N	N	N	6,006	2,315	3,667	1,205	CC	AA
47.	C	69	F	Y	N	N	N	3,926	1,764	1,897	1,179	CC	GG
48.	C	66	M	N	Y	Y	N	6,968	3,921	4,103	0,974	CC	GG
49.	C	60	F	Y	N	N	N	4,966	1,382	3,103	1,154	CC	GG
50.	P	64	F	N	N	Y	Y	4,316	1,056	2,538	1,231	CA	AG
51.	P	58	F	Y	Y	N	N	9,100	4,045	5,846	1,282	CC	GG
52.	P	74	F	Y	N	N	Y	6,266	1,730	4,154	1,231	CC	AG
53.	C	71	M	N	N	N	N	4,290	1,573	2,051	1,410	CC	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
54.	P	80	F	Y	N	N	Y	3,770	0,966	2,308	0,974	CC	AA
55.	P	62	M	Y	Y	N	N	4,888	1,202	3,282	1,000	CA	GG
56.	C	68	M	N	N	N	N	5,460	1,292	3,154	1,641	CC	GG
57.	C	65	F	N	N	N	N	5,200	1,764	3,154	1,179	CA	GG
58.	C	72	F	Y	N	N	N	6,084	1,674	4,051	1,179	CC	AG
59.	C	65	M	N	N	N	N	3,042	0,899	1,641	0,949	CC	GG
60.	C	70	F	N	N	N	N	4,862	1,483	3,026	1,103	CA	AG
61.	C	70	F	Y	N	N	N	5,252	1,180	3,333	1,308	CA	GG
62.	C	65	F	Y	N	N	N	5,954	1,764	3,769	1,308	CC	AA
63.	P	63	M	Y	N	N	N	3,406	1,000	1,846	1,051	CC	GG
64.	C	65	F	Y	N	N	N	4,524	1,270	2,179	1,692	CC	GG
65.	C	78	F	Y	N	N	N	4,394	1,247	2,385	1,385	CC	AA
66.	P	68	M	Y	N	N	N	4,446	0,899	2,436	1,538	CC	GG
67.	C	47	M	N	N	N	N	6,266	3,056	3,333	1,462	CC	GG
68.	P	77	F	Y	N	N	N	5,070	1,067	3,513	1,000	CC	AA
69.	C	77	F	Y	Y	N	N	4,056	0,978	2,641	0,923	CC	GG
70.	P	80	F	Y	N	N	N	5,226	1,056	3,077	1,590	CA	AG
71.	C	71	M	N	N	N	N	4,368	1,472	2,308	1,333	CA	AA
72.	C	73	M	Y	N	N	N	3,640	2,472	1,949	0,513	CC	GG
73.	C	61	M	N	N	Y	N	4,680	0,393	2,923	1,513	CC	GG
74.	P	62	M	N	N	N	N	3,692	1,011	1,385	1,103	CA	AA
75.	P	77	M	N	N	N	N	3,822	0,607	1,744	1,744	CA	AG
76.	P	24	M	N	N	N	N	6,110	2,865	3,667	1,051	CC	AG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
77.	P	53	F	N	N	Y	N	2,496	2,157	0,590	0,897	CA	AA
78.	P	61	M	N	N	N	N	4,836	1,596	3,103	0,949	CC	GG
79.	C	52	M	N	N	N	N	6,942	1,157	4,692	1,615	CC	GG
80.	P	78	M	Y	N	Y	N	5,226	2,056	3,077	1,128	CC	AG
81.	C	65	M	N	N	N	Y	4,160	0,854	2,897	0,821	CC	GG
82.	P	81	M	Y	N	N	N	3,458	3,034	0,949	1,077	CA	AG
83.	P	80	F	Y	N	N	Y	2,730	1,270	1,462	0,641	CC	AA
84.	C	50	F	Y	N	N	N	3,198	0,854	2,154	0,615	CA	AG
85.	C	87	F	Y	N	N	Y	2,756	1,022	1,385	0,872	CA	AG
86.	C	67	M	N	N	N	N	4,368	1,146	2,103	0,154	CA	GG
87.	C	38	M	N	N	Y	N	4,784	0,562	2,846	1,615	CC	GG
88.	C	50	M	N	N	N	N	3,822	0,618	2,026	1,462	CA	AA
89.	P	26	M	N	N	N	N	4,212	1,067	2,051	1,615	CA	AG
90.	P	55	M	Y	N	N	N	5,044	0,933	3,154	1,385	CA	AG
91.	P	26	M	N	N	N	N	4,056	0,719	2,923	0,744	CA	AG
92.	P	73	F	Y	N	N	N	4,004	1,135	2,538	0,897	CA	AG
93.	C	80	F	N	Y	N	N	3,978	1,382	1,974	1,308	CC	AA
94.	P	36	M	N	N	N	N	4,862	1,573	2,974	1,103	CC	AA
95.	P	56	M	N	N	N	N	4,342	1,225	2,564	1,154	CC	GG
96.	P	47	F	N	N	N	N	4,784	1,382	3,154	0,923	CC	GG
97.	P	73	M	Y	Y	N	N	5,148	2,517	3,026	0,897	CA	AA
98.	P	73	F	Y	N	N	N	4,004	1,135	2,538	0,897	CA	AG
99.	P	66	F	N	N	N	N	3,354	2,787	1,487	0,538	CC	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
100.	P	74	F	Y	N	N	N	5,980	1,427	3,949	1,308	CC	GG
101.	C	44	M	N	N	Y	N	5,200	2,382	2,462	1,590	CC	GG
102.	C	51	F	N	N	N	N	4,368	0,573	2,821	1,231	CC	AG
103.	C	67	M	Y	N	N	N	4,472	1,663	2,590	1,051	CA	AG
104.	C	73	F	Y	Y	N	N	5,902	2,315	3,667	1,103	CC	AG
105.	C	88	F	Y	N	N	N	4,004	0,798	2,564	1,026	CC	AA
106.	P	66	M	Y	Y	N	N	3,354	2,854	1,256	0,744	CA	AG
107.	P	61	F	Y	Y	N	N	3,224	2,000	1,615	0,641	CC	AA
108.	P	78	M	N	N	Y	N	3,380	1,236	1,795	1,026	CA	AA
109.	C	59	M	N	N	N	N	4,082	1,236	2,308	1,154	CA	AG
110.	C	69	F	N	Y	N	N	6,266	1,337	4,026	1,538	CA	AG
111.	P	80	M	Y	N	N	N	7,904	0,831	6,128	1,282	CC	AG
112.	P	76	F	Y	Y	N	N	4,108	1,236	2,513	0,974	CC	GG
113.	P	79	F	Y	N	N	N	5,252	1,528	3,308	1,179	CC	GG
114.	C	51	F	N	N	N	N	2,860	1,011	1,385	0,974	CC	GG
115.	P	21	M	N	N	N	N	3,874	0,933	2,564	0,821	CA	AG
116.	P	76	M	N	N	N	Y	5,018	1,191	3,385	1,026	CA	AG
117.	P	28	M	N	N	Y	N	3,926	1,640	2,000	1,128	CC	GG
118.	P	20	M	N	N	Y	N	4,316	0,899	2,564	1,282	CA	AG
119.	P	80	F	Y	N	N	N	5,018	2,820	2,205	1,462	CA	AG
120.	P	64	F	Y	N	N	N	3,770	1,618	1,974	1,000	CC	AG
121.	P	67	F	Y	Y	N	Y	10,166	2,539	7,615	1,256	CA	AG
122.	P	58	F	Y	Y	N	N	7,124	2,876	4,333	1,385	CC	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
123.	C	77	M	N	N	N	N	4,654	1,876	2,051	1,179	CC	AA
124.	P	49	M	N	N	Y	N	5,876	2,292	3,564	1,179	CC	AA
125.	P	78	F	N	N	N	N	4,628	0,989	2,641	1,462	CC	GG
126.	P	65	M	N	N	N	N	4,004	1,843	2,026	1,077	CC	GG
127.	P	75	M	Y	N	N	N	5,070	1,596	3,333	0,949	CC	AG
128.	P	79	M	N	N	Y	N	2,730	0,899	1,385	0,897	CA	AG
129.	P	73	F	Y	Y	N	N	7,566	3,539	5,256	0,590	CC	AG
130.	P	25	M	N	N	N	N	4,602	1,573	2,744	1,103	CA	AA
131.	P	73	M	Y	N	N	N	5,070	0,888	3,051	1,538	CA	AA
132.	P	74	M	Y	N	N	N	3,328	1,326	1,795	0,872	CA	AA
133.	P	56	M	Y	Y	N	N	4,602	1,764	2,692	1,051	CC	GG
134.	P	67	M	Y	N	N	N	4,290	1,730	2,282	1,103	CA	AG
135.	P	74	M	Y	N	N	N	4,394	2,258	2,026	1,282	CA	GG
136.	P	64	M	Y	Y	N	N	4,368	1,910	2,385	1,051	CA	AG
137.	P	73	M	Y	Y	N	N	4,628	1,944	2,564	1,103	CC	AA
138.	C	52	F	N	N	N	N	3,822	1,124	1,872	1,385	CC	AG
139.	P	57	M	N	N	Y	N	4,862	1,674	2,974	1,051	CA	AA
140.	P	76	M	N	N	N	N	4,186	1,921	2,410	0,846	CA	AA
141.	P	61	M	N	N	Y	N	4,862	1,393	2,974	1,179	CC	AA
142.	C	77	F	Y	N	N	N	4,212	2,000	2,026	1,205	CA	AG
143.	P	62	F	N	Y	N	N	4,082	1,629	2,333	0,949	CA	AG
144.	P	73	M	N	N	Y	N	7,904	2,978	5,231	1,205	CA	AG
145.	P	63	M	N	N	Y	N	3,458	1,157	1,692	1,179	CC	AA

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
146.	P	52	M	N	N	Y	N	4,108	1,101	2,769	0,795	CC	AG
147.	C	79	M	N	N	N	N	5,252	0,865	3,846	0,949	CC	AA
148.	P	61	F	Y	N	N	N	3,146	1,393	1,590	0,872	CA	AG
149.	C	46	M	Y	N	Y	N	7,904	2,831	5,308	1,205	CA	AG
150.	P	45	M	N	N	Y	N	3,926	3,742	1,308	0,846	CC	AA
151.	P	56	F	N	Y	N	N	7,020	3,191	4,333	1,128	CA	AG
152.	P	67	M	Y	Y	N	N	6,916	2,843	4,385	1,128	CA	AG
153.	P	53	M	Y	N	N	N	7,384	3,393	4,513	1,231	CC	GG
154.	C	38	M	N	N	Y	N	4,628	1,449	2,564	1,333	CC	AA
155.	P	80	M	N	N	N	N	5,720	1,337	3,744	1,282	CA	AG
156.	P	62	M	Y	Y	N	N	5,564	1,236	3,667	1,179	CA	AG
157.	P	83	F	Y	N	N	N	3,640	1,101	1,974	1,103	CA	AG
158.	C	41	F	N	N	N	N	3,900	1,461	2,154	1,026	CC	AG
159.	P	80	M	N	N	N	N	5,278	1,685	3,154	1,282	CC	AA
160.	P	61	M	N	N	Y	N	3,406	1,348	1,923	0,872	CC	AG
161.	P	67	F	N	Y	N	N	4,940	1,517	3,205	0,974	CC	GG
162.	C	48	F	N	N	N	Y	2,600	0,966	1,410	0,718	CA	AG
163.	P	65	F	N	N	Y	N	5,772	1,180	3,821	1,333	CC	GG
164.	P	79	F	Y	Y	N	N	4,264	1,685	2,641	0,795	CA	AG
165.	P	61	M	Y	N	Y	N	4,706	1,202	3,051	1,051	CA	GG
166.	P	36	F	N	N	Y	N	6,396	1,944	3,795	1,615	CA	AG
167.	P	80	M	Y	Y	N	N	7,514	4,236	4,436	1,051	CC	AA
168.	P	61	M	N	Y	Y	N	3,718	1,146	2,154	1,000	CA	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
169.	P	69	M	Y	N	N	N	4,056	0,865	2,692	0,923	CA	AG
170.	P	76	F	Y	Y	N	N	4,108	0,764	2,667	1,026	CA	AG
171.	P	69	F	N	N	N	N	3,198	1,112	1,795	0,846	CC	GG
172.	C	43	F	N	N	Y	N	5,772	1,843	3,513	1,333	CA	AG
173.	P	77	M	Y	Y	Y	N	3,848	0,820	2,615	0,795	CA	AG
174.	P	82	M	N	N	N	N	3,770	1,663	2,051	0,897	CA	AA
175.	C	45	F	N	N	N	N	5,226	2,753	2,590	1,308	CC	GG
176.	C	38	F	N	N	N	N	5,720	1,292	3,564	1,487	CC	AG
177.	P	78	F	Y	N	N	N	4,888	0,697	3,179	1,333	CA	AG
178.	C	64	F	Y	N	N	Y	6,994	1,494	4,769	1,436	CC	AA
179.	P	58	F	N	N	N	N	4,316	1,101	2,410	1,333	CA	AG
180.	C	66	M	N	N	N	N	5,304	0,697	3,462	1,462	CA	AA
181.	P	53	M	N	N	Y	N	3,614	0,371	2,205	1,179	CC	AG
182.	C	42	M	N	N	Y	N	4,628	2,742	2,462	0,846	CC	GG
183.	C	65	M	Y	N	N	N	3,848	1,292	1,949	1,256	CC	GG
184.	C	87	M	Y	Y	N	N	2,704	1,236	0,974	1,128	CC	GG
185.	C	75	F	N	N	Y	Y	4,784	0,910	2,897	1,410	CA	AG
186.	P	54	M	N	N	N	N	4,420	0,663	3,103	0,949	CC	GG
187.	C	77	M	Y	N	N	N	3,614	0,674	2,436	0,821	CC	AA
188.	C	69	F	N	N	N	N	4,628	1,292	2,718	1,256	CA	AG
189.	P	75	F	N	Y	N	N	4,914	0,978	3,026	1,385	CC	AA
190.	P	40	F	N	N	Y	Y	4,992	1,056	3,256	1,179	CA	GG
191.	P	48	M	Y	N	N	N	5,486	0,809	3,769	1,282	CA	AG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
192.	P	80	F	Y	Y	N	N	4,784	0,865	2,872	1,385	CC	GG
193.	P	41	M	N	N	N	N	3,770	1,348	2,256	0,846	CC	AG
194.	P	77	F	Y	Y	N	N	4,992	1,449	3,154	1,103	CA	AG
195.	P	63	F	Y	Y	N	Y	3,926	0,775	2,564	0,949	CC	GG
196.	P	55	M	Y	N	Y	N	7,202	1,831	5,308	0,949	CA	AG
197.	P	79	M	Y	Y	N	N	3,120	0,764	1,564	1,154	CA	AG
198.	P	67	F	Y	Y	N	N	5,122	1,427	2,923	1,487	CC	AA
199.	P	63	M	Y	Y	N	N	3,770	1,764	2,128	0,795	CA	AG
200.	P	75	F	Y	N	N	N	5,928	1,899	3,897	1,077	CC	GG
201.	C	65	M	N	N	N	N	4,134	0,697	2,641	1,128	CA	AA
202.	C	78	M	N	N	N	N	4,108	0,921	2,231	1,410	CA	AA
203.	C	81	M	Y	N	N	N	2,808	0,607	1,231	1,256	CA	AA
204.	C	56	M	N	Y	N	N	4,602	1,371	2,821	1,103	CC	GG
205.	C	64	F	Y	Y	N	N	4,316	2,764	2,205	0,795	CC	GG
206.	C	79	F	Y	N	N	N	4,706	1,236	2,821	1,256	CC	GG
207.	C	67	F	N	N	N	N	7,748	1,146	5,846	1,282	CC	AG
208.	C	76	F	Y	N	N	N	4,316	1,236	2,179	1,513	CC	AG
209.	C	64	M	N	N	Y	N	3,380	1,022	2,205	0,667	CA	AG
210.	C	77	M	N	Y	N	N	8,788	1,775	6,231	1,615	CC	AG
211.	C	75	M	N	N	N	N	4,966	1,674	2,795	1,333	CA	AG
212.	P	54	F	N	N	N	N	5,226	3,382	2,923	0,692	CC	GG
213.	P	61	F	Y	N	N	N	4,680	1,236	3,154	0,897	CC	AG
214.	P	71	M	Y	N	N	N	3,250	0,798	1,872	0,974	CC	AG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
215.	C	68	F	Y	N	N	Y	4,836	1,910	2,487	1,410	CC	AA
216.	P	74	F	Y	N	N	N	5,486	1,854	3,154	1,410	CA	AG
217.	P	59	M	N	N	N	N	3,328	1,427	1,538	0,590	CC	AG
218.	P	62	M	N	N	Y	N	4,004	0,787	2,641	0,949	CC	GG
219.	P	82	F	Y	Y	N	N	4,654	1,180	3,077	0,974	CA	AG
220.	C	71	F	Y	N	N	N	3,874	1,090	2,308	1,026	CC	GG
221.	C	52	F	N	N	N	N	4,004	0,798	2,564	1,026	CC	GG
222.	C	78	M	Y	N	N	N	4,134	1,393	2,487	0,949	CA	AG
223.	P	70	M	Y	N	N	N	5,148	0,854	3,436	1,256	CC	GG
224.	P	69	M	Y	N	N	N	4,420	0,933	2,846	1,077	CA	GG
225.	C	80	M	Y	N	N	N	5,122	0,966	3,231	1,385	CC	AG
226.	P	57	F	N	Y	N	N	5,460	1,730	3,462	1,128	CC	GG
227.	P	80	M	Y	N	N	N	3,536	0,697	2,000	1,179	CC	AA
228.	C	78	F	N	N	N	N	4,082	0,944	2,513	1,051	CA	AG
229.	C	57	F	N	N	N	N	2,444	1,045	0,487	1,436	CA	AG
230.	C	60	F	Y	N	N	N	5,356	2,225	2,872	1,385	CC	AA
231.	C	63	F	Y	N	N	N	6,682	2,764	4,103	1,231	CC	AG
232.	P	74	M	N	Y	N	N	3,380	0,730	1,667	1,333	CC	GG
233.	P	50	M	Y	N	N	N	2,002	0,843	0,615	0,974	CC	AA
234.	C	58	M	Y	N	N	N	3,900	2,247	2,051	0,769	CC	GG
235.	C	54	M	Y	N	N	N	4,316	1,157	2,410	1,308	CA	AA
236.	C	78	M	N	N	N	N	3,692	0,674	2,051	1,282	CC	GG
237.	P	57	M	N	Y	N	N	5,018	1,427	3,564	0,744	CC	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
238.	C	75	M	Y	Y	N	N	4,810	0,798	2,949	1,436	CC	AG
239.	C	77	M	Y	N	N	N	3,068	0,989	1,641	0,923	CC	AG
240.	P	62	M	Y	N	N	N	5,070	2,618	3,051	0,744	CC	AG
241.	P	81	F	Y	Y	N	N	4,186	0,955	2,462	1,231	CC	AA
242.	P	77	F	Y	Y	N	N	3,016	2,101	1,564	0,462	CA	AG
243.	P	54	M	Y	Y	N	N	4,134	1,337	2,846	0,615	CA	AG
244.	P	82	F	Y	Y	N	N	4,342	1,011	2,615	1,205	CA	AG
245.	P	71	F	Y	Y	N	N	4,238	1,933	2,333	0,974	CC	GG
246.	P	80	F	Y	N	N	N	4,290	1,562	2,308	1,205	CC	GG
247.	C	59	F	N	N	N	N	5,538	1,416	3,231	1,590	CC	GG
248.	C	57	F	N	N	N	N	4,368	1,865	2,564	0,897	CA	GG
249.	C	79	F	Y	Y	N	N	4,238	1,652	2,205	1,231	CA	AG
250.	C	52	F	N	N	N	N	6,058	1,281	4,179	1,205	CC	GG
251.	C	79	F	Y	N	N	N	1,534	1,112	0,590	0,410	CA	AG
252.	P	69	M	Y	Y	N	N	5,382	2,506	2,795	1,359	CC	GG
253.	C	54	M	N	Y	N	N	7,332	2,472	4,897	1,205	CC	GG
254.	P	54	F	Y	Y	N	N	5,148	1,202	2,590	1,385	CC	GG
255.	P	70	F	Y	N	N	N	4,706	1,629	2,436	1,462	CC	GG
256.	C	67	F	N	N	N	N	4,160	0,787	2,359	1,333	CA	GG
257.	C	50	F	N	N	N	N	3,406	2,764	1,744	0,359	CA	AG
258.	P	31	F	N	N	N	N	6,396	3,011	3,615	1,308	CC	GG
259.	P	55	F	N	N	N	N	7,098	0,697	4,872	1,821	CA	AG
260.	C	90	F	N	Y	N	N	5,564	1,640	3,410	1,333	CC	AG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
261.	P	71	M	Y	Y	N	N	5,616	2,000	3,744	0,872	CA	GG
262.	C	77	F	Y	N	N	N	8,164	1,944	5,179	1,974	CC	AG
263.	P	83	F	Y	N	N	N	4,186	0,775	2,051	1,718	CC	AA
264.	C	57	M	Y	Y	N	N	4,524	0,551	2,641	1,564	CA	AG
265.	C	68	M	N	N	N	N	3,536	0,584	1,846	1,385	CC	GG
266.	P	74	F	Y	Y	N	N	7,410	3,112	4,436	1,462	CC	GG
267.	P	47	M	Y	Y	N	N	4,550	1,449	2,436	1,385	CA	AG
268.	P	67	M	Y	Y	N	N	5,512	0,978	3,872	1,128	CC	GG
269.	P	69	F	N	N	N	N	6,448	1,652	4,359	1,256	CC	GG
270.	C	60	M	Y	N	N	N	4,004	1,000	2,359	1,128	CA	AG
271.	P	74	M	N	N	Y	N	4,004	1,079	2,718	0,744	CA	AG
272.	P	71	M	Y	Y	Y	N	5,954	2,629	3,615	1,051	CA	AA
273.	P	74	M	Y	N	N	N	3,640	1,371	2,051	0,923	CC	GG
274.	C	44	M	N	N	N	Y	4,446	1,225	2,821	1,000	CC	GG
275.	P	68	F	Y	Y	Y	N	3,042	1,225	1,410	1,026	CC	AA
276.	C	52	F	Y	N	N	N	5,200	1,944	2,846	1,385	CA	AG
277.	P	68	F	Y	N	N	N	5,330	1,034	3,615	1,179	CA	AG
278.	C	58	F	N	N	N	N	4,082	1,674	2,308	0,949	CC	GG
279.	P	57	M	Y	Y	Y	N	6,162	2,843	3,897	0,872	CC	GG
280.	C	79	M	Y	Y	N	N	5,174	1,944	3,308	0,897	CA	AG
281.	C	73	F	N	N	N	N	5,278	1,247	3,615	1,026	CC	AG
282.	P	34	F	N	N	N	N	5,798	1,955	2,641	0,615	CA	AG
283.	P	61	M	Y	N	N	N	4,576	1,483	2,923	0,923	CC	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
284.	P	62	M	N	N	N	N	4,706	2,112	2,692	0,974	CC	GG
285.	P	62	M	Y	N	N	Y	5,694	1,517	3,795	1,128	CC	GG
286.	P	63	F	Y	Y	N	N	6,396	1,843	4,513	0,949	CA	AG
287.	P	68	M	Y	N	Y	Y	4,862	1,056	3,590	0,718	CC	GG
288.	P	71	F	N	N	N	N	5,954	1,101	4,436	0,923	CC	GG
289.	P	70	M	Y	Y	Y	N	5,590	1,011	3,821	1,231	CA	AG
290.	P	69	F	Y	N	N	N	5,356	0,517	3,821	1,231	CA	AG
291.	P	71	M	Y	N	N	N	5,668	1,382	4,077	0,872	CC	AG
292.	P	83	M	Y	N	N	N	3,744	0,921	2,179	1,103	CC	GG
293.	P	79	M	Y	Y	Y	Y	4,368	1,427	3,051	0,615	CC	GG
294.	P	38	M	Y	N	Y	N	6,630	1,685	4,718	1,051	CC	GG
295.	P	71	F	Y	N	N	Y	5,252	1,416	3,205	1,333	CC	GG
296.	P	73	M	Y	N	Y	Y	6,370	1,281	4,538	1,154	CC	GG
297.	P	63	M	N	N	Y	Y	4,472	0,775	2,590	1,462	CA	AG
298.	P	30	M	N	N	N	Y	4,030	1,326	2,487	0,872	CA	GG
299.	P	68	M	Y	N	Y	Y	4,550	1,124	3,026	0,949	CA	AG
300.	P	61	F	Y	N	N	Y	4,940	1,292	2,564	1,641	CC	GG
301.	P	75	M	N	N	N	N	3,640	0,685	2,256	1,026	CC	GG
302.	P	42	M	N	N	N	N	5,980	0,876	4,282	1,205	CC	AG
303.	P	59	F	Y	N	N	Y	6,812	1,820	4,051	1,846	CA	AG
304.	P	83	M	Y	N	N	N	2,392	0,921	1,000	0,949	CC	GG
305.	P	73	M	Y	N	Y	Y	6,188	1,966	3,897	1,308	CA	AG
306.	P	78	M	N	N	N	Y	4,706	1,326	3,103	0,923	CC	AG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
307.	P	71	M	Y	Y	Y	Y	5,720	3,775	3,692	1,000	CC	GG
308.	P	76	F	Y	N	Y	Y	5,122	2,067	3,333	0,769	CC	GG
309.	P	74	F	Y	Y	N	Y	5,616	3,337	3,410	0,615	CC	GG
310.	P	79	M	Y	Y	N	Y	5,070	1,079	3,769	0,744	CA	GG
311.	P	59	M	Y	N	N	Y	4,394	1,281	2,795	0,949	CC	AG
312.	P	70	F	Y	Y	N	Y	5,954	1,798	4,000	1,051	CC	AG
313.	P	52	M	N	N	N	N	5,278	0,685	3,333	1,308	CC	GG
314.	P	77	F	Y	Y	Y	Y	6,786	1,640	4,744	1,205	CA	AG
315.	P	77	M	Y	N	Y	N	3,328	1,607	1,897	1,051	CC	AG
316.	P	79	F	Y	N	N	N	4,550	0,809	3,051	1,077	CA	AG
317.	P	55	M	Y	N	Y	Y	5,824	3,831	3,154	0,846	CA	AG
318.	P	68	M	Y	N	Y	Y	6,344	1,180	4,641	1,077	CA	AG
319.	P	79	F	Y	Y	N	Y	6,422	1,708	4,615	0,949	CA	AG
320.	P	46	M	Y	Y	N	Y	5,018	1,157	3,154	1,256	CC	AG
321.	P	73	M	Y	N	Y	Y	4,368	1,607	2,590	1,000	CC	GG
322.	P	70	M	Y	Y	N	Y	6,604	2,180	4,513	1,000	CC	GG
323.	P	73	F	Y	Y	N	N	3,354	1,079	2,179	0,641	CA	AG
324.	P	79	F	N	N	Y	Y	6,630	1,034	3,974	2,103	CA	GG
325.	P	81	F	N	N	N	N	5,642	2,348	3,538	0,949	CA	AG
326.	P	66	M	Y	Y	Y	N	3,952	4,449	1,308	0,564	CC	GG
327.	P	75	M	Y	N	Y	Y	6,474	2,775	4,000	1,128	CC	GG
328.	P	83	F	Y	Y	N	N	5,122	2,011	3,231	0,897	CA	AG
329.	P	64	M	Y	N	Y	Y	2,808	0,865	1,769	0,615	CA	AG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
330.	P	81	M	Y	N	Y	Y	2,522	0,831	1,462	0,641	CC	GG
331.	P	72	F	Y	N	Y	N	5,070	1,079	3,179	1,385	CA	AG
332.	P	64	F	Y	Y	N	Y	4,550	0,921	3,103	0,974	CC	AG
333.	P	71	M	Y	Y	Y	Y	4,316	1,157	3,026	0,692	CC	AG
334.	P	64	F	Y	N	N	Y	5,954	1,258	4,179	1,128	CC	GG
335.	P	51	M	Y	N	Y	Y	4,524	1,921	2,923	0,667	CA	GG
336.	P	65	M	Y	Y	Y	Y	5,382	3,753	2,487	1,103	CC	GG
337.	P	60	F	N	N	Y	N	5,876	1,202	4,000	1,256	CC	AA
338.	P	53	M	Y	N	Y	Y	5,330	1,820	3,308	1,128	CC	GG
339.	P	75	M	Y	N	Y	Y	4,966	1,112	2,923	0,923	CC	AG
340.	P	70	F	Y	N	N	Y	5,538	0,955	3,385	1,641	CA	AG
341.	P	82	F	Y	N	Y	N	4,966	1,292	3,205	1,103	CC	GG
342.	P	76	F	Y	N	N	Y	5,980	1,955	4,026	0,974	CC	GG
343.	P	32	M	N	N	Y	N	3,796	1,236	2,282	0,897	CC	GG
344.	P	52	F	N	N	N	N	5,642	1,618	3,641	1,179	CA	GG
345.	P	80	F	Y	N	N	Y	5,018	1,292	3,128	1,333	CC	GG
346.	C	55	F	N	N	N	N	4,498	1,157	2,538	1,359	CC	AG
347.	C	52	F	N	N	N	N	4,420	1,371	2,692	1,077	CC	AG
348.	C	51	M	N	N	N	N	3,952	1,180	2,359	1,026	CA	GG
349.	P	61	F	Y	N	Y	N	4,472	1,191	2,513	1,308	CC	AG
350.	C	66	M	N	N	N	N	5,044	0,854	3,282	1,308	CC	AG
351.	C	66	F	N	N	N	N	6,318	1,528	4,154	1,385	CC	GG
352.	C	54	M	N	N	N	N	5,460	1,876	3,256	1,359	CC	GG

Table E.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	Ala140Asp SNP	Asn142Asp SNP
353.	C	55	F	N	N	N	N	6,448	2,764	3,974	1,128	CA	AG
354.	P	71	F	Y	Y	N	Y	7,566	1,472	0,000	0,000	CA	AG
355.	P	66	M	Y	Y	N	N	5,148	1,202	2,974	1,410	CC	GG
356.	P	56	F	N	N	N	N	4,940	1,169	3,282	1,051	CA	AG
357.	P	46	M	N	Y	N	N	4,368	1,315	2,744	0,949	CC	GG
358.	P	51	M	N	N	Y	Y	3,770	1,146	2,179	1,000	CC	GG
359.	C	61	M	N	N	Y	N	6,188	1,494	4,103	1,282	CC	GG
360.	P	72	M	N	N	N	N	6,838	0,719	4,077	2,333	CA	AG
361.	C	74	M	N	N	N	N	6,448	2,011	4,205	1,231	CC	GG
362.	C	52	M	N	N	N	N	5,642	2,011	3,308	0,923	CA	AG
363.	C	64	F	N	N	N	N	6,864	1,483	4,436	1,667	CC	GG
364.	P	81	F	Y	N	N	N	4,420	1,854	2,179	1,538	CC	GG
365.	C	62	F	N	N	N	N	5,590	1,876	3,308	1,359	CC	AG
366.	P	74	F	Y	Y	N	Y	6,214	1,124	4,872	0,744	CC	AA
367.	P	77	M	Y	N	N	N	4,940	1,056	3,333	1,051	CC	GG
368.	P	78	F	Y	N	N	Y	4,368	1,079	2,821	0,974	CA	AG
369.	C	65	M	Y	Y	N	N	5,692	1,011	2,986	1,006	CA	AG