GENETIC POLYMORPHISM OF VITAMIN D3 METABOLISING CYTOCHROME P450 (CYPS) ENYZMES AND RISK OF ISCHEMIC STROKE IN TURKISH POPULATION

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ABSTRACT

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Stroke, as a neurological disorder, is defined as cessation or severe reduction of blood flow to the brain due to a clot or burst of blood vessel in the brain. Atherosclerosis is the hardening of the arteries due to accumulation of plaques in the vessels. Vitamin D deficiency is known as important risk factor in pathogenesis of atherosclerosis, which contributes to stroke development. Incidence of stroke is affected by environmental and genetic risk factors. So, genetic variations including single nucleotide polymorphisms (SNPs) in enzymes involved in Vitamin D metabolism can affect susceptibility to the development of the disease. Therefore, the primary aim of this study was to investigate the association between polymorphisms of Vitamin D metabolizing enzymes (rs927650 SNP in CYP24A1 and rs10741657 SNP in CYP2R1 genes) and the ischemic stroke risk.

The study population was comprised of 256 ischemic stroke patients and 132 control subjects. There was no significant difference between two groups with regards to age and gender. It was found that when compared to control group, number of individuals with hypertension, diabetes, obesity and smoking was significantly higher in the group

of ischemic stroke patients. Also, the average concentration of triglyceride and LDL-cholesterol were higher in patients than controls. On the other hand, HDL-cholesterol level was significantly lower than controls.

The frequency of risky T allele was almost same among ischemic stroke patients and control group for CYP24A1 rs927650 polymorphism. For CYP2R1 rs10741657 polymorphism, the frequency of risky G allele was found as 0.660 in patients and was nearly the same for controls. The study revealed no significant difference for distribution of genotypes for CYP24A1 rs927650 and CYP2R1 rs10741657 polymorphisms in any subgroups when compared between the stroke patients and controls (all P values were higher than 0.05). Detailed stratification analysis for CYP24A1 rs927650 SNP showed that, risk of having ischemic stroke for diabetic individuals was higher in risky T allele carrying individuals (OR=2.395) when compared with wild type genotype group (OR=2.275). In addition, the risk of smoking-related ischemic stroke was higher in risky T allele carriers (OR= 3.727). For CYP2R1 rs10741657 SNP, the risk of hypertensive, diabetic and obese individuals having ischemic stroke was significantly higher in G allele carriers when compared with wild type group (OR= 3.419, OR= 2.804 and OR= 4.817, respectively). Hypertension, smoking, obesity and LDL-cholesterol were found as significant predictors of ischemic stroke in logistic regression analysis; however it was revealed that HDL-cholesterol had protective effect on stroke.

The association between rs927650 of CYP24A1 and rs10741657 of CYP2R1 polymorphisms and ischemic stroke risk in Turkish population was investigated in this study for the first time and it was concluded that there was no significant difference between patient and control groups with regard to C and T allele frequencies in CYP24A1 rs927650 polymorphism and A and G allele frequencies in CYP2R1 rs10741657 polymorphism.

Keywords: CYP24A1, CYP2R1, polymorphism, vitamin D, ischemic stroke, rs927650, rs10741657, SNP, Turkish population

ÖZ

VİTAMİN D3 METABOLİZMASINDA ROL ALAN CYTOCHROME P450 (CYPS) ENZİMLERİNİN GENETİK POLİMORFİZİMLERİ İLE TÜRK POPULASYONUNDA İSKEMİK İNME RİSKİ İLİŞKİSİNİN ARAŞTIRILMASI

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İnme, nörolojik bir hastalık olarak, pıhtı veya beyin içerisindeki damarların patlaması sonucu beyine giden kan akışının durması veya ciddi şekilde azalması olarak tanımlanır. Ateroskleroz damarlarda plak birikiminden dolayı arterlerin sertleşmesidir. Vitamin D eksikliği inmenin oluşmasına katkı sağlayan aterosklerozun patojenezinde önemli bir risk faktörü olarak bilinmektedir. Toplumda inme görülme sıklığı çevresel ve genetik risk faktörlerinden etkilenir. Bu yüzden Vitamin D metabolizmasına katılan enzimlerdeki tek nükleotid polimorfizmleri (SNP) de dahil olmak üzere genetik varyasyonlar bu hastalığa yatkınlığı etkileyebilir. Bu nedenle bu çalışmanın birincil amacı, Vitamin D'yi metabolize eden enzimlerdeki polimorfizmler (sırasıyla CYP24A1 ve CYP2R1 genlerindeki rs927650 ve rs10741657 polimorfizmler) ile iskemik inme ilişkisini incelemektir.

Çalışma popülasyonu 256 iskemik inmeli hastadan ve 132 kontrolden oluşmuştur. Gruplar arasında yaş ve cinsiyet açısından istatiksel olarak anlamlı bir farklılık gözlemlenmemiştir. Kontrol grubu ile karşılaştırıldığında, iskemik inmeli hastalarda

yüksek tansiyonlu, diyabetli, obez ve sigara içen kişi sayısı anlamlı olarak yüksek bulunmuştur. Ayrıca, ortalama trigliserid ve LDL-kolesterol konsantrasyonları da hastalarda kontrol grubundan daha yüksektir. Bununla birlikte HLD-kolesterol kontrol grubundan anlamlı bir şekilde daha düşük görülmüştür.

CYP24A1 rs927650 polimorfizmi için, T alel frekansı iskemik inmeli hastalarda ve kontrol grubunda neredeyse aynıydı. CYP2R1 rs10741657 polimorfizmi için, hastalarda G alel frekansı 0.660 olarak bulunmuştur ve kontrol grubu için de bu değer neredeyse aynıydı. Bu çalışma, inmeli hastalar ve kontrol grubu arasında herhangi bir alt grup içerisinde CYP24A1 rs927650 ve CYP2R1 rs10741657 polimorfizmlerinin genotip dağılımlarında anlamlı bir fark olmadığını göstermiştir (bütün P değerleri 0.05'ten küçüktür). CYP24A1 rs927650 tek nükleotid polimorfizmi için yapılan ayrıntılı analizlerde, iskemik inme riskinin, yabanıl tip genotipli diyabetli bireylere göre (eşitsizlik oranı=2.275), T aleli taşıyan diyabetikli bireylerde (eşitsizlik oranı=2.395) yüksek olduğu belirlenmiştir. Ayrıca mutant ''T'' aleli taşıyan ve sigara kullanan bireylerde iskemik inme riskinin yüksek olduğu görülmüştür (eşitsizlik oranı=3.727). CYP2R1 rs10741657 polimorfizmine bakıldığında, G aleli taşıyan hipertansiyonlu, diyabetik ve obez kişilerde iskemik inme riskinin daha yüksek olduğu görülmüştür (sırasıyla eşitsizlik oranı=3.419, eşitsizlik oranı=2.804 ve eşitsizlik oranı=4.817).

Lojistik regresyon analizinde hipertansiyon, sigara kullanımı, obezite ve kötü huylu kolesterolün inme için önemli tahmin unsurları olduğu, diğer taraftan iyi huylu kolesterolün inme için koruyucu bir etkiye sahip olduğu görülmüştür.

rs927650 CYP24A1 ve rs10741657 CYP2R1 polimorfizimleri ile Türk popülasyonundaki iskemik inme riski arasındaki ilişki bu çalışma ile ilk kez incelenmiştir. Hasta ve kontrol grupları arasında, rs927650 CYP24A1 polimorfiziminde C ve T alel frekanslarına göre ve rs10741657 CYP2R1 polimorfizminde A ve G alel frekanslarına göre anlamlı bir fark olmadığı sonucuna varılmıştır.

Anahtar kelimeler: CYP24A1, CYP2R1, polimorfizm, vitamin D, iskemik inme, rs927650, rs10741657, SNP, Türk popülasyonu

Dedicated to my beloved family,

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LIST OF ABBREVIATIONS

CI	Confidence interval
CT	Computed tomography
CAD	Coronary artery disease
CYP2R1	Vitamin D 25-hydroxylase
CYP24A1	25-hydroxyvitamin D-24-hydroxylase
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
GST	Glutathione S-transferases
HDL	High density lipoprotein
LDL	Low density lipoprotein
NO	Nitric oxide
OR	Odds ratio
OS	Oxidative stress
PCR	Polymerase chain reaction
PON1	Human paraoxonase 1
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
T1D	Type 1 diabetes

CHAPTER 1

INTRODUCTION

1.1. Stroke

Stroke, serious and life-threatening neurological medical condition, also known as brain attack, occurs when the blood flow to an area of the brain is interrupted or reduced which is generally caused by a clot or blood vessel that bursts or leaks. When blood flow is cut off, supply of oxygen and nutrients to brain cells is blocked. Brain cells begin to die after just a few minutes without nutrient or oxygen supply.

A stroke may be caused by a blocked artery (ischemic stroke) or the leaking or bursting of a blood vessel (hemorrhagic stroke). Some people may experience only a temporary disruption of blood flow to their brain known as transient ischemic attack, or TIA.

Effect of stroke varies in terms of proportion of the affected area and location of the damaged part of the brain. There are some main clinical indicators related with effect of the stroke. These are headache with vomiting or altered consciousness, presence of weakness or numbness of face, arm or leg especially on one side of the body, loss of coordination and having difficulty in walking, seeing also confusion accompanied by slurred speech and understanding.

According to the federal statistics, stroke is the fifth-leading cause of death. When compared to the past, ranking of the stroke has dropped from fourth-leading cause of death. This difference has been formed through measures taken against and treatment efforts for stroke (http://www.stroke.org/).

Stroke kills almost 130,000 of the 800,000 Americans who die from cardiovascular disease each year (http://www.cdc.gov). Also, it is a common cause of most of adult disability. Living 1300 million people are maimed in varying degrees due to stroke (Wolf et al., 1993; Murray et al.,1997). This ratio puts the disease to most disabling and causing addiction category (Bonita et al., 1992; Hankey et al., 1999).

1.1.1. Classification of Stroke

There are two main pathologic types of stroke including ischemic and hemorrhagic. Ischemic stroke which is caused due to occlusion in artery by clot is responsible for nearly 85% of deaths and hemorrhagic stroke due to bleeding in the brain accounts 15% of deaths (Hickey et al., 2011).

1.1.1.1. Ischemic Stroke

Occlusion in a blood vessel supplying blood to the brain results in ischemic stroke. Forming of fatty deposits covering the vessel walls is the primary reason for this type of obstruction. This condition is called atherosclerosis. Two types of obstruction can be formed by these fatty deposits (Figure 1.1).

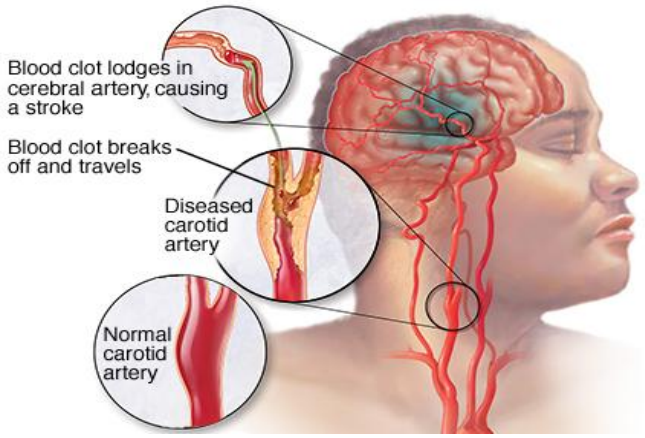

Figure 1.1 Ischemic Stroke (image taken from http://www.mayoclinic.org).

The most common types of ischemic stroke include cerebral thrombosis and cerebral embolism. Cerebral thrombosis occurs when blood clot develops in one of the arteries that supplies blood to the brain. On the other hand, cerebral embolism occurs generally at another location in the circulatory system, away from brain; usually the heart and large arteries of the upper chest and neck. Irregular heartbeat is the second cause of embolism, known as atrial fibrillation. Similar to an ischemic stroke, transient ischemic attack (TIA) often referred to as mini-stroke, warning stroke, or transient stroke occurs when a clot or debris blocks blood flow to part of your brain. But in TIA, blockage is temporary and does not have chronic symptoms (http://www.medscape.com).

1.1.1.2. Hemorrhagic Stroke

If there is leak or rupture of blood vessel in brain, hemorrhage stroke can result (Figure 1.2). Many conditions trigger this effect in blood vessels, including hypertension, overtreatment with anticoagulants and weak spots in blood vessel walls; named as aneurysms. There are two main sub-types of hemorrhagic stroke including intracerebral hemorrhage and subarachnoid hemorrhage. In an intracerebral hemorrhage, a blood

vessel in the brain bursts and spills into the surrounding brain tissue, damaging brain cells. Also brain cells beyond the leak are deprived of blood and also damaged. In a subarachnoid hemorrhage, an artery on or near the surface of your brain bursts and pours out area between the surface of brain and skull (http://www.strokeassociation.org and http://emedicine.medscape.com).

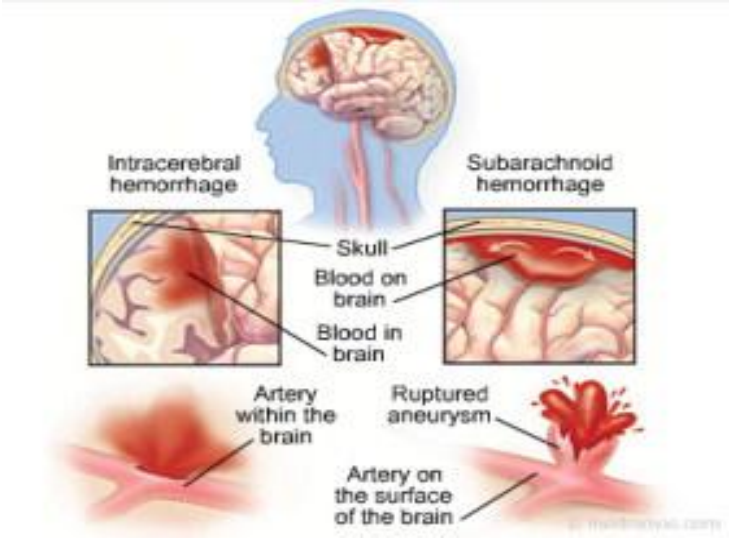

Figure 1.2 Hemorrhagic Stroke (image taken from http://ctrnd.med.ufl.edu).

1.1.2. Risk Factor of Stroke

The risk factors of stroke can be categorized into two groups. Non-modifiable risk factors cannot be controlled by outside whereas modifiable risk factor is the ones that can be controlled.

1.1.2.1. Non-Modifiable Risk Factors

Age, heredity, gender, race and TIA are considered as non-modifiable risk factors for stroke. These factors cannot be modified so the risk for stroke cannot be decreased by changing life style or treatment.

Age is the most important risk factor for stroke. After age 55, risk of stroke doubles in both men and women for each 10 years (Wolf et al., 1992). Risk of stroke is strongly related with gender. Stroke incidence rate in men is higher 1.25 times than women (Sacco et al., 1997). Families with history of stroke also have higher risk for stroke. It is due to genetic predisposition for stroke and other stroke risk factors, and a common familial exposure to environmental or lifestyle risks. Stroke risk factors are also effected by race-ethnicity (Sacco et al., 2001). When compared to whites, the incidence of stroke is higher in blacks and Hispanics (White et al., 2005). Asians, especially Chinese and Japanese, have high incidence rates for stroke (Sacco et al., 1997). Moreover, transient ischemic attack (TIA) was reported as important risk factor for ischemic stroke (Harmsen et al., 2006).

1.1.2.2. Modifiable Risk Factors

It is important to decrease the stroke incidence rate and mortality. 50% of stroke is avoidable through by control of modifiable risk factors and lifestyle changes (Di Legge et al., 2012) because these factors can be controlled, treated or modified to prevent stroke.

Hypertension is one of the most important, independent modifiable risk factor for ischemic stroke (Rosamond et al., 2008). When compared to persons with a systolic blood pressure of less than 140 mm Hg, individuals having systolic hypertension above 180 mm Hg are under three times higher risk of ischemic stroke (Ellekjaer et al., 1992)

also 10 mm Hg reduction in systolic or a 5 mm Hg reduction in diastolic blood pressure would provide 40% reduction in stroke death (Lewington et al., 2002).

Diabetes mellitus is the other independent, important risk factor for stroke. Diabetic people are also under risk of hypertension (Bell et al., 1994). Diabetic patients have a worse prognosis, with a two-fold increase in the likelihood of subsequent strokes (Hankey et al., 1998).

Risk for heart disease and atherosclerosis also increases with hyperlipidemia (high LDL-cholesterol and TG level in the blood). Some studies have also showed that cholesterol-lowering drugs decrease the risk of stroke (American Academy of Neurology, 2007). Besides, the American Heart Association states that abstaining from the foods containing high amount of cholesterol reduces risk of stroke.

The studies have showed that cardiac diseases increase the incidence of stroke. Abnormal heart rhythm named as atrial fibrillation causes non-effective blood flow to the body, which can lead to formation of clot and blood pool. When the clot detaches and enters in the bloodstream, it settles to the main artery in brain, causes the stroke. As a major risk factor, its prevalence increases with advancing age (Marini et al., 2005). Also coronary heart disease, heart failure, dilated cardiomyopathy, heart valve disease and some types of congenital heart defects increase the risk of stroke (http://www.strokeassociation.org).

The smoking is also considered as a risk factor for stroke. The cohort studies revealed that a dose-response relationship between the amount of cigarettes smoked per day and the relative risk of stroke (Higa et a., 1991) and the other study has also showed that the smoking induces about fifty percent increase in risk of stroke (Shinton et al., 1989). Hypertension, diabetes and hyperlipidemia are common risk factors for obesity. Obesity and poor physical activity are risk factors for stroke. The relation between obesity and increased risk of cardiovascular events, disability and mortality was revealed (Flegal et al., 2005). Also, the risk of ischemic stroke increases with obesity (Suk et al., 2003). On the other hand, there is inverse association between physical activity and stroke risk.

Physical Activity Guidelines Advisory Committee Report has been estimated that physical activity provides 25–30% risk reduction for stroke (Physical Activity Guidelines Advisory Committee, 2008).

Diet have an influence on stroke by effecting insulin resistance, inflammation, thrombosis, endothelial function, and oxidation (Lakkur et al., 2015). So, consuming food sources containing antioxidant, vitamins, potassium, calcium, vegetables, fruits, and whole grains intake decreases the risk of stroke (Foroughi et al., 2013).

1.1.2.3. Uncertain Risk Factor

Alcohol and drug abuse, socioeconomic factors and geographic location are uncertain risk factors of stroke due to not enough documentation.

1.1.3. Atherosclerosis

Atherosclerosis is known as hardening of the arteries, in which arteries are become narrowed and hardened due to accumulation of plaque inside the arteries. Cholesterol, fatty substances, cellular waste products, calcium and fibrin constitute the formation of the plaques. Atherosclerosis is the common cause of cardiovascular diseases including stroke (http://www.heart.org). Interaction between different factors and cell types, including cells of the immune system such as T cells, B cells, natural killer cells, monocytes/macrophages, dendritic cells and cells of the vessel wall (endothelial cells, vascular smooth muscle cells) take part in the development process of atherosclerosis (Kassi et al., 2013). The endothelial dysfunction, progress in inflammation and vulnerable plaque formation take place in stage of atherosclerosis respectively (Szmitko et al., 2003).

In the previous studies carried out in our laboratory, human paraoxonase 1 (PON1) 192RR genotype were found to be significant determinant of ischemic stroke by contributing the development of atherosclerosis. PON1, as an antiatherogenic enzyme, prevents HDL and LDL oxidation, which is the early step in the development of atherosclerosis. The study showed that ischemic stroke patients having 192RR genotype had decreased PON1 activity so increased plaque formation in arteries (Can Demirdöğen et al., 2008). Besides increased oxidative stress has been linked to endothelial dysfunction in atherosclerosis. Glutathione S-transferases (GSTs) prevent oxidative stress by detoxifying the oxygen species. Concerning that, they studied association of the polymorphisms in Glutathione S-transferases (GSTs) and GST activity with ischemic stroke. They revealed that GSTT1 and GSTM1 null genotypes, together with hypertension, may increase the incidence of ischemic stroke risk (Türkanoğlu et al., 2010).

Vitamin D deficiency is appearing as risk factor for stroke among the other risk factors like hypertension, smoking, hyperlipidemia, obesity, age, and gender. Vitamin D takes part in bone and calcium metabolism. Apart from these well-documented roles, vitamin D has been identified as an important factor in cardiovascular health (Kassi et al., 2013).

Vitamin D has a direct role in systemic inflammatory process (Danik et al., 2012; Wallis et al., 2008). Endothelial cells (EC) have vitamin D receptors and express 1α-hydroxylase to produce calcitriol 1α-25$(OH)_2D_3$ (Merke et al., 1989). Besides, Vitamin D takes part in an inflammatory process by applying protective effects on endothelial activation and dysfunction. Decrease in nitric oxide (NO) and increase in oxidative stress are the main contributor to endothelial disfunctioning (Vanhoutte et al., 1997). It has been revealed that Vitamin D stimulates NO synthesis (Molinari et al., 2011; Queen et al., 2006) also reduces oxidative stress in endothelium by suppressing the superoxide synthesis (Hirata et al., 2013). Vascular tone is also regulated by vitamin D by modulating discharge of endothelium derived contracting factors (Wallis et al., 2008) and vitamin D prevents the proliferation of vascular smooth muscle cells by inhibiting release of epidermal growth factor and endothelin (Carthy et al., 1989; Chen et al.,

2010). The study of Riek et al., 2013 also revealed that vitamin D decreases the foam-cell formation by effect on macrophages. Moreover, vitamin D has an effect on regulation of coagulation process through upregulating the expression of the anticoagulant and down regulating the expression of a critical coagulation factors in monocytes (Koyama et al., 1998).

1.1.4. Prevention of Stroke

By focusing on main risk factors including hypertension, diabetes mellitus, hyperlipidemia, cardiac diseases, obesity, physical activity, and diet, %80 of stroke can be precluded (http://www.strokeassociation.org). Clinical trials have showed that the antihypertensive treatment is an effective prevention strategy for stroke. According to trials involving 50000 patients, it can be concluded that systematic treatment of hypertension provided a 38% reduction in all stroke and a 40% reduction in fatal stroke (MacMahon et al., 1994). Atrial fibrillation (AF) is the important treatable cardiac precursor of stroke. So, anticoagulants such as warfarin, antiplatelet agents such as aspirin may be used for the treatment for preventing of clot formation. According to the American College of Physicians ("Guidelines for medical treatment for stroke prevention. American College of Physicians.," 1994), to avoid stroke in AF, the current studies recommend the usage of warfarin to patients who are candidate anticoagulation and also to administer aspirin for young subjects at low risk of stroke or for patients who is unsuitable for warfarin treatment. The study of the Framingham and the Nurses' Health, cessation of smoking reduce stroke risk immediately (Wolf et al., 1988; Kawachi et al., 1993). Also, moderate consumption of alcohol may reduce cardiovascular disease, including stroke. A pooled analysis of four pravastatin trials revealed that 46% reduction in risk of stroke with usage of pravastatin for treatment of high cholesterol (Byington et al., 1995). Early diagnosis and therapy of hypertension provides to prevention of stroke in patients with diabetes (Bell et al., 1994).

Physical activity decreases blood pressure, weight, pulse rate, level of LDL-cholesterol and platelet agreeability; also increases HDL-cholesterol and insulin sensitivity and improves glucose tolerance so it has good impact in terms of decreasing risk factors for atherosclerosis (Sacco et al., 1997). Also consuming of fish, green tea, and milk has a protective effect on stroke on the other hand consuming foods including high amount of fat and cholesterol could be harmful (Abbott et al., 1996).

1.2. Properties and Functions of Vitamin D

Vitamin D is one of the oldest steroid hormones with the identification of the hydroxylated metabolites of vitamin D, including the principal circulatory form 25-OH-D_3 and the hormonal form, 1α -25-$(OH)_2D_3$ (Blunt et al., 1968). There are two different forms of vitamin D. Plants and some fish contain vitamin D_2, known as ergocalciferol and vitamin D_3, known as cholecalciferol is produced in the skin by the help of sunlight. So, sources of vitamin D can be being exposed to ultraviolet-B light or plant or animal originated dietary intake. But vitamin D is biologically inactive and needs two hydroxylations to be metabolically active (Holick et al., 2007).

Vitamin D, obtained from diet or synthesized from skin by way of ultraviolet-B light is metabolized to 25-hydroxyvitamin D in the liver. This form is still inactive form of vitamin D. Hydroxylation of vitamin D from C-25 in the liver is carried out by one or more cytochrome P450 vitamin D 25 hydroxylases including CYP2R1, CYP27A1, CYP2D11 and CYP2D25. After that, 25-hydroxyvitamin is hydroxylated in kidney at the position of carbon 1 by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) to form hormonally active form of vitamin D. The hydroxylation is performed by CYP27B1 predominantly in kidney. In addition to kidney, extra renal sites, including osteoclasts, skin, colon, brain, and macrophages present the enzyme. This situation may provide wide -ranging effects of vitamin D (Brannon et al., 2008).

Inactivation of vitamin D is carried out by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1). So, concentrations of 1α-25-$(OH)_2D_3$ and 25-OH-D_3 regulate expression of CYP24A1 gene closely. CYP24A1 limits the amount of 1α-25-$(OH)_2D_3$ in target tissues by converting 1α-25-$(OH)_2D_3$ to 1-24-25$(OH)_3D_3$ (calcitroic acid) or 24-25$(OH)_2D_3$ which causes the decrease in the pool of 25-OH-D_3 available for 1α hydroxylation (Christakos et al., 2012) (Figure 1.3).

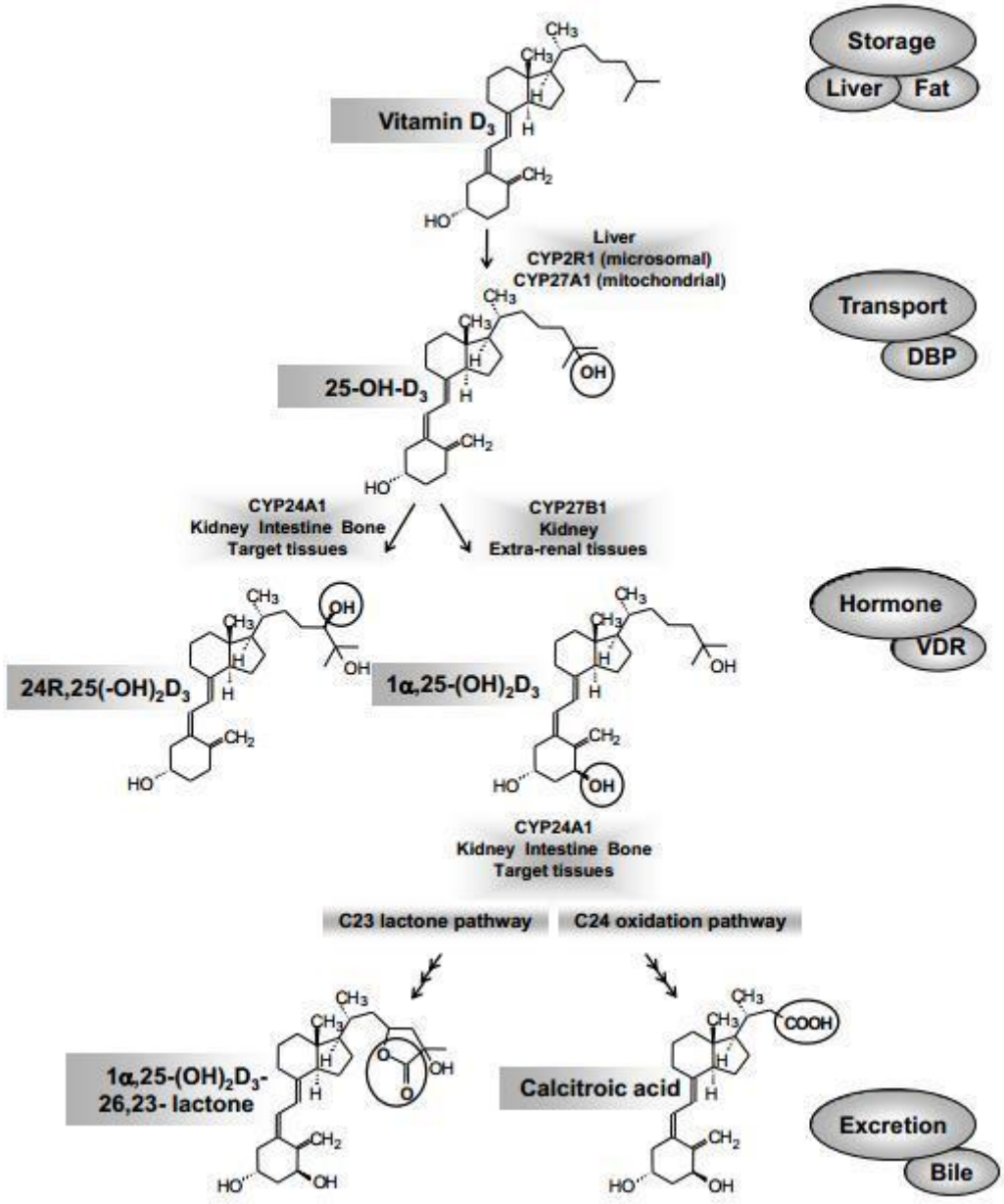

Figure 1.3 The metabolic pathway for vitamin D (image modified from "Cytochrome P450-mediated metabolism of vitamin D – April 2013/ Vitamin D Wiki," n.d.).

Vitamin D takes part in regulation of the renin-angiotensin-aldosterone system, regulating blood pressure and parathyroid hormone release required for calcium homeostasis. Vitamin D is inversely associated with plasma renin activity and parathyroid hormone. So, vitamin D deficiency may increase incidence of hypertension, inflammation and so increased cardiovascular risk (Lee et al., 2008) (Figure 1.4).

Rickets and osteomalacia are well-known disorders due to vitamin D deficiency in children and adults, respectively. In addition to calcium regulation, it has been shown that vitamin D has an important role in cell differentiation, inhibition of cell proliferation and modulation of the immune system. According to the recent epidemiologic studies, increased risk for cardiovascular diseases, cancer and autoimmune diseases such as type 1 diabetes has been observed due to low concentration of vitamin D.

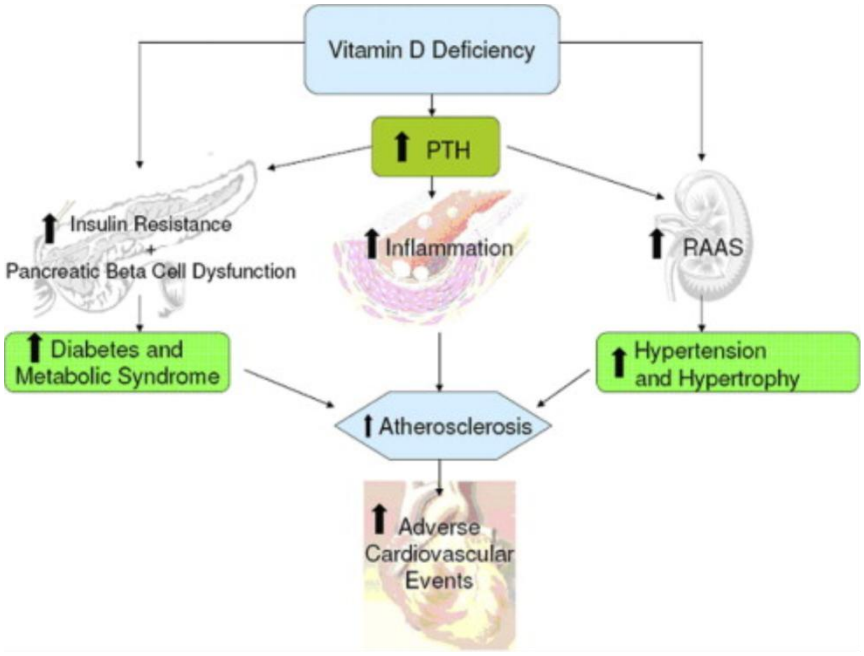

Figure 1.4 Possible mechanisms of increased cardiovascular (CV) risk from vitamin D deficiency (image taken from Lee et al., 2008).

1.3. Cytochrome P450s

The cytochrome P450 enzymes contain a large superfamily of enzymes. Actually, all organisms from bacteria to humans and plants can have them. When they bind the carbon monoxide, they give absorption peak at 450 nm. The name comes from this feature (Munro et al., 2007; Werck et al., 2000)

The cytochrome P450 enzymes are monooxgenases and generally, introduce oxygen usually in the form of a hydroxyl group to the substrate. On the other hand, in addition to hydroxylation, the cytochrome P450 enzymes are able to perform the other reactions such as dealkylation, carbon-carbon bond cleavage, dehydrogenation and epoxidation (Munro et al., 2007).

1.3.1. CYP24A1

CYP24A1 is a mitochondrial enzyme which is present in kidney, may function to balance systemic 25-OH-D_3 and 1α-25-$(OH)_2D_3$ levels, the other cells resides out of kidney which may act in conjunction with CYP27B1 to ‘‘fine-tune’’ target tissue exposure to 1α-25-$(OH)_2D_3$ hormone (Jones et al., 1987). CYP24A1 is capable of catalyzing multiple hydroxylation reactions at carbons C-24 and C-23 of the side chain of both 25-OH-D_3 and its hormonal form, 1 α-25-$(OH)_2D_3$ (Prosser et al., 2004).

Primary role of CYP24A1 whose expression is activated by negative feedback by 1α-25-$(OH)_2D_3$ is to limit or reduce the action of 1α,25-$(OH)_2D_3$ on target cells (Lohnes et al., 1992). Moreover, analysis of the CYP24A1 was showed that the vitamin D response element exists in the upstream promoter, which intervene this induction at the transcriptional level (Ohyama et al., 1993). It can be concluded the excess vitamin D response pathway activation is prevented by this way.

1.3.2. CYP2R1

Vitamin D needs to be hydroxylated at 1α and 25 carbons to be hormonally active by cytochrome P450 enzymes in the kidney and liver, respectively. CYP27A1 and CYP2R1 are the two most viable candidates for the vitamin D 25-hydroxylases (Cheng et al., 2003).

CYP2R1, a hepatic, microsomal enzyme, catalyzes the hydroxylation of carbon 25. The product of this process is 25-hydroxyvitamin D_3 (25-OH-D_3), which is the primary circulatory form of the vitamin. The mitochondrial enzyme CYP27B1 catalyzes 1a-hydroxylation in the kidney. CYP2R1 alone or other unidentified enzyme performs this hydroxylation. According to the studies, there was no change in vitamin D metabolism though mutations existed in the human and mouse genes encoding the mitochondrial CYP27A1. However, these mutations caused the deterioration of bile acid synthesis (Skrede et al., 1986; Cali et al., 1991; Rosen et al., 1998; Repa et al., 2000).

Also, in the study of Cheng et al., 2013, low circulating levels of 25-OH-D_3 and classic symptoms of vitamin D deficiency was found in a patient with homozygous for a transition mutation which substituted leucine into proline in conserved region of exon 2 of the CYP2R1 gene on chromosome 11p15.2 (Cheng et al., 2003).

1.4. Genetic Polymorphisms

Genetic polymorphisms in which multiple allele exists as stable components of the population are variance in the genome among population. They are natural variations in a gene and refer to inherited differences between individuals (Dawkins et al., 2013).

Polymorphism provides genetic diversity in population. Characteristics of height and hair color may be influenced by these variants. So, some variants affect phenotype but others do not. Also, susceptibility of diseases and drug metabolism may also be affected

by them. Depending on the nature of polymorphism, the biological function of the organism may be affected or not.

1.4.1. Single Nucleotide Polymorphism

When alleles are compared, change in a single nucleotide is called a single nucleotide polymorphism ("Genes IX," 2007). The least common allele must have a frequency of 1 percent or more in the population, to be classified as a single nucleotide polymorphism. If the frequency is lower than this, the allele is regarded as a mutation.

The vast majority of SNPs are located in the non-coding regions and are believed not to be under natural selection (Shastry et al., 2002). However at least 5% of the human genome are under stabilizing selection (Waterston et al., 2002). Intergenic regions and transcription factor binding sites are the regulatory parts of the genome. So, in addition to functional changes caused by variations on the protein coding sequence, alteration, deletion, or destruction in regulatory sites can cause disturbance the use of its target genes or increase or decrease in gene expression (Knight et al., 2003). So polymorphisms which present in the in regulatory sites of a gene may have an influence on expression of the target gene by changing the binding affinity for a particular transcription factor, leads to recognition by a completely different transcription factor, or destroy the binding site all together (Fried et al., n.d.).

1.4.2. Polymorphism of CYP24A1 and CYP2R1

Some studies have investigated association between the different polymorphism in CYP24A1 and vitamin D levels of patients with type 1 diabetes and some cancer types. But there is no study, which has investigated direct relation of CYP24A1 rs927650 polymorphism on stroke. The current study is the first time to explore this association. In the study of Mauf et al., 2015, association between the lower level of vitamin D and incidence of type 1 diabetes (T1D) was examined. T1D is a chronic autoimmune disease

which is mainly carried out by immunogenic dendritic cells (Zhou et al., 1995). It was suggested increased plasma levels of 25-OH-D_3 may inhibit formation of dendritic cell (DC) and increase immunomodulatory cells so decrease incidence of T1D. So, 25-OH-D_3 shows immunomodulatory effects on a cellular level in patients with T1D. For CYP24A1 rs927650 polymorphism, when compared to TT genotyped individuals, individuals with CC genotype showed a significantly higher increase of immunomodulatory cells (Mauf et al., 2015).

The study of Rose et al., 2013 is also in accord with the previous study. Individuals with TT genotype in CYP24A1 rs927650 SNP had an increased frequency of mature dendritic cells, which increase the risk of T1D. Besides, the maturation of DCs is disrupted in the presence of 1α-25$(OH)_2D_3$ (Van Etten et al., 2004) and also 1α-25$(OH)_2D_3$ treatment has been shown to induce apoptosis of mature DCs (Van Halteren et al., 2004).

Moreover, a potential interaction between CYP24A1 rs927650 polymorphism and plasma level of 25-OH-D_3 was revealed in individuals with breast cancer. The risk was increased among the carriers of T allele due to low concentration of plasma 25-OH-D_3. Because CYP24A1 which degrades 1α-25$(OH)_2D_3$ may repeal the growth control of CYP24A gene intervened by 1α-25$(OH)_2D_3$. So certain CYP24A1 polymorphisms potentially increases breast cancer risk among women (Reimers et al., 2015).

For CYP2R1, two different studies showed the association of the G variant of the rs10741657 SNP with type 1 diabetes (Hussein et al., 2012; Ramos-Lopez et al., 2007). Moreover, the study of Ramos-Lopez et al., 2007 demonstrated that individuals having G allele had lower level of 25-OH-D_3 concentration than AA genotyped individuals.

Also, the other studies have revealed the association of the CYP2R1 rs10741657 SNP with incidence of pancreatic cancer (Anderson et al., 2013) as well as hepatitis C virus-related hepatocellular carcinoma (Lange et al., 2013). The association of the CYP2R1 rs10741657 SNP with coronary artery disease incidence was found in individuals having

the G variant in their genotypes. Also, individuals with AA genotype had higher concentration of 25-OH-D_3 than AG and GG genotyped individuals (Hassanein et al., 2014).

1.5. Aim of the Study

Ischemic stroke is an interruption of blood circulation in brain due to blockage or rupture in the vessels feeding the brain. Brain cells are damaged when brain is not able to get nutrients and oxygen supply and effect of this damage depends on primarily which area in the brain injured and how long brain cells exposure. Age, heredity, gender, race, high cholesterol, smoking, diabetes mellitus are important risk factors for stroke incidence. Evolving datas show the role of Vitamin D in coronary artery diseases including atherosclerosis. Vitamin D is involved in regulation of inflammatory process, endothelial disfunctioning, vascular tone, coagulation process and proliferation, which are quite related to atherosclerosis. So vitamin D deficiency may have an effect on development of stroke.

CYP24A1 and CYP2R1 are important cytochrome P450 enzymes, located in the kidney and liver, respectively get included in vitamin D metabolism. CYP2R1 takes place in the biologically activation process of Vitamin D on the other hand CYP24A1 carries out the degradation of vitamin D. Genetic variation in the genes, which express CYP24A1 and CYP2R1 enzymes can change the enzyme activity in organism. So concentration of vitamin D can vary according to the effect of these variations on the gene.

In the present study, rs927650 polymorphism in CYP24A1 gene and rs10741657 polymorphism in CYP2R1gene were investigated for the risk of stroke. Because the enzymes take part in metabolism of vitamin D, the alteration in enzyme function may cause formation a disease or increasing the severity of a disease. According to study of Reimers et al., 2015, concentration of 25-OH-D_3 was decreased with individuals having T variant compared to carriers of common allele for rs927650 SNP in CYP24A1 gene.

The variation in CYP24A1 gene caused decrease in growth control of 1α-25$(OH)_2D_3$ and potentially increased breast cancer risk. Also the study of Penna-Martinez et al., 2012 reported that individuals with papillary thyroid carcinoma had lower level of circulating 1α-25$(OH)_2D_3$ with T variant in rs927650 polymorphisms. When considered in this way, this SNP may increase the enzyme activity that cause an increase in degradation of vitamin D and induces vitamin D deficiency. So, CYP24A1 rs927650 SNP can be a potential risk factor for stroke.

For CYP2R1 rs10741657 polymorphism, the study of Zhang et al., 2012 showed that rs10741657 was significantly associated with plasma 25-OH-D_3 levels. Ramos-Lopez et al., 2007 also reported that individuals carrying G variant in their genome had significantly lower levels of 25-OH-D_3 when compared to those with AA genotype. Additionally, in the study of Nissen et al., 2014, it was revealed that the carriers of risky allele had significantly lower serum 25-OH-D_3 concentrations. Therefore, it can be suggested that due to decreasing Vitamin D level, G variant may have close relationship with ischemic stroke.

The aim of this study was to investigate association of CYP24A1 rs927650 and CYP2R1 rs10741657 SNPs with ischemic stroke risk in Turkish population and to determine whether these polymorphisms are associated with ischemic stroke. The steps given below are followed to success this aim.

- Gathering total blood samples from ischemic stroke patients and healthy controls,
- Isolation of genomic DNA in intact form from blood samples,
- Amplification of desired regions in CYP24A1 gene and CYP2R1 gene by PCR method,
- Performing restriction endonuclease digestion to amplified regions to determine the genotype of each individual
- Determination of genotype and allele frequencies for CYP24A1 and CYP2R1 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 and conventional risk factors for stroke.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Population and Blood Sampling

Blood samples were collected by the Gülhane Military Medical Academy Hospital Neurology Department, Ankara, from October 2005 to April 2011. The blood samples were obtained from 256 adult with the diagnosis of acute hemispheric ischemic stroke and 132 symptom-free adults as controls. All the participants in control and patient groups were Caucasian people and they were from the same geographic region, central Anatolia, Turkey. Informed consent forms were obtained from all participants before procedures being applied. A copy of the informed consent is provided in Appendix A. All the study procedures were conducted in compliance with Decleration of Helsinki. Also, the Ethical Committee of the Gülhane Military Medical Academy approved the research study and the approval is available in Appendix B.

The patients that are included in the study group were selected according to the inclusion criteria. Inclusion criteria that should be met were,

- Having anterior circulation stroke outcome of carotid artery atherosclerotic disease,
- Not having familial background for major illnesses, including autoimmune diseases, neoplasms, coagulopathies, hepatic or renal failure, hematological, autoimmune or chronic inflammatory diseases,

- No known embolism (aortic arch, cardiac or carotid),
- Not having heart attack within 3 weeks or transient ischemic attack or stroke at any time.

Neurological examination and brain computer tomography (CT) scan were performed for initial diagnosis of the cerebral infarction, then transthoracic echocardiographic examination and Holter study and Transcranial Doppler emboli detection procedures were applied for identifying the presence of emboli source.

Subjects of the control group were selected at random from neurology policlinics. These subjects did not experience stroke or transient ischemic attack throughout life. All exclusion criteria applied for patient group were also considered for controls in addition to not having carotid stenosis > 50% or ulcerated carotid plaque.

Bilateral carotid Doppler Ultrasound (CUSG) and Transthoracic Echocardiographic were carried out for all subjects. All participants were asked by colleagues from Gülhane Medical Faculty Neurology Department to get their history of conventional vascular risk factors and conditions in detail. Hypertension was described as a systolic blood pressure > 140 mm Hg or as 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 as measuring body mass greater than or equal to 30. Smoking status of the participants stated as ‘’yes ‘’ ıf the subject was a current smoker or stopped smoking less than three months ago.

Routine laboratory tests, including electrocardiogram, chest X-ray, complete blood count, leukocyte differential, erythrocyte sedimentation rate, routine biochemistry tests including fasting glucose, lipid profile (triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, VLDL-cholesterolL-C), creatinine, sodium, potassium, bilirubin, and liver function tests, routine urine tests and rheumatologic screening tests were performed for all participants. All laboratory measurements were done blinded to clinical characteristics.

4-5 mL of blood samples from ischemic stroke patients and controls were taken in EDTA-containing vacuumed tubes and stored at -20°C till use for DNA isolation. Blood samples were kept in 4°C while they were in active use.

2.1.2. Chemicals and Enzymes

To perform the experimental procedures, some chemicals were required. Agarose (A-9539), bromophenol blue (B-5525), ethidium bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), sodium chloride (NaCI; S-3014), sodium dodecyl sulfate (SDS; L-4390), 2-amino-2 (hydroxymethyl)-1,3-propandiol (Tris; T-1503) were products of Sigma Chemical Company, Saint Louis, Missouri, USA.

Borate (11607) and absolute ethanol (32221) were products of Riedel de Haën, Seelze, Germany.

Triton X-100 (11869) was the product of Merck & Co., Inc., Whitehouse Station, NJ, USA. Potassium chloride (A2939) and Magnesium chloride (A4425) were purchased from AppliChem, Ottoweg, Darmstadt. Sucrose (7653), Taq DNA Polymerase (supplied together with $MgCl_2$ and amplification buffer) (#EP0407), dNTP mix (#R0191), Gene RulerTM 50 bp DNA Ladder (#SM0371) and restriction enzymes BglII (#ER0081, supplied with their buffers) and MnII (#ER1071, supplied with their buffers) were ordered from Fermentas, USA. Purity of the chemicals, which were purchased from commercial source and used in the study were highest and they were of molecular grade.

2.1.3. Primers

Primers used throughout the study were selected by literature search and were derived from known sequences of human. The primer pairs were purchased from Iontek (Iontek, Istanbul, Turkey). Primer stocks were brought to 100 pmol/µL concentration and stored

at -20°C. Aliquots of 10 pmol/µL concentration were prepared and used for PCR. The sequences of forward and reverse primers used for analysis of rs927650 polymorphism of CYP24A1 gene and rs10741657 polymorphism of CYP2R1 are given in Table 2.1.

Table 2.1 The sequences of forward and reverse primers used for analysis of rs927650 polymorphism in CYP24A1 and rs10741657 polymorphism in CYP2R1.

Gene and SNP Name	Forward and Reverse Primer Sequences	Reference
CYP24A1, rs927650	5'-GGGAAGAGCAATGACATGGA-3' 5'-GCCCTGGAAGACTCATTTTG-3'	Ramos-Lopez et al., 2007
CYP2R1, rs10741657	5'-TGGTTGCATAACACA AACCTA-3' 5'-CTGAAAGCCAGTAACAATGGT-3'	Martinez et al., 2012

2.2. Methods

Buffers that are used for isolation of DNA from whole samples included Tris-HCl buffer containing EDTA, pH 8.0, TKME buffer saturated with NaCl, pH 7.6 and TE buffer, pH 8.0.TBE buffer, pH 8.3 was used in agarose gel electrophoresis of PCR and restriction digestion products. Preparation of reagents and chemicals used for DNA isolation from human whole blood samples, PCR, restriction endonuclease digestion and agarose gel electrophoresis were described in Appendix C.

2.2.1. Preparation of Human Genomic DNA Sample for PCR

2.2.1.1. Isolation of Genomic DNA from Human Whole Blood Samples

Lahiri and Schnabel, 1993 (Lahiri et al., 1993) method with slight modification was carried out for isolation of DNA from whole blood samples. In this method, 750 µL of whole blood samples collected EDTA-containing vacuumed tubes to eliminate blood clotting was transferred into eppendorf tube and was treated with the same volume of low-salt TKM Buffer, pH 7.6 to lyse the cells. 20 mL of Triton X-100 was added inside the tubes and mixed by inversions to disintegrate the cells. These suspensions were centrifuged at 1000 g for 10 minutes at room temperature by using Sigma 1-15 benchtop microfuge (Sigma Postfach 1713-D-37507, Osterode). Two distinct layers were formed after centrifugation. Upper part named as supernatant was discarded. Bottom layer named as pellet included the genomic DNA. The pellets was washed three more times with 750 µL TKM Buffer and the final pellet was resuspended in 200 µL of TKM Buffer by tapping.10 µL of 10% SDS (Sodium Dodecyl Sulfate) was added to suspensions and whole suspensions was mixed thoroughly and the mixtures were incubated at 58°C for 10 minutes.

After that, 75 µL of cold saturated NaCl (~ 6 M) was added to the suspensions and the tubes were mixed well. The centrifugation process was performed again at 14000 g at 4°C for 10 minutes and supernatant fraction, which contained the DNA, was taken to a separate eppendorf tube. Two-time volume ice-cold absolute ethanol was added to the supernatant to precipitate DNA. The tubes were stored at -20°C for almost one hour and then centrifugation at 10000 g for 10 minutes at 4°C was carried out to settle down DNA to pellet. After centrifugation, supernatant was discarded and the pellet was air-dried. DNA containing pellet was solubilized with 100 µL of TE Buffer, pH 8.0. Then 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

Shimadzu UV160-A double-beam spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) was used to determine the concentration of DNA in the sample. Measurement was performed at 260 nm and 280 nm absorbance values and in quartz cuvettes. Because the nucleic acids give maximum absorbance at 260 nm; this wavelength was used to calculate the concentration of DNA in the sample. Based on the knowledge that an optical density of 1.0 corresponded to approximately 50 µg/mL for double-stranded DNA, the concentration of DNA was calculated using the formula given below:

Concentration (µg/mL) = A260nm x 50 (µg/mL) x Dilution Factor

2.2.1.3. Qualification of Genomic DNA Samples by Spectrophotometry

Purity assessment of the nucleic acid was estimated the ratio between OD values at 260 nm and 280 nm (OD260/OD280 ratio). Pure DNA preparations give the ratio of 1.8 while the higher and lower values means that there is either RNA or protein contamination.

2.2.1.4. Qualification of Genomic DNA Samples by Agarose Gel Electrophoresis

0.5% agarose gel electrophoresis using Biogen horizontal gel electrophoresis system was performed to assess the intactness of DNA samples. This system forms 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 C.

First step of 0.5% agarose gel preparation was adding 0.5X TBE buffer pH8.3 to 0.15 g agarose. Then the mixture was heated in microwave oven untill all of the grains of the agarose dissolved. Meanwhile the mold, plastic tray and comb were cleaned with 70%

ethanol before the pouring the gel. The solution was cooled to approximately 60°C with the help of magnetic stirrer, which provided continuous stirring for homogenous cooling. Ethidium bromide was added from a stock solution of 10 mg/mL in water to a final concentration of 0.5µg/mL when cooled enough and the solution was mixed thoroughly. The warm agarose solution was then poured into the mold and any air bubbles were removed with the help of the pipette tip. The gel was allowed to solidify completely for approximately 20-40 minutes at room temperature.

After the agarose gel was solidified, it was taken to the electrophoresis tank, which was also filled with 0.5X TBE buffer. The slots of the gel were mounted facing the negative pole-cathode. Before loading DNA samples, 5 µL DNA was mixed with 1 µL of gel loading dye by sucking in and out of a micropipette. The mixture was slowly loaded to the wells of the gel. After loading, the lid of the tank was closed and the electrical leads were attached to the power supply. The power supply was set to a constant voltage so that not more than a voltage of 5V/cm (measured as the distance between the electrodes) was applied (corresponds to a maximum of 150 volts for Scie-Plas HU13W horizontal gel electrophoresis unit). The gel was run approximately 40 minutes; until the bromophenol blue reached to the bottom of the gel. Gel imagining was performed under UV light and the photograph of the gel was taken by Vilber Lourmat Gel Imaging System (Marne La Vallee, Cedex, France) and InfinityCapt (version 12.9) computer software. Single band in agarose gel electrophoresis shows pure DNA preparations; on the other hand two bands mean RNA contamination. A smear shows that the DNA is degraded.

2.2.2. Genotyping of rs927650 Single Nucleotide Polymorphisms in CYP24A1 Gene and rs10741657 Polymorphism in CYP2R1

Genetic polymorphisms of CYP 24A1 gene (rs927650) and CYP2R1 gene (rs10741657) were investigated in this study. PCR-RFLP (restriction fragment length polymorphism)

analysis was performed for detection of the polymorphisms. Firstly, the desired region of gene was copied by PCR and the procedure was followed by RFLP (Table 2.2). Techne Progene (Cambridge, UK) and Eppendorf Mastercycler (Hamburg, Germany) thermocyclers carried out PCR and DB-120 Heat block (Biosan Ltd., Latvia) was used to incubate samples during digestion. In the following sections, the details of the procedures are given.

Table 2.2 Genes including SNPs, Polymorphism, regions of amplification, PCR product size, restriction endonucleases and fragment sizes for CYP241 and CYP2R1 genes.

Gene	**Polymorphism**	**Region of Amplification**	**PCR Product Size**	**Restriction Endonuclease**	**Fragments Size (bp)**
CYP24A1	(C→T)	Intron Region	311 bp	Bg1II	CC:311 CT:311-212-99 TT:212-99
CYP2R1	(A→G)	5'flanking region	287 bp	MnII	AA:248-39 AG:248-150-98-39 GG: 150-98-39

2.2.2.1. Genotyping of rs927650 Single Nucleotide Polymorphisms on CYP24A1 Gene

2.2.2.1.1 Polymerase Chain Reaction for rs927650 SNP

The reagents for PCR protocol of rs927650 single nucleotide polymorphism found in intronic region of CYP24A1 are (details were given in Appendix C),

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

Optimization for reaction conditions and components of PCR was done to obtain single specific product for desired SNP regions. In this part, three concentrations of $MgCl_2$ (2 nm, 3 nm, 4 nm) and three concentrations of primer (30 pmole, 40 pmole and 50 pmole) were tested to obtain best concentration. The concentrations of compounds used for optimized PCR mixture were given in Table 2.3.

Table 2.3 The components of PCR mixture for rs927650 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	0.2 mM
Forward Primer	10 pmole/µL	3 µL	30 pmole
Reverse Primer	10 pmole/µL	3 µL	30 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 that was used for amplification of rs927650 SNP was given in Table 2.4.

Table 2.4 The thermal cycling program to amplify rs927650 SNP.

	Temperature	Time	
Initial Denaturation	94 °C	2 min	
Denaturation	94 °C	30 sc	35 cycles
Annealing	60 °C	30 sc	
Extension	72 °C	1 min	
Final Extension	72 °C	3 min	

The analysis of amplified PCR products was done on 2% agarose gel. Before loading the samples, 10 µL PCR product was mixed by 3 µL of gel loading dye and then the wells of the gel were filled with these mixtures. 6 µL of DNA ladder was loaded to one well to determine the size of the PCR products. The gel was run for 45 min at 120 V until the bromophenol blue reached to the bottom of the gel, analysis of the gel was done through by UV light and the gel photograph was taken.

2.2.2.1.2 Restriction Endonuclease Digestion of CYP24A1 rs927650 Polymorphism

Restriction fragment length polymorphism technique (RFLP) was used to determine genotypes of rs927650 polymorphism in CYP24A1 gene. PCR products of rs927650 were digested by Bg1II, which recognizes the individuals carrying T allele. So, if base

substitution occurs (C>T), the enzyme recognizes the cut site and DNA is divided into the fragments. If the fragments are composed of 212 bp and 99 bp, this indicates homozygote polymorphic subjects, whereas 311 bp, 212 bp, 99 bp fragments show heterozygote individuals for this SNP. Single band of 311 bp means homozygote wild type individuals (Figure 2.1).

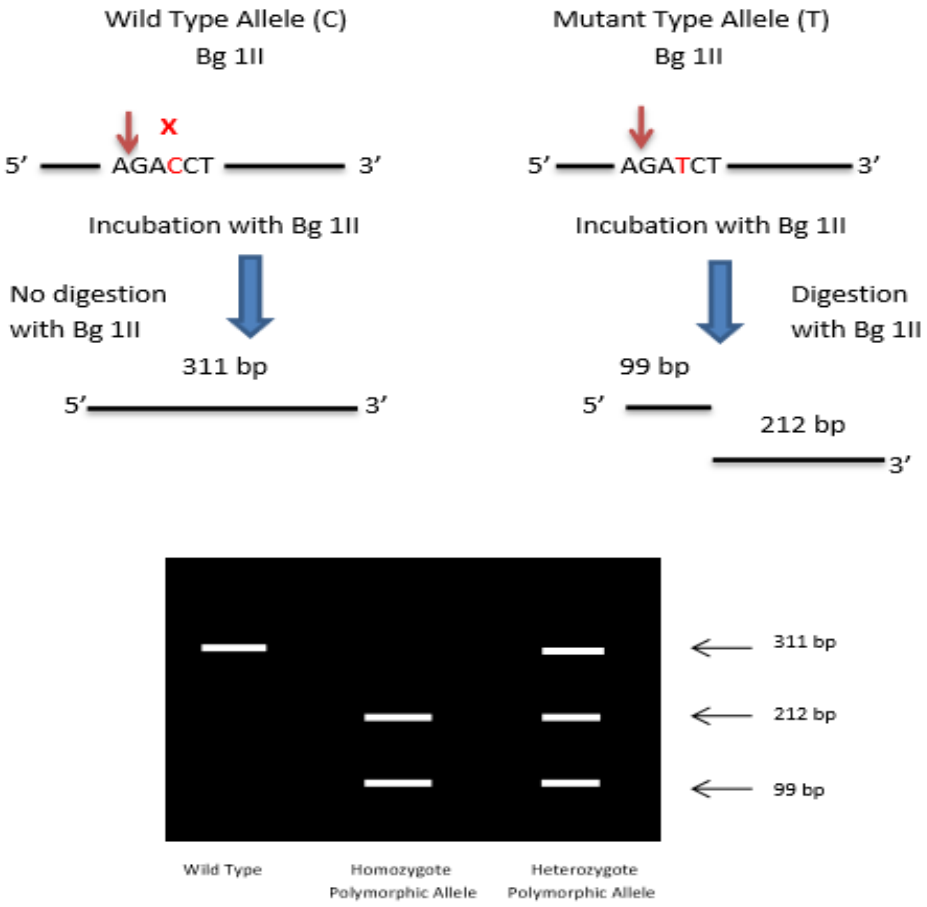

Figure 2.1 Schematic presentation of the determination of genotypes of CYP24A1 rs927650 single nucleotide polymorphism.

By testing different concentrations of restriction enzyme, optimized condition was obtained. The components of restriction enzyme digestion mixture are given in Table 2.5. The reagents used for determination of genotypes of CYP24A1 rs927650 single nucleotide polymorphism were given in Appendix C.

Table 2.5 Restriction endonuclease digestion mixture constituents for rs927650 polymorphism.

Constituent	**Stock Concentration**	**Volume to be Added**	**Final Concentration in 30µL Reaction Mixture**
PCR Product		10µL	
Buffer 0	10X	3µL	1X
Bg1II	10U/µL	0.2µL	2U
Sterile apyrogen H_2O		16.8µL	

10 µL of PCR product was incubated in 2U Bg1II restriction enzyme at 37°C for 18 to 24 hours. 6 µL loading dye was added to digestion product after incubation then the mixture was loaded to well of 3% agarose gel to examine the length of digestion products. 6 µL of 50 bp ladder was also loaded to one well to determine the length of fragments. The gel was run for 1 hour at 120 V until the bromophenol blue reached to the bottom of the gel.

Amplified region of CYP24A1 gene including rs927650 single nucleotide polymorphism was illustrated in Figure 2.2 with highlighted forward and reverse primers, recognition site of endonuclease and single nucleotide substitution. 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 AGATCT, the enzyme can cut. In mutant type, the enzyme cuts between ‘A’ and ‘G’. In the wild type, 102nd allele, highlighted as yellow, is ‘C’ and the enzyme could not cut from this allele (The nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

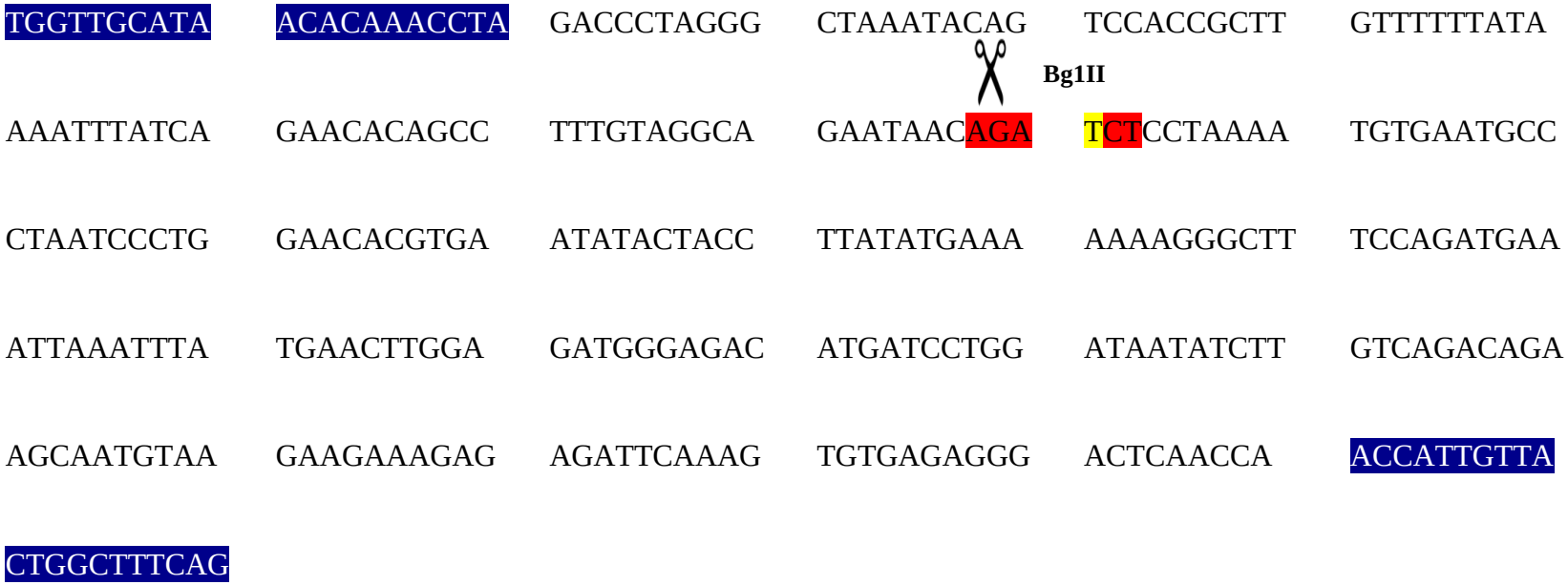

Figure 2.2 Schematic representation of the CYP24A1 gene that includes rs927650 single nucleotide polymorphism. The blue highlighted sequences are the forward and reverse primers used for PCR procedure. The red region is enzyme recognition site; when the sequence is AGATCT, the enzyme can cut. In mutant type, the enzyme cuts between 'A' and 'G'. In the wild type, 102nd allele, highlighted as yellow, is 'C' and the enzyme could not cut from this allele (The nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

2.2.2.2. Genotyping of rs10741657 Single Nucleotide Polymorphism on CYP2R1 Gene

2.2.2.2.1 Polymerase Chain Reaction for rs10741657 SNP

The reagents that were involved in PCR protocol of rs10741657 single nucleotide polymorphism found in 5' flanking region of CYP2R1 are (details were given in Appendix C),"

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

Optimization for reaction conditions and components of PCR was done to obtain single specific product for desired SNP regions. In this part, three concentrations of $MgCl_2$ (2 nm, 3 nm, 4 nm) and three concentrations of primer (30 pmole, 40 pmole and 50 pmole) were tested to obtain best concentration. The concentrations of compounds used for optimized PCR mixture were given in Table 2.6.

Table 2.6 The components of PCR mixture for CYP2R1 rs107416570 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	0.2 mM
Forward Primer	10 pmole/µL	3 µL	30 pmole
Reverse Primer	10 pmole/µL	3 µL	30 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 CYP2R1 rs10741657 SNP was given in Table 2.7.

Table 2.7 The thermal cycling conditions to amplify CYP2R1 rs 10741657 SNP.

	Temperature	**Time**	
Initial Denaturation	94 °C	2 min	
Denaturation	94 °C	30 sc	35 cycles
Annealing	60 °C	30 sc	
Extension	72 °C	1 min	
Final Extension	72 °C	3 min	

The analysis of amplified PCR products was done on 2% agarose gel. Before loading the samples, 10 µL PCR product was mixed by 3 µL of gel loading dye and then the wells of the gel were filled with this mixture. 6 µL of DNA ladder was loaded to one well to determine the size of the PCR products. The gel was run for 45 min at 120 V until the bromophenol blue reached to the bottom of the gel, analysis of the gel was done through by UV light and gel photograph was taken.

2.2.2.2.2 Restriction Endonuclease Digestion of CYP2R1 rs10741657 Polymorphism

Restriction fragment length polymorphism technique (RFLP) was used to determine genotypes of rs10741657 polymorphism in CYP2R1 gene. PCR products of rs10741657

were digested by MnII, which recognizes the individuals with G variant. So, if base substitution occurs (A>G), the enzyme recognizes the cut site and DNA is divided into the fragments. If fragments composed of 150 bp, 98 bp and 39 bp, this indicates homogeneous mutant subjects, whereas 248 bp, 150 bp, 98 bp and 39 bp fragments show heterozygote individuals for this SNP. 248 bp and 39 bp fragments mean wild type allele containing individuals (Figure 2.3).

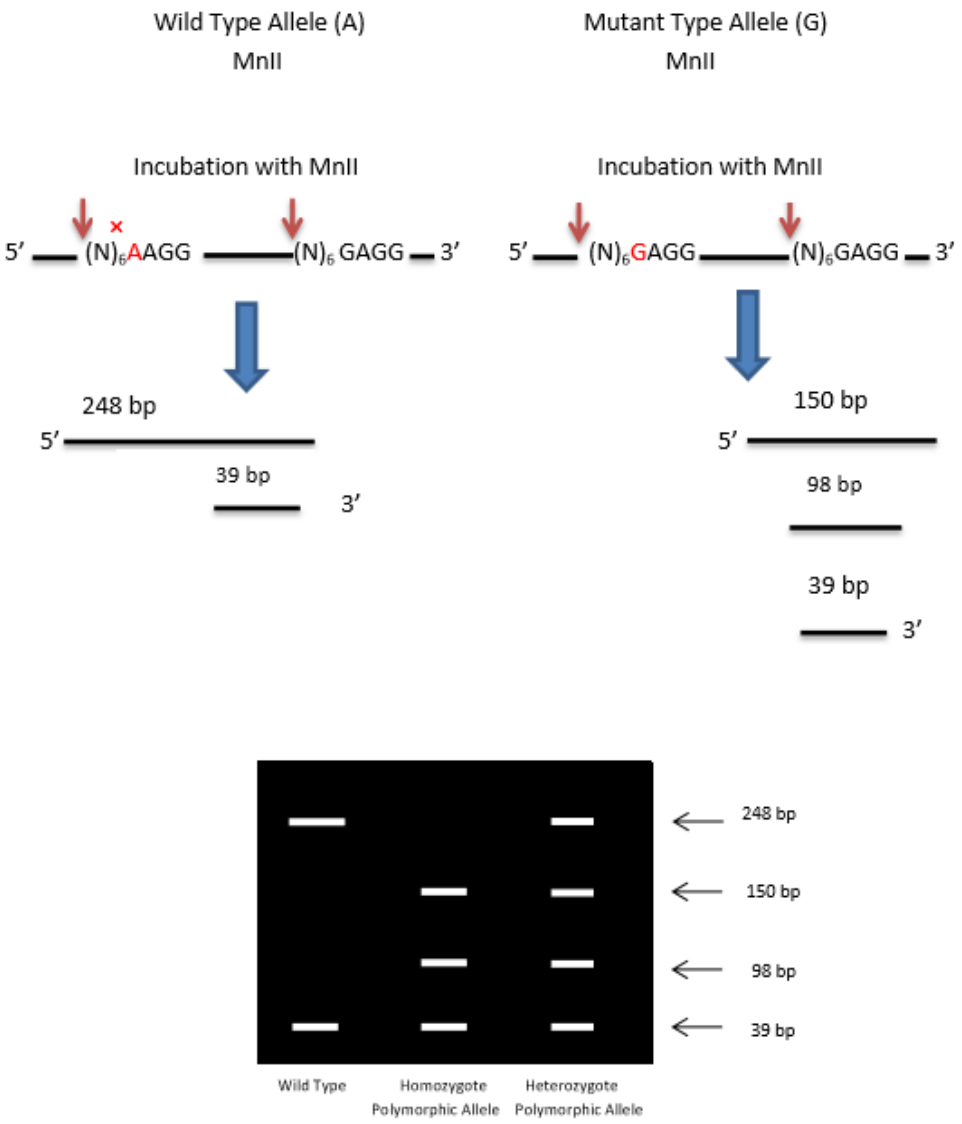

Figure 2.3 Schematic presentation of the determination of genotypes of CYP2R1 rs10741657 single nucleotide polymorphism.

By testing different concentrations of restriction enzyme, optimized condition was obtained. The components of restriction enzyme digestion mixture are given in Table 2.8. The reagents used for determination of genotypes of CYP2R1 rs10741657 single nucleotide polymorphism were given in Appendix C.

Table 2.8 Restriction endonuclease digestion mixture constituents for rs10741657 polymorphism.

Constituent	Stock Concentration	Volume to be Added	Final Concentration in 30µL Reaction Mixture
PCR Product		10µL	
Buffer 0	10X	3µL	1X
MnII	10U/µL	0.2µL	2U
Sterile apyrogen H_2O		16.8µL	

10 µL of PCR product was incubated in 2U MnII restriction enzyme at 37°C for 18 to 24 hours.6 µL loading dye was added to digestion product after incubation then the mixture was loaded to well of 3% agarose gel to examine the length of digestion products. 6 µL of 50 bp ladder was also loaded to one well to determine the length of fragments. The gel was run for 1 hour at 120 V until the bromophenol blue reached to the bottom of the gel.

Amplified region of CYP2R1 gene including rs10741657 single nucleotide polymorphism was illustrated in Figure 2.4 with highlighted forward and reverse

primers, recognition site of endonuclease and single nucleotide substitution. The blue highlighted sequences are the forward and reverse primers used for PCR procedure. If A/G allele change occurs, two sites will be formed to be recognized by the enzyme. The red regions are enzyme recognition site; when the sequence is GGAG, the enzyme can cut. In polymorphic allele, the enzyme cuts from 'GGAG$(N)_6$'. In the wild type, 105th allele, highlighted as yellow, is 'A' and the enzyme could not cut from as a second site (The nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

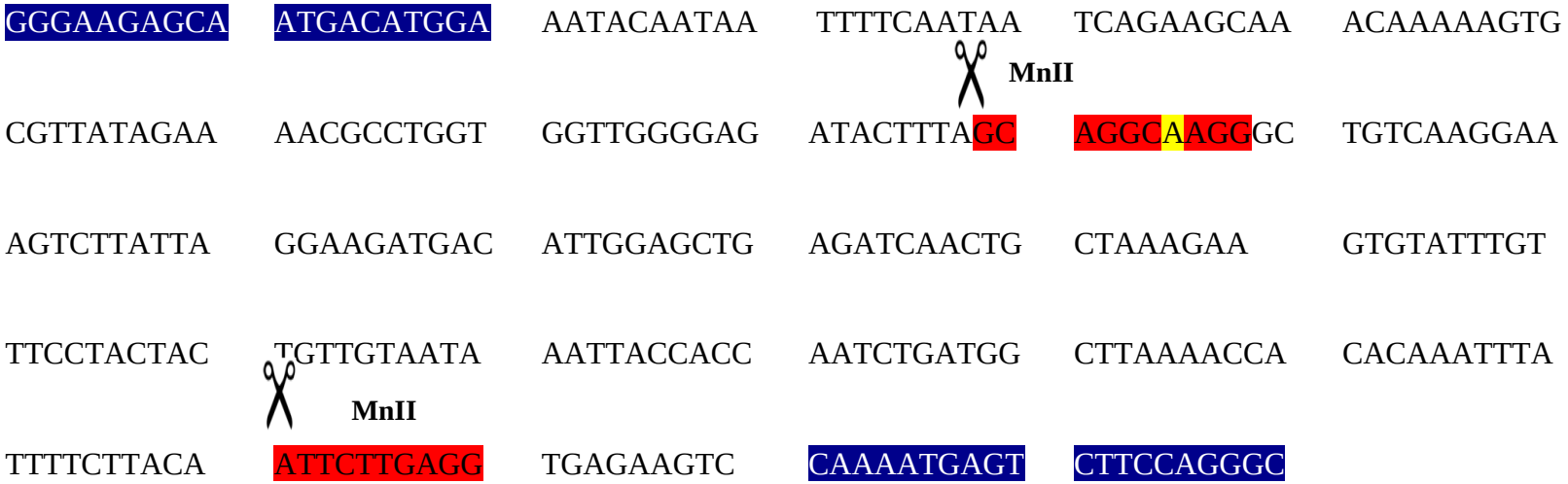

Figure 2.4 Schematic representation of the CYP2R1 gene that includes rs10741657 single nucleotide polymorphism. The blue highlighted sequences are the forward and reverse primers used for PCR procedure. If A/G allele change occurs, two sites will be formed to be recognized by the enzyme. The red regions are enzyme recognition site; when the sequence is GGAG, the enzyme can cut. In polymorphic allele, the enzyme cuts from 'GGAG$(N)_6$'. In the wild type, 105th allele, highlighted as yellow, is 'A' and the enzyme could not cut from as a second site (The nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

2.2.3. Statistical Analysis

SPSS 20.0 (developed by SPSS, Chicago, IL, USA) was applied to perform statistical analysis of the study. Kolmogorov-Smirnov test was carried out to investigate the normality of sample distribution of each continuous variable and continuous variables were stated as mean ± SD. Moreover, Independent Samples t-test or Mann-Whitney U test was used to determine differences of continuous variables depending on the shape of distribution curves. Expression of categorical variables was indicated in proportions and comparison was carried out by applying Chi-square test ($\chi 2$).

Determination of the effects of vascular risk factors, lipid parameters, CYP24A1 and CYP2R1 genetic polymorphisms in the prediction of ischemic stroke, logistic regression analysis with backward selection was applied. 2-tailed probability values with 95% confidence intervals were estimated for each odds ratio. Calibration of the test was carried out by using The Hosmer-Lemeshow goodness of fit test.

Evaluation of the results was done according to the P-values in terms of whether results were significant or insignificant. If P value was less than 0.05, the result was evaluated as statistically significant or vice versa.

CHAPTER 3

RESULTS

3.1. Study Population

256 ischemic stroke patients and 132 control subjects without any symptoms of ischemic stroke constituted the study population. PCR-RFLP method was used for determination of the polymorphisms in CYP24A1 and CYP2R1 genes and then calculation of the genotypes and allele frequencies were performed. Measurement of serum total cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol levels were carried out by GATA Biochemistry laboratory. Also, GATA Neurology Department recorded the traditional risk factors such as hypertension, diabetes, obesity and cigarette usage for all ischemic stroke patients and these are represented in Appendix D. The results of clinical laboratory tests and demographic features and also some risk factors of acute ischemic stroke patients and control subjects are given in Table 3.1.

The age of study population varied between 37 to 90 for control group and 20 to 83 for patients. The means of ages were 65.57±13.40 for patients and 64.42±12.19 for controls. Since age is a risk factor for stroke, we should eliminate the significant difference of ages between patients and controls (P=0.096). There were 112 female and 144 male among stroke patients. Number of males was found to be higher in patient group (56.3%) when compared to control group (48.5%; P=0.163).

The prevalence of conventional risk factors for stroke such as hypertension, diabetes, cigarette usage and obesity were higher in patients than control group (Table 3.2). Also,

these risk factors were found significant risk factors for ischemic stroke (P values are lower than 0.05).

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

Parameter	**Patients (n=256)**	**Control (n=132)**	***P***
Age (years)[a]	65.57±13.40	64.42±12.19	0.096
Male, n (%)[b]	144 (56.3)	64 (48.5)	0.163
Hypertension, n (%)[b]	173 (67.6)	51 (38.6)	0.000
Diabetes mellitus, n(%)[b]	88 (34.4)	24 (18.2)	0.001
Smokers, n (%)[b]	70 (27.3)	16 (12.1)	0.000
Obesity, n (%)[b]	60 (23.4)	8 (6.1)	0.000
Total cholesterol (mmol/L)[c]	4.87±1.27	4.72±1.26	0.268
Triglycerides (mmol/L)[c]	1.55 ±0.74	1.47±0.66	0.277
HDL-cholesterol (mmol/L)[c]	1.08±0.28	1.20±0.31	0.000
LDL-cholesterol (mmol/L)[c]	2.97±1.06	2.74±1.01	0.041

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

[a]Mann Whitney U test is applied

[b]Chi-square test is applied

[c]Independent Samples T-test is applied

Table 3.2 The effects of conventional risk factors on ischemic stroke.

Parameter	**OR (%95 CI)**	***P***
Male, n (%)[a]	1.366 (0.896-2.082)	0.163
Hypertension, n (%)[a]	3.310 (2.138-5.126)	0.000
Diabetes mellitus, n (%)[a]	2.357 (1.413-3.933)	0.001
Smokers, n (%)[a]	2.728 (1.512-4.925)	0.001
Obesity, n (%)[a]	4.745 (2.194-10.260)	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 CYP24A1 and CYP2R1 Genes

Genotype frequencies of CYP24A1 and CYP2R1 were determined by carrying out PCR-RFLP method. In this procedure, firstly desired region of DNA was amplified by PCR, which was followed by RFLP, then the products were visualized by agarose gel electrophoresis.

3.2.1. Genotyping for rs927650 Single Nucleotide Polymorphism of CYP24A1 gene

Determination of rs927650 single nucleotide polymorphism of CYP24A1 gene was achieved by PCR-RFLP method. The polymerase chain reaction was followed by restriction enzyme digestion. The specific enzyme for this procedure is Bg1II.

3.2.1.1. Polymerase Chain Reaction Results for CYP24A1 rs927650 Single Nucleotide Polymorphism

CYP24A1 rs927650 SNP, a transition mutation, is found in intronic region (intron 11) of DNA and converts C allele to T allele (http://www.humgenomics.com and http://www.thermofisher.com). This region was amplified by allele specific PCR method. The PCR was optimized to avoid non-specific bands and to obtain single band, which included rs927650 polymorphism. The optimized PCR mixture was given in section 2.2.2.1.1. Approximately 200 ng DNA, 0.2mM dNTPs, 30 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 which was prepared by adding 4 g agarose to 200 mL of 0.5 x TBE buffer which is described in Appendix C. 10 µL of ethidium bromide was added to final agarose gel mixture. For loading PCR samples to wells of agarose gel, 10 µL of each PCR product was mixed with 3µL of gel loading dye. 6 µL ladder (50-1000 bp) was applied to one of the wells. The gel was run for 1 hour at 100 V.

Product size of the amplified region was 311 bp due to selected primers. Figure 3.1 represents the gel image of PCR result of rs927650 SNP.

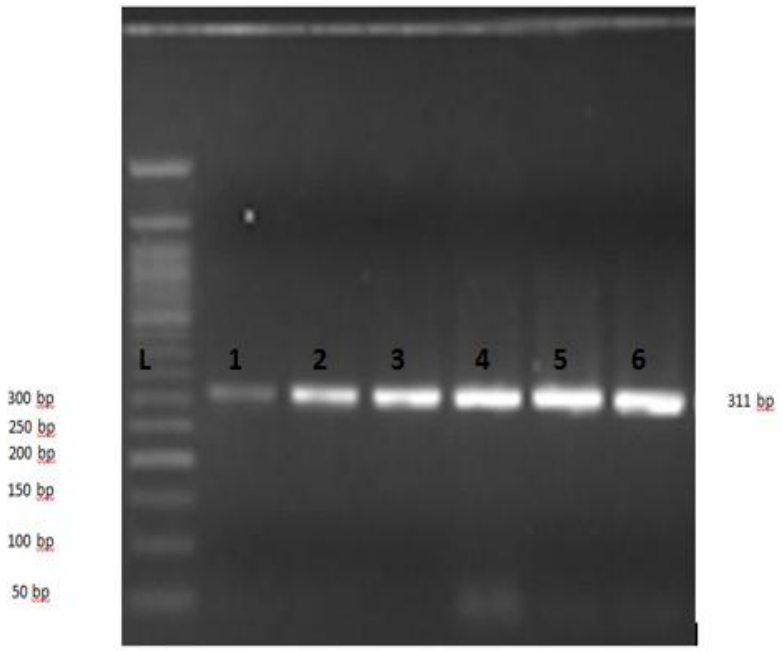

Figure 3.1 Agarose gel electrophoresis image for polymerase chain reaction products of rs 927650 SNP in CYP24A1 gene.

3.2.1.2. Restriction Endonuclease Digestion Results for CYP24A1 rs927650 Single Nucleotide Polymorphism

Restriction fragment length polymorphism technique (RFLP) was carried out to analyze the genotypes of rs927650 polymorphism in CYP24A1 gene. After amplification by PCR, the products were digested with Bg1II, which recognizes the individuals carrying T variant. So, if C allele substitutes for T allele, T allele constitutes the cut site, which is identified by the enzyme leading to fragmentation of DNA. If the product of PCR includes wild type genotype for rs927650 SNP, the enzyme cannot recognize the cut site; so 311 bp fragments are seen. Heterozygous polymorphic allele product yields 311 bp, 212 bp, 99 bp on the other hand subjects with homozygous polymorphic allele forms 212 bp, 99 bp fragments.

By testing different concentrations of restriction enzyme, optimized condition was obtained. Details are given in section 2.2.2.1.2. The components of restriction enzyme digestion mixture are given in Table 2.5. The reagents used for determination of genotypes of CYP24A1 rs927650 single nucleotide polymorphism were given in Appendix C. The result of the RFLP for CYP24A1 rs927650 is given in Figure 3.2.

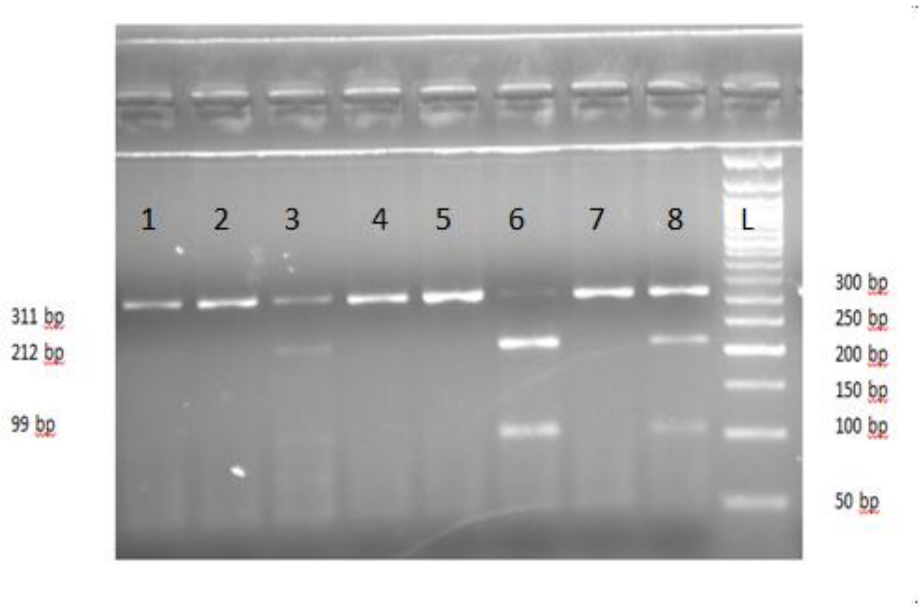

Figure 3.2 Agarose gel electrophoresis image for restriction endonuclease digestion with Mn1II of amplified CYP24A1 gene.

3.2.2. Genotyping for rs10741657 Single Nucleotide Polymorphism CYP2R1 Gene

rs10741657 SNP of CYP24A1 gene was investigated by PCR-RFLP method. The polymerase chain reaction was followed by restriction enzyme digestion. The specific enzyme used for this procedure is MnII.

3.2.2.1. Polymerase Chain Reaction Results for CYP2R1 rs10741657 Single Nucleotide Polymorphism

rs10741657 is located on 5' flanking region of CYP2R1 gene. A allele is converted to G allele in this polymorphism. The region was amplified by allele specific PCR method. To avoid from non-specific band and obtain just single band belonging to this region, different parameters such as $MgCl_2$ and primer concentration were tested. The optimized reaction conditions are given in section 2.2.2.2.2. 1.5 mM $MgCl_2$, 0.2 mM dNTP, 30 pmole each primer, 2.5 unit TaqDNA polymerase and approximately 200 ng DNA were added to reaction mixture.

PCR products were analysed on 2% agarose prepared by adding 4 g agarose to 200 ml of 0.5x TBE buffer whose preparation is given in Appendix C. Ethidium bromide was added to whole mixture of the agarose gel. 10 µL of each PCR product was mixed with gel loading dye before loading the mixture to wells of gel. Also, 6µL ladder (50-1000 bp) was applied to the one of the wells. The gel was run for 1 hour at 100 V. Product size of the amplified region was 287 bp due to selected primers. Figure 3.3 represents the gel image of PCR result of rs10741657 SNP.

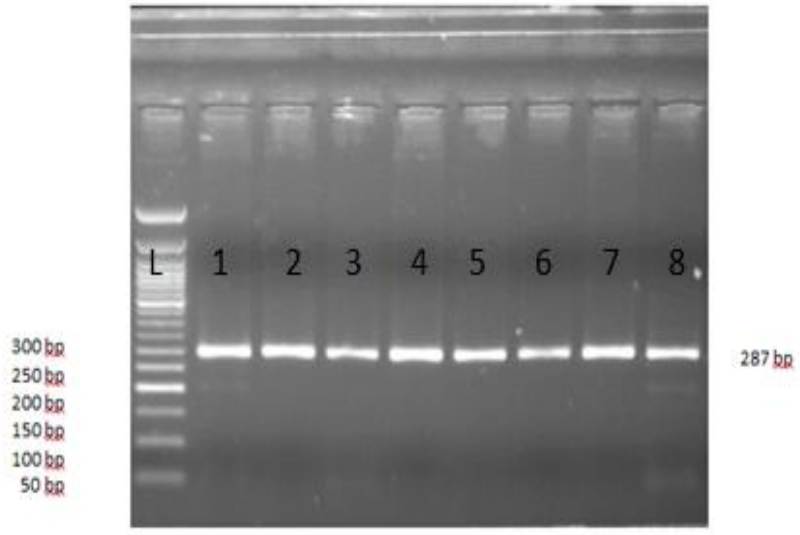

Figure 3.3 Agarose gel electrophoresis image for polymerase chain reaction product of CYP2R1 gene.

3.2.2.2. Restriction Endonuclease Digestion Results for CYP2R1 rs10741657 Single Nucleotide Polymorphism

Restriction fragment length polymorphism technique (RFLP) was performed to determine the genotypes of rs10741657 SNP. If substitution occurs in 5'flanking region (A>G), MnII recognizes the individuals with G variant. After recognition by the enzyme, DNA is divided into the fragments. If individuals carry homozygous polymorphic allele, the fragments are composed of 150 bp, 98 bp and 39 bp whereas 248 bp, 150 bp, 98 bp and 39 bp fragments show heterozygote individuals for this SNP. However, the wild type genotype do not have recognition site for the enzyme so 248 bp and 39 bp fragments mean individuals with homozygous wild type genotype.

By testing different concentrations of restriction enzyme, optimized condition was obtained. Details are given in section 2.2.2.2.2. The components of restriction enzyme digestion mixture are given in Table 2.8. The reagents used in restriction enzyme

polymorphism for CYP24R1 rs10741657 single nucleotide polymorphism were given in Appendix C. The result of the RFLP for rs10751657 is given Figure 3.4.

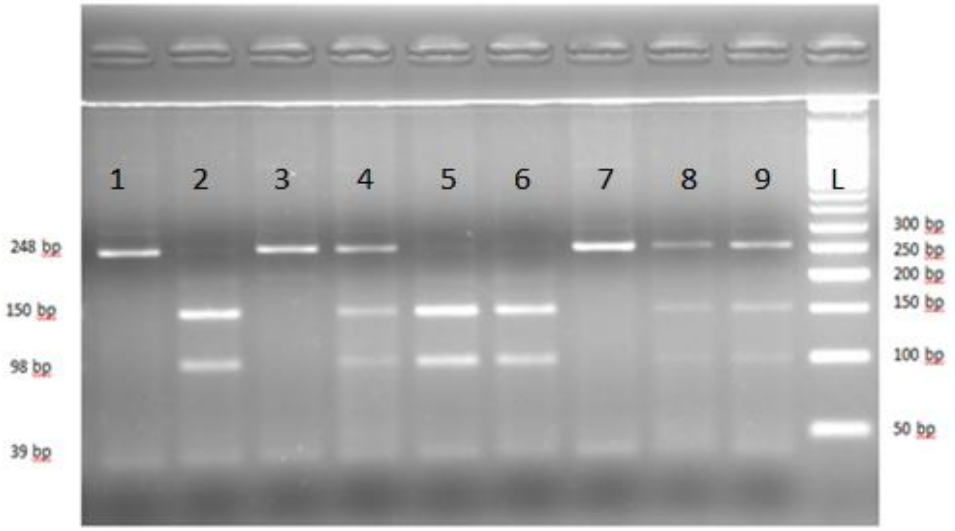

Figure 3.4 Agarose gel electrophoresis image for restriction endonuclease digestion with BgII of amplified CYP2R1 gene.

3.3. Genotypes and Allele Frequencies of rs927650 Single Nucleotide Polymorphism of CYP24A1 gene and rs10741657 Single Nucleotide Polymorphism of CYP2R1 gene

Genotype and allele frequencies of 256 patient and 132 control individuals were investigated for rs927650 SNP in CYP24A1 gene and rs10741657 SNP in CYP2R1 gene in this study. Aim of the analysis was to determine the effect of the polymorphisms on risk of ischemic stroke. Odds ratio was calculated according to the groups that were formed according to the carried alleles. If genotype contains at least one allele as a mutant, this genotype is grouped as risky group because both of the polymorphisms

result in vitamin D deficiency. On the other hand, the homozygous wild type individuals were counted as non-risky group.

Odds ratio is calculated by the formula indicated at below.

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

3.3.1. Genotypes and Allele Frequencies of rs927650 Single Nucleotide Polymorphism of CYP24A1 gene

The distribution of genotypes and allele frequencies for rs927650 single nucleotide polymorphism of CYP24A1 gene within ischemic stroke patient and healthy controls groups are given in Table 3.3.

Table 3.3 Genotypes and allele frequency distribution of patients and controls for CYP24A1 rs927650 single nucleotide polymorphism.

	Patients (n=256)	**Controls (n=132)**	**OR (95%CI)**	***P***
rs927650 polymorphism Genotypes, n (%)				
CC	80 (31.3%)	40 (30.3%)		
CT	140 (54.7%)	80 (60.6%)	0.957[a] (0.606-1.509)	0.848
TT	36 (14.0%)	12 (9.1%)		
Allele frequency				
C	0.586	0.606	1.087[b] (0.803-1.472)	0.589
T	0.414	0.394		

[a]CT+TT vs. CC, [b]C vs. T

256 patient and 132 control individuals were examined in terms of conversion of 'C' allele to 'T' allele in the scope of the study. 80 (31.3%) patients and 40 (30.3%) controls were homozygous wild type (CC), while 140 (54.7%) patients and 80 (60.6%) controls were heterozygote (CT) and the number of homozygote polymorphic genotype (TT) was 36 (14.0%) in patients and 12 (9.1%) in controls among study population. There is no significant difference between the percentage of the individuals with CT and TT in patients and controls (OR=0.957, 95%CI: 0.606-1.509 and P=0.848). Likewise, the percentage of T allele frequencies in stroke patient and control groups did not differ significantly (OR=1.087, 95%CI: 0.803-1.472 and P= 0.589).

3.3.2. Genotypes and Allele Frequencies of rs10741657 Single Nucleotide Polymorphism of CYP2R1 gene

The distribution of genotypes and allele frequencies for rs10741657 single nucleotide polymorphism of CYP2R1 gene within ischemic stroke patient and healthy control groups are given in Table 3.4.

Table 3.4 Genotypes and allele frequency distribution of patients and controls for CYP2R1 rs10741657 single nucleotide polymorphism.

	Patients (n=256)	**Control (n=132)**	**OR (95%CI)**	***P***
rs10741657 polymorphism Genotypes, n (%)				
AA	33 (12.9)	22 (16.7)		
AG	108 (42.2)	49 (37.1)	1.352[a] (0.752-2.428)	0.312
GG	115 (44.9)	61 (46.2)		
Allele frequency				
A	0.340	0.352	1.056[b] (0.773-1.443)	0.720
G	0.660	0.648		

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

256 patient and 132 control individuals were investigated for conversion of 'A' allele to 'G' allele in the CYP2R1 gene. 12.9% of patient group and 16.7% of control group had

homozygous AA genotype. The percentages of heterozygous AG genotype were 42.2 % and 37.1% in the stroke patients and controls respectively while the percentages of subjects with homogenous GG genotype were 44.9% and 46.2%. The heterozygous and homozygous mutant type individuals were considered as risky. When AG and GG were attributed as a single group and compered to the wild type AA genotype, it was concluded as no significant difference in patients and controls (OR=1.352, 95%CI: 0.752-2.428 and P=0.312). Similarly, there was no significant difference between G allele frequencies of patients and control groups (OR=1.056, 95%CI: 0.773-1.443, and P= 0.720).

3.4. CYP24A1 and CYP2R1 Genotypes in Different Subgroups of Stroke Patient and Control Group

Genotype analysis of CYP24A1 rs927650 SNP and CYP2R1 rs10741657 SNP for four different subgroups having different conventional risk factors were done to compare between ischemic stroke patients and control groups. These groups were composed of hypertensive versus normotensive, diabetic versus non-diabetic, obesity versus non-obesity and smoker versus non-smoker.

While making comparison between the genotypes of CYP24A1 rs927650 SNP for different subgroups, CT and TT genotypes were attributed as a one group against homozygous wild type CC genotype. Similarly, AG and GG genotypes were ascribed as a single group against to AA genotype to make comparison for rs10741657 polymorphism in CYP2R1.

3.4.1. CYP24A1 and CYP2R1 Genotypes in Hypertensive vs. Normotensive Subgroup of Stroke Patients and Controls

Table 3.5 represents the distribution of CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes between stroke patients and controls with regard to hypertensive and normotensive subgroups. Hypertensive group consisted of 173 stroke patients and 51 controls and percentages for the CYP24A1 rs927650 homozygous mutant TT genotypes was 13.9 % and 9.8 % for patients and control groups, respectively. The heterozygous and homozygous mutant types were considered as risky genotypes and the proportion of risky genotypes (CT +TT) was 68.2% for patient group and 70.6% for control group. When compared to T allele carriers to wild type CC genotype, no significant difference between stroke patients and controls in hypertensive subgroup was seen (OR=0.89, 95%CI: 0.452 -1.768 and P=0.747).

There were 83 stroke patients and 81 controls among normotensive subgroup and similarly T allele carriers in patients is also almost same with controls when compared to the wild type genotype (OR=1.036, 95%CI: 0.533-2.014 and P=1) for normotensive individuals.

For CYP2R1 rs10741657, number of homozygous mutant GG genotype was 78 for stroke patients and 26 for controls in hypertensive subgroup. Risky genotype frequencies (AG+GG) are 89.6% and 86.3% for stroke patients and controls, respectively. The proportion of so-called risky genotypes was higher in patient group than control group. When G allele carriers was compared to the wild type AA genotype, stroke patient was more susceptible to carrying G allele but no significant difference between stroke patients and controls was seen for hypertensive subgroup (OR=1.37, 95%CI: 0.538-3.49 and P=0.58).

Risky genotype frequencies (AG+GG) are 81.9% for stroke patients and 81.5% for controls among normotensive subgroup. The frequencies of AA and AG+GG genotypes were almost the same in the stroke and control groups. The difference was not

significant between patients and controls when G allele carriers were compared to the wild type genotype (AA) carriers (OR=1.03, 95%CI: 0.467- 2.274 and P=1).

Table 3.5 Genotype distributions of CYP24A1 rs927650 and CYP2R1 rs10741657 single nucleotide polymorphisms in hypertensive vs. normotensive subgroup of stroke patients and controls.

	Hypertensive (n=224)				**Normotensive (n=164)**			
	Patient (n=173)	**Control (n=51)**	**OR (95% CI)**	***P***	**Patient (n=83)**	**Control (n=81)**	**OR (95% CI)**	***P***
rs927650								
CC	55 (31.8%)	15 (29.4%)			25 (30.1%)	25 (30.9%)		
CT	94 (54.3%)	31 (60.8%)	0.89[a]	0.747	46 (55.4%)	49 (60.5%)	1.036 [a]	1
TT	24 (13.9%)	5 (9.8%)	(0.452-1.768)		12 (14.5%)	7 (8.6%)	(0.533 -2.014)	
rs10741657								
AA	18 (10.4%)	7 (13.7%)			15 (18.1%)	15 (18.5%)		
AG	77 (44.5%)	18 (35.3%)	1.37 [b]	0.508	31 (37.3%)	31 (38.3%)	1.03[b]	1
GG	78 (45.1%)	26 (51.0%)	(0.538 -3.49)		37 (44.6%)	35 (43.2%)	(0.467 - 2.274)	

[a]CT+TT vs. CC

[b]AG+GG vs. AA

3.4.2. CYP24A1 and CY2R1 Genotypes in Diabetic vs. Non-diabetic Subgroup of Stroke Patients and Controls

The distribution of CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes in diabetic/non-diabetic subgroups of stroke patients and controls is given Table 3.6. 112 diabetic subjects and 276 non-diabetic subjects were analyzed for this investigation. For rs927650 SNP among diabetes, the frequency of CT and TT risky genotypes in the stroke patients was 67.0% and 66.7% in the controls. When compared to the wild type CC genotype, T allele carriers were almost same in patients and controls and there was no significant difference between them (OR=1, 95%CI: 0.39-2.65 and P=1). This situation was almost the same for the non-diabetic individuals too (P=1).

For rs10741657, the frequency of AG and GG genotypes called risky genotype were higher in the stroke patients (89.8%) when compared to controls (75.0%) among diabetes subgroup. When compared to the wild type AA genotype, the G allele carriers (GA+AA) were higher in control group than the C allele carriers in the stroke patient group (P=0.060). Among nondiabetes subgroup, when compared to the wild type AA genotype, G allele carriers were almost same in patients and controls and there was no significant difference between them (OR=1.043, 95%CI: 0.53-2.07 and P=1)

Table 3.6 Genotype distributions of CYP24A1 rs927650 and CYP2R1 rs10741657 single nucleotide polymorphisms in Diabetic vs. Non-diabetic Subgroup of Stroke Patients and Controls.

	Diabetic (n=112)				**Non-Diabetic (n=276)**			
	Patient (n=88)	**Control (n=24)**	**OR (95% CI)**	***P***	**Patient (n=168)**	**Control (n=108)**	**OR (95% CI)**	***P***
rs927650								
CC	29 (33.0%)	8 (33.3%)			51 (30.4%)	32(29.7%)		
CT	47 (53.4%)	13 (54.2%)	1[a]	1	93 (55.4%)	67 (62.0%)	0.966[a]	1
TT	12 (13.6%)	3 (12.5%)	(0.39-2.652)		24 (14.2%)	9 (8.3%)	(0.57 -1.638)	
rs10751657								
AA	9 (10.2 %)	6 (25.0%)			24 (14.3%)	16 (14.8%)		
AG	39 (44.3%)	10 (41.7%)	2.926[b]	0.060	69 (41.1%)	39 (36.1%)	1.043[b]	1
GG	40 (45.5%)	8 (33.3%)	(0.92-9.27)		75 (44.6%)	53 (49.1%)	(0.53-2.07)	

[a]CT+TT vs. CC

[b]AG+GG vs. AA

3.4.3. CYP24A1 and CYP2R1 Genotypes in Obesity vs. Non-obesity Subgroup of Stroke Patients and Controls

Genotype distribution of CYP24A1 rs927650 and CYP2R1 rs10741650 in obese and non-obese subgroups of stroke patients and controls are summarized in Table 3.7. 68 obese subjects and 320 non-obese subjects were involved for this analysis.

For rs927650, among obese group, the frequency of CT and TT genotypes in the stroke patients (85%) is almost same with the frequency of same genotypes in control group (87.5%). The difference was not significant between patients and controls when T allele carriers were compared to the wild type genotype (CC) carriers (OR=0.81, 95%CI: 0.09-7.39 and P=1). For non-obese subjects, CT+TT genotypes frequencies were 63.8% for patients. Similar to obese group, no significant difference was seen between stroke patients and controls also in non-obese group (OR=0.808, 95%CI: 0.501-1.303, and P=0.381).

For rs10741657, the proportion of AG and GG genotypes were observed as (91.6%) and (87.5%) for stroke patients and controls, respectively. When compared wild type AA genotype, G allele carriers (AG+GG) were higher in stroke patients than controls (P=0.543). Among 320 non-obese individuals, the proportion of AG and GG genotypes were lower in controls (83.1%) when compared to the proportion of AG and GG genotypes in ischemic stroke patients (95.7%). But, the difference was not significant when compared to the wild type genotype among non-obese subjects (P=0.521).

Table 3.7 Genotype distributions of CYP24A1 rs927650 and CYP2R1 rs10741657 single nucleotide polymorphisms in Obesity vs. Non-obesity Subgroup of Stroke Patients and Controls.

	Obesity (n=68)				**Non-Obesity (n=320)**			
	Patient (n=60)	**Control (n=8)**	**OR (95% CI)**	***P***	**Patient (n=196)**	**Control (n=124)**	**OR (95% CI)**	***P***
rs927650								
CC	9 (15.0%)	1 (12.5%)			71 (36.2%)	39 (31.5%)		
CT	44 (73.3%)	6 (75.0%)	0.81[a]	1[c]	96 (49.0%)	74 (59.7%)	0.808[a]	0.381
TT	7 (11.7%)	1 (12.5%)	(0.09-7.39)		29 (14.8%)	11 (8.9%)	(0.501-1.303)	
rs10741657								
AA	5 (8.4%)	1 (12.5%)			28 (14.3%)	21 (16.9%)		
AG	26 (43.3%)	4 (50.0%)	1.571[b]	0.543[c]	82 (41.8%)	45 (36.3%)	1.223[b]	0.521
GG	29 (48.3%)	3 (37.5%)	(0.16 -15.465)		86 (43.9%)	58 (46.8%)	(0.66 -2.266)	

[a]CT+TT vs. CC

[b]AG+GG vs. AA

[c] Fisher Exact Test is applied

3.4.4. CYP24A1 and CYP2R1 Genotypes in Smoker vs. Non-smoker Subgroup of Stroke Patients and Controls

Table 3.8 summarizes the genotype distribution of CYP24A1 rs927650 and CYP2R1 rs10741657 in smoker/non-smoker patient and control groups. The frequencies of homozygous wild type CC genotype among smokers are 21.4 % and 37.5% for stroke patients and controls respectively. Proportion of T allele carriers (CT+TT) was higher in stroke patients (78.6%) than the controls (62.5%) among smokers (P=0.177). On the other hand, the percentage of T allele carriers was found to be lower in stroke patients (65.1%) than the controls (70.6%) in non-smoker subgroup (P=0.310).

For rs10741657, among smoker subgroup, frequency of AA homozygous wild type genotype (17.1%) in stroke patients was higher than same genotype of the controls (6.3 %). However, the frequency of G allele carriers (AG+GG) is higher in the control group than stroke patient group. In terms of rs10741657 polymorphism genotype frequencies, there were no statistically differences in obese stroke patients and controls (P=0.446). Among non-smoker group, rather to smoking group, the frequency of the G allele carriers was higher in stroke patients (88.7%) than the controls (83.1%). Also, there was no significantly difference in genotype distribution among non-smoker subgroup for stroke patients and controls (P=0.096).

Table 3.8 Genotype distributions of CYP24A1 rs927650 and CYP2R1 rs10741657 single nucleotide polymorphisms in Smoker vs. Non-smoker Subgroup of Stroke Patients and Controls.

	Smoker (n=86)				**Non-Smoker (n=302)**			
	Patient (n=70)	**Control (n=16)**	**OR (95% CI)**	***P***	**Patient (n=186)**	**Control (n=116)**	**OR (95% CI)**	***P***
rs927650								
CC	15 (21.4%)	6 (37.5%)			65 (34.9%)	34 (39.4%)		
CT	48 (68.6%)	10 (62.5%)	2.2[a]	0.177	92 (49.5%)	70 (60.3%)	0.772[a]	0.310
TT	7 (10.0%)	0 (0%)	(0.688 -7.032)		29 (15.6%)	12 (10.3%)	(0.468-1.273)	
rs10741657								
AA	12 (17.1%)	1 (6.3%)			21 (11.3%)	21 (16.9%)		
AG	26 (37.2%)	5 (31.2%)	0.322[b]	0.446[c]	82 (44.1%)	44 (36.3%)	1.737[b]	0.096
GG	32 (45.7%)	10 (62.5%)	(0.039-2.678)		83 (44.6%)	51 (46.8%)	(0.902- 3.345)	

[a]CT+TT vs. CC

[b]AG+GG vs. AA

[c] Fisher Exact Test is applied

3.5. Effects of Conventional Vascular Risk Factors in Different CYP24A1 and CYP2R1 Genotypes of Ischemic Stroke Patients and Controls

In this analysis, the conventional vascular risk factors such as hypertension, diabetes, obesity and smoking were examined with regard to ratio of ischemic stroke patients to control group considering risky (heterozygous or homozygous mutant type) and non-risky group (wild type). The heterozygous genotypes (CT for rs927650 SNP and AG for rs10741657 SNP) and homozygous mutant genotypes (TT for rs927650 SNP and GG for rs10741657 SNP) were considered as one group, which was compared versus wild type genotypes (CC rs927650 SNP and AA for for rs10741657 SNP). The analysis showed the odds ratio and evaluation of the results was done according to P value. If P value was lower than 0.05, the result was evaluated as significantly meaningful.

3.5.1. Effects Conventional Vascular Risk Factors in Different CYP24A1 rs927650 Single Nucleotide Polymorphism Genotypes of Ischemic Stroke Patients and Control Group

Proportion of ischemic stroke patients to controls within the risky genotype group and non-risky genotype group were analyzed for conventional risk factors such as hypertension, diabetes, obesity and smoking. While making comparison between the genotypes of CYP24A1 rs927650, the CT and TT were attributed as a single group, also called as risky group. Wild type genotype CC was ascribed as the other group. Table 3.9 shows that in each genotype group of CYP24A1 rs927650 polymorphism, the proportion of stroke patients to controls was increased in hypertensive, diabetic, smoker and obese group when compared to normotensive, non-diabetic, non-smoker and non-obese group. When compared to normotensives, the risk of having stroke is 3.165 times higher (P=0.000) in hypertensives carrying risky T allele in CYP24A1 rs927650 SNP. The risk of having stroke is also 3.667 times higher for individuals carrying CC genotype than

normotensives (P=0.01). Consequently, the risk of stroke increases with hypertension for all genotypes.

Among diabetics, individuals with CT+TT genotype, the risk of having stroke was significantly higher in diabetic subjects than non-diabetics (OR=2.395, 95%CI: 1.284-4.468, P=0.005). Proportion of stroke patients to controls with CC genotypes was insignificantly 2.275 times higher in diabetics than non-diabetics (P=0.069). Similar to diabetics, individuals with CT+TT genotypes in obese subgroup had significant risk for having stroke when compared to non-obese subgroup (P=0.001). Also, the number of obese subjects having both CC genotype and stroke was 4.944 times higher than non-obese subjects, but insignificantly (P=0.162). For smoker subgroup, in CT+TT genotype group, the proportion of stroke patient was found significantly 3.727 times higher when compared to non-smoker group (P=0.00). In genotype group of CC, the proportion of stroke patients in smoker subgroup is almost similar to non-smoker subgroup (OR= 1.308; 95%CI: 0.465-3.676, P=0.610).

Table 3.9 Stratification of hypertensive/normotensive, diabetic/non-diabetic, smoker/non-smoker, obesity /non-obesity individuals according to CYP24A1 rs927650 genotypes.

	CC Genotype (n=120)	**OR (95%)**	***P***	**CT+TT Genotype (n=268)**	**OR (95%)**	***P***
	Stroke/Control			**Stroke/Control**		
Hypertensive (n=224)	55/15	3.667[a]	0.001	118/36	3.165[a]	0.000
Normotensive(n=164)	25/25	(1.655-8.126)		58/56	(1.875-5.342)	
Diabetic (n=112)	29/8	2.275[b]	0.069	59/16	2.395[b]	0.005
Non-diabetic (n=276)	51/32	(0.926-5.588)		117/76	(1.284-4.468)	
Obesity (n=68)	9/1	4.944[c]	0.162[e]	51/7	4.138[c]	0.001
Non- obesity (n=320)	71/39	(0.604-40.476)		125/85	(1.783-9.605)	
Smoker (n=86)	15/6	1.308[d]	0.610	55/10	3.727[d]	0.000
Non-smoker (n=302)	65/34	(0.465 -3.676)		121/82	(1.797- 7.733)	

[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 CYP2R1 rs10741657 SNP Genotypes of Ischemic Stroke Patients and Control Group

Table 3.10 summarizes the proportion of stroke patients to controls in terms of different genotypes of rs10741657 polymorphism for hypertensives, diabetics, smokers and obese people. Subjects with of AG+GG genotype in hypertensive subgroup had nearly 3.4 times higher risk of stroke when compared to normotensive subjects (P=0.000).

The proportion of stroke patients in hypertensive subgroup was higher than the normotensive subgroup in genotype group of AA (P=0.097). When compared to non-diabetics individuals having AG+GG genotype, the risk of having stroke was found 2.8 times significantly higher for diabetic individuals with same genotype (P=0.000). The proportion of diabetics to non-diabetics individuals having AA genotype was same (P=1).

As in represented in Table 3.10, among obese subjects, subjects with G allele had higher risk of ischemic stroke (OR=4.817, 95%CI: 2.113-10.98, P=0.00), on the other hand having AA genotype did not significantly increase the risk of stroke (OR=3.75, 95%CI: 0.407 -34.539, P=0.384). In both genotype groups (AA/AG+GG), the proportion of stroke patients to controls was significantly higher in smoker group when compared to non-smoker group (P=0.009 for AA genotype, P=0.010 for AG+GG genotypes).

Table 3.10 Stratification of hypertensive/normotensive, diabetic/non-diabetic, smoker/non-smoker, obesity /non-obesity individuals according to CYP2R1 rs10741657 genotype.

	AA Genotype (n=55)	**OR (95%)**	***P***	**AG+GG Genotype (n=333)**	**OR (95%)**	***P***
	Stroke/Control			**Stroke/Control**		
Hypertensive (n=224)	18/7	2.571[a]	0.097	155/44	3.419[a]	0.000
Normotensive(n=164)	15/15	(0.832-7.951)		68/66	(2.124-5.505)	
Diabetic (n=112)	9/6	1[b]	1	79/18	2.804[b]	0.000
Non-diabetic (n=276)	24/16	(0.298- 3.358)		144/92	(1.578-4.982)	
Obesity (n=68)	5/1	3.75[c]	0.384[e]	55/7	4.817[c]	0.000
Non- obesity (n=320)	28/21	(0.407 -34.539)		168/103	(2.113-10.98)	
Smoker (n=86)	12/1	12[d]	0.009 [e]	58/15	2.226[d]	0.010
Non-smoker (n=302)	21/21	(1.429-100.755)		165/95	(1.196 -4.144)	

[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

To determine the effects of age, sex, hypertension, diabetes, obesity, smoking, lipid parameter, CYP24A1 and CYP2R1 genotypes in the prediction of ischemic stroke, logistic regression analyses with backward selection method (Backward likelihood ratio) was carried out. Different binary logistic regression models were constituted with different combinations of parameters in the overall population or in different subgroups.

Model 1:

Model 1 included all subjects in study population and was established by adding age, sex, hypertension, smoking status, diabetes, obesity, lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP24A, CYP2R1 genotypes as covariates and the results obtained by logistic regression are summarized in Table 3.11. The analysis showed that hypertension (OR=3.387, 95%CI: 2.095-5.475, P=0.000), smoking (OR=3.074, 95%CI: 1.598-5.915, P=0.001), obesity (OR=2.730, 95%CI: 1.211-6.155, P=0.015) and LDL-cholesterol (OR=1.397, 95%CI: 1.086-1.796 P=0.009) were the significant determinants of ischemic stroke. However, HDL-cholesterol (OR=0.276, 95%CI: 0.119-0.640, P=0.003) was served as preventive factor in terms of determinants of stroke. The model predicted 71.1% of cases correctly and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=7.237; 8 degrees of freedom; P=0.511).

Table 3.11 Model 1**,** Logistic regression analysis of conventional risk factors (age, hypertension, smoking status, diabetes, obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) and CYP24A1, CYP2R1 genotypes.

Parameters	**OR**	**95%CI**	***P***
Hypertension	3.387	2.095-5.475	0.000
Smoking	3.074	1.598-5.915	0.001
Obesity	2.730	1.211-6.155	0.015
HDL-cholesterol	0.276	0.119-0.640	0.003
LDL-cholesterol	1.397	1.086-1.796	0.009

Model 2:

In the second model, only male subjects were involved in the analysis. Vascular risk factors (age, hypertension, smoking, diabetes and obesity), lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol), CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes were added as covariates. The results of the analysis are given in Table 3.12. Hypertension (OR=3.601, 95%CI: 1.800-7.205, P=0.000), smoking (OR=2.209, 95%CI: 1.038-4.702, P=0.040) and obesity (OR=5.851, 95%CI: 1.294-26.464, P=0.022) were found as significant risk factors of ischemic stroke. However, age (OR=0.976, 95%CI: 0.953-1.0, P=0.069) was not statistically important risk factor for ischemic stroke. The model predicted 71.6% of cases correctly and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=5.882; 8 degrees of freedom; P=0.660).

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 CYP24A1, CYP2R1 genotypes in males.

Parameters	**OR**	**95%CI**	***P***
Hypertension	3.601	1.800-7.205	0.000
Age	0.976	0.953-1.0	0.053
Smoking	2.209	1.038-4.702	0.040
Obesity	5.851	1.294-26.464	0.022

Model 3:

Only female subjects were analyzed in this model. Vascular risk factors such as age, hypertension, smoking, diabetes and obesity, lipid parameters such as total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol and CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes were added as covariates. According to the model 3 analysis, hypertension, diabetes, smoking, LDL-cholesterol were found as significant predictors of ischemic stroke. Also, HDL-cholesterol was found to be as protective factor in ischemic stroke (Table 3.13). The model predicted 75.6% of cases correctly and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=8.215; 8 degrees of freedom; P=0.413).

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 CYP24A1, CYP2R1 genotypes in females.

Parameters	**OR**	**95%CI**	***P***
Hypertension	5.796	2.639-12.729	0.000
Diabetes	3.119	1.310-7.428	0.010
Smoking	10.485	2.278-48.255	0.003
LDL-cholesterol	2.236	1.436-3.482	0.000
HDL-cholesterol	0.185	0.050-0.684	0.011

CHAPTER 4

DISCUSSION

A stroke occurs when the blood flow to the brain is obstructed or when a blood vessel in the brain ruptures, causing brain cells to die. Stroke is a leading cause of serious long-term disability. Environmental and genetic factors contribute to the progress of the stroke. Stroke mortality is declining currently in different subgroups such as both sexes and all race and age groups in last decades. The most effective contributor of the decline in stroke mortality is the control of hypertension, which has been started in the 1970s. In addition to controlling hypertension also control of diabetes mellitus and high cholesterol and smoking cessation also seem to have influenced the decline in stroke mortality (Mozaffarian et al., 2014).

Vitamin D deficiency has been found to trigger hypertension, heart attack, (Poole et al., 2006) and also cardiovascular-related diseases (Wang et al., 2008), like diabetes (Zittermann et al., 2003), heart failure (Zittermann et al., 2006), atherosclerosis (Kassi et al., 2013), peripheral vascular disease and pathological state of the endothelium (Alyami et al., 2014) and many studies reported that low level vitamin D increases stroke risk and stroke severity (Anderson et al., 2010; Poole et al., 2006).

All Vitamin D taken from diet or by the action of sunlight on the skin is biologically inactive and needs to have hydroxylation of carbon 25 and 1α for metabolic activation in the body. There are several hepatic cytochrome P-450s taking part in 25-hydroxylation. CYP2R1 is one of these enzymes. This enzyme is a microsomal Vitamin D hydroxylase and hydroxylation process of Vitamin D occurs in the liver. CYP2R1 seems to be one of

the most important 25-hydroxylase involved in Vitamin D metabolism. Functions of activated vitamin D are numerous. Regulation of minerals, effects on growth of cancer cells and protection against certain immune disorders can be counted as the functions in the body. Clinically active vitamin D status in the body is determined by the measurement of 25-OH-D_3.

Deactivation of 1α-25$(OH)_2D_3$ is also important for homeostasis in the body. Catabolism of 1α,25$(OH)_2D_3$ is carried out by CYP24A1 enzyme (24-hydroxylase), in target tissues and products of the this pathway are 1α-24,25$(OH)_3D_3$ or 24-25$(OH)_2D_3$. So 24-hydroxylase decreases the availability of 25-OH-D_3 for 1α-hydroxylation. Thus alteration in activity of the enzymes taking part in vitamin D activation or inactivation affects the ischemic stroke risk. The objective of this study is to investigate the possible association of CYP24A1 polymorphism rs927650 and CYP2R1 polymorphism rs10741657 in terms of risk factors with ischemic stroke in Turkish population. In the sub-group analysis, conventional risk factors and lipid parameters were also evaluated in stroke patients and healthy control groups.

The study population consisted of 256 stroke patients and 132 healthy subjects having no symptoms of stroke. Before making comparison between the patient and control groups, evaluation of the some criteria was carried out to prevent significant differences. All subjects were included to the study according to the all inclusion and exclusion criteria.

As people get older, the risk for developing stroke increases so age is one of the most important non-modifiable risk factor for stroke. Difference in mean age between stroke patients and controls was reduced to minimum level for prevention the effect of age on results. The age of study population ranged from 32 to 90 and the mean of ages were 65.57 for ischemic stroke patients and 64.42 for healthy controls. The results showed that there is no statistically significant difference in mean age between the two groups (Table 3.1).

The gender is another non-modifiable risk factor for ischemic stroke and it was reported that male gender has 1.25 times higher incidence rate of stroke risk than female (Sacco et al., 1997). In this study, number of male subjects was 144 in stroke patient group (n=256), and they had 1.366 times higher risk of stroke when compared to females. The number of males in the patient group (56.3%) was higher than the number of males in the control group (48.5%). It was found that there was no statistically significant difference between the groups with respect to gender (P=0.163).

The conventional risk factors such as hypertension, diabetes mellitus, obesity and smoking are modifiable risk factors for stroke (Sacco et al., 1995; Sacco et al., 1998). As expected, when compared to control group, it was found that these factors significantly increased the risk of having stroke in this study also (P<0.05) (Table 3.1 and Table 3.2).

The frequency of hypertensive individuals are 38.6 % and 67.6% for controls and stroke patients respectively and the risk of having stroke was found approximately 3.3 times higher for hypertensive individuals for stroke (P=0.000). There were also significant effects of diabetes and smoking on risk of having stroke, they showed nearly 2.5 fold relative risk for stroke (P=0.001 for both them). Obesity was found to be higher in the patient group (23.4%) than control group (6.1 %) and risk of having stroke is significantly 4.7 times higher in obese people than non-obese people (P=0.000).

Association of blood lipid profile and stroke risk has been investigated in some studies (Iso et al., 1989; Harmsen et al., 2006; Hachinski et al., 1996) but there are some paradoxes in the relation according to these studies. Some studies reported positive association between plasma total cholesterol and stroke (Harmsen et al., 2006; Hachinski et al., 1996) but others showed inverse relation (Olsen et al., 2007) or no association (Bots et al., 2002). In this study, stroke patients (4.87 ±1.27 mmol/L) and controls (4.72±1.26 mmol/L) had almost same total cholesterol levels. As it can be seen from the result, the total cholesterol is not associated with stroke risk (P=0.268).

The level of triglycerides in patients (1.55 ±0.74mmol/L) was slightly higher than in controls (1.47±0.66 mmol/L, P=0.277) and this minor difference was found as insignificant risk factor for stroke development. Also patients had significantly higher level of LDL-cholesterol (2.97±1.06mmol/L) when compared to the controls (2.74±1.0106mmol/L). Previous studies also support this result related with LDL-cholesterol (Sanossian et al., 2008). On the other hand, higher level of HDL-cholesterol has protective effect on stroke (Sanossian et al., 2006) and as expected the control group has significantly higher level of HDL-cholesterol (1.20±0.31mmol/L) than stroke patients (1.08±0.28mmol/L) in this study.

Investigation of CYP24A1 rs927650 polymorphism in control group was performed because the frequency of this polymorphism differs among different population. The frequency of minor allele T in control group was found as 0.394 in this study. The interested allele was found as 0.45 for Caucasian population in the study of Schäfer et al., 2012 however the study of Penna-Martinez et al., 2012 obtained the frequency of T allele as 0.26 in German population. So, the frequency of CYP24A1 rs927650 can be different among population of different ethnicity. The results obtained from this study are coherent with studies performed on Germans and Caucasians.

The frequency of CYP2R1 rs10741657 polymorphism also shows variations between different ethnic groups. In the present study, the frequency of G allele was found as 0.648% in control group. When viewed the literature, similarly the studies of Penna-Martinez et al., 2012 and Ramos-Lopez et al., 2007 also got the frequency of G as 0.62 % in healthy subjects among German population, on the other hand the study of Hassenein et al., 2014 was found the distribution of G allele as 0.37 % in Egyptian population.

In the present study, genotype distribution of rs927650 polymorphism in CYP24A1 was 31.3%, 54.7% and 14.0 % for CC, CT and TT, respectively in patients while the frequencies of control group were 30.3% for CC, 60.6% for CT and 9.1% for TT genotypes. In stroke patients, allele frequencies were 58.6 % and 41.4% for wild type allele C and mutant allele T, in order of. The results showed no significant difference

between patients and controls in regards to allele and genotype frequencies (P= 0.848 for genotypes and P=0.589, Table 3.3).

For CYP2R1 rs10741657 genotype analyses, in patients, distributions of the wild type 'AA', heterozygous 'GA' and homozygous mutant 'GG' genotypes were found as 12.9%, 42.2% and 44.9%, respectively. The frequencies of same genotypes were 16.7%, 37.1% and 46.2% for control group. When compared the allele frequencies, in stroke patients, the wild type 'A' allele frequency is 0.340 in patients and 0.352 in controls, also; the mutant allele frequency ''G'' was 0.660 and 0.648 in stroke patients and controls respectively. It was concluded there was no significant differences between patients and controls with regards to comparison of genotype and allele frequency (P=0.312 for genotypes and P=0.720 for allele, Table 3.4). So, similar to rs927650 polymorphism, rs10741657 could not be considered as a risk factor for ischemic stroke.

The association between vitamin D status and cardiovascular risk have been investigated in some small cross-sectional studies (Wang et al., 2008; Scragg et al., 1990; Poole et al., 2006; Zitterman et al., 2003). Scragg et al., 1990 has explained the relation of low vitamin D status to stroke. The connection between vitamin D deficiency and cardiovascular disease may be explained by several mechanisms in the number of studies conducted. The study of Li et al., 2002 showed that direct involvement of 1α- $25(OH)_2D_3$ in suppression of renin gene provides the regulation of renin-angiotensin system directly suppressing renin gene expression. Disruption in renin-angiotensin system affects development of heart diseases (Xiang et al., 2005). Modulation of calcium and phosphate homeostasis, smooth muscle cell proliferation, inflammation, thrombosis can be included to the functions and physiological actions of vitamin D. Some of cytochrome enzymes have significant role in metabolism of vitamin D. Change in normal activity of the enzymes taking part in activation or inactivation of vitamin D can affect the risk of having stroke. The study of Kasuga et al., 2002 indicated the overexpression of 24-hydroxlyase (CYP24A1), taking part in catabolism of 1α-$25(OH)_2D_3$ increases the risk of atherosclerosis. Transgenic rats generated for the study were constitutively expressing

CYP24A. Atherosclerotic lesions were examined in aorta of rats either they were fed normal diet or high fat diet. Besides, Hassanein et al., 2014 observed that the patient with coronary artery disease had significantly lower level of 25-OH-D_3 when compared to control group without coronary artery disease. The study also revealed that the association of rs927650 with CAD incidence. So it can be concluded the polymorphism in CYP2R1 also increase the incidence of coronary artery disease due to decreased level of Vitamin D.

In the literature, there is no study investigating the association of CYP24A1 rs927650 polymorphism and CYP2R1 rs10741657 polymorphism with stroke in terms of affecting the vitamin D metabolism in Turkish population or any other populations, so the present study is the first to analyze these associations.

Sub-group analysis of CYP24A1 rs927650 and CYP2R1 rs10741657 was carried out also to find whether they were risk factor for stroke or not. Comparison of genotypes between the patients group and control group was performed in hypertension (Table 3.5), diabetes mellitus (Table 3.6), obesity (Table 3.7) and smoking (Table 3.8) sub-groups. The analysis done for all study population indicated that these sub-groups were significant risk factors for ischemic stroke (Table 3.1). On the other hand, no significant difference was found for distribution of genotypes CYP24A1 rs927650 and CYP2R1 rs10741657 in any subgroups when compared between the stroke patients and control (all P values are higher than 0.05).

In stratification part, the results showed that the proportion of stroke patients to controls was higher in hypertensive, diabetics, smokers and obese subgroups than normotensive, non-diabetic, non-smoker and non-obese subgroup regardless of genotype group (Table 3.9 and Table 3.10). While comparison of genotypes between patients and controls, it was performed according to individuals having wild type genotype or a mutant allele.

For sub-group analysis of CP24A1 rs927650 polymorphism, individuals having T allele (CT genotype and TT genotype) were grouped as risky group so comparison was done

according to the individuals having wild type CC genotype. When compared to individuals of control group having CC genotype, proportion of stroke patients with same genotype is nearly 3.7 times higher in hypertensive group than normotensive group (P=0.001). Among the individuals with CT and TT genotypes, the risk of having stroke in hypertensive group was significantly 3.2 fold higher normotensive group (P=0.000). The proportion of CT and TT genotypes were expected to be higher for stroke patients in hypertensive group but there was no important difference when compared to individuals with CC genotype. In diabetic and non-diabetic analysis, the number of wild type individuals with diabetic was 2.3 fold higher than non-diabetic (P=0.069). When viewed the risky group; risk of having stroke with diabetes was significantly higher than non-diabetic individuals (P=0.005). The proportion of individuals with wild type genotype was nearly 5 times higher in obese group than non-obese group (P=0.162). Also individuals having risky T allele in obese group had risk of stroke nearly 4 times higher than non-obese individuals (P=0.001) so having wild type genotype in obese people may decrease the risk of having stroke. The number of smoker individuals with wild type genotype was 1.3 times higher than non-smokers. However, risk of having stroke was nearly 4 times higher in smokers than non-smoker when individuals having risky T allele (P=0.000). The risk of having stroke in smoker group was 2.728 for all study population (Table 3.2). However, risk of having stroke increased to 3.727 in individuals having risky T allele among smoker group.

For CYP2R1 rs10741657, risk of having stroke in hypertensive individuals with AG+GG genotypes was 3.419 times significantly higher than normotensive individuals (P=0.000). Risk of having stroke among hypertension was 3.310 for all study population (Table 3.2). So, the risk increased from 3.310 to 3.419 when individuals having AG+GG genotypes and hypertension. Also the number of AA genotyped individuals was nearly 2.6 times higher in hypertensive group than normotensive group (P=0.097). The stratification analysis of diabetic and non-diabetic groups indicated that the proportion of diabetic individuals having wild type genotype AA was same with non-diabetic individuals (P=1). On the other hand, risk of stroke in diabetics having AG+GG genotypes was 2.8 times

significantly higher than non-diabetic with same genotype (P=0.000). Risk of stroke in diabetic was 2.357 for all study population (Table 3.2). However, the risk increased to 2.804 in diabetic individuals when carrying risky G allele. For obesity and non-obesity group, the number of obese individuals with wild type (AA) genotype was 3.75 times than non-obesity (P=0.384). Individuals with AG+GG genotype in obesity had 4.817 times significantly higher risk for stroke than non-obesity (P=0.000). In smoker group, individuals having wild type genotype had significantly higher risk for stroke than non-smoker group (OR=12, 95%CI: 1.429-100.755 and P=0.009). Also for the other genotype group (AG+GG), risk of having stroke is 2.226 times significantly higher in smoker group than non-smoker (P=0.010). In briefly, the proportion of stroke patients to controls was significantly higher in smoker group when compared to non-smoker group for both of the genotype groups AA/AG+GG.

Logistic regression analysis was carried out to detect the effect of conventional risk factors such as hypertension, diabetes, smoking, obesity, sex, and age, lipid parameters (total cholesterol, low density lipoprotein, high density lipoprotein and triglycerides) and CYP24A1 rs927650 and CYP2R1 rs10741657 genotype polymorphisms on stroke. In the first model, whole population was included to the analysis. Results of the analysis showed that hypertension, smoking, obesity and LDL-cholesterol were the significant determinants of ischemic stroke also HDL-cholesterol had preventive effect on stroke. Same analysis was also performed just for male subject. Hypertension, smoking and obesity were found as significant determinants of ischemic stroke. In the third model including just female subject, hypertension, diabetes, smoking, LDL-cholesterol were found to be as significant predictors of ischemic stroke on the other hand HDL-cholesterol had protective effect for ischemic stroke.

CHAPTER 5

CONCLUSION

CYP24A1 and CYP2R1 have important roles in the metabolism of Vitamin D. Single nucleotide polymorphisms in these genes might be associated with change in vitamin D concentration. But, the effects of polymorphic variations in CYP24A1 and CYP2R1 genes encoding proteins involved in 1α-$25(OH)_2D_3$ homeostasis are not fully understood. One of the main causes of the stroke is atherosclerosis, which may be associated with the variable concentration of vitamin D level due to these polymorphisms. So, polymorphisms in the enzymes may be associated with the development of atherosclerosis and inevitably stroke.

256 ischemic stroke patients and 132 control subjects were included in the study population and there was no significant difference between two groups with regard to age and gender. When compared to control group, number of individuals with hypertensive, diabetics, obesity and smoking was significantly higher in ischemic stroke patients. Also, average concentrations of total cholesterol, triglyceride and LDL-cholesterol level were higher in patients than controls.

Risky T allele frequency was almost same among patients and control group for CYP24A1 rs927650 polymorphism. So, it might be said that having T allele does not pose

risk for stroke. For CYP2R1 rs10741657, the frequency of risky G allele frequency was almost same among patients and controls. Any significant risk factor was not found for different genotypes and alleles for ischemic stroke.

Sub-group analysis was also carried out for CYP24A1 rs10741657 SNP and CYP2R1 rs10741657 SNP among groups of hypertensive/normotensive, diabetic/non-diabetic, obesity/non-obesity and smoker/non-smoker. The analysis showed that the sub-groups did not pose risk for stroke when considered the rs927650 genotypes and rs10741657 genotypes (Table 3.5, Table 3.6, Table 3.7, Table 3.8).

Stratification analysis of CYP24A1 rs927650 showed that risk of smokers having ischemic stroke was 3.727 higher in individuals carrying risky T allele when compared to wild type genotype while this risk was 2.728 fold higher for smoker group than non-smoker in all study population. Therefore the stroke risk becomes higher when the individuals are smoker and have CT and TT genotype in CYP24A1 gene.

In CYP2R1 rs10741657 stratification analysis, we found that stroke risk for hypertensive individuals was significantly higher in individuals having G allele when compared to individuals with wild type genotype. Risk of having stroke in hypertensive individuals is 3.310 fold higher when compared to normotensive group for all study population. The stroke risk ratio increased to 3.419 for heterozygote or homozygote mutant individuals in hypertensive group. Therefore, we can conclude that, risky G allele carriers among hypertensives had increased stroke risk. Risk of diabetic group having stroke was 2.357 fold higher when compared to non-diabetic without any group regarding different genotypes. However risk of having stroke for diabetes group increased to 2.804 for individuals with AG+GG genotype. In the condition of obesity, having AG+GG genotypes was risk factor for stroke among obese individuals.

According to the logistic regression analysis, hypertension, smoking, obesity and LDL-cholesterol were revealed as the significant determinants of ischemic stroke when all population included to the analysis. When the analysis included just males, hypertension,

smoking and obesity were found as significant risk factors of ischemic stroke. Finally, the analysis carried out on female subjects, concluded that hypertension, diabetes, smoking and LDL-cholesterol were the significant predictors of ischemic stroke. HDL-cholesterol had protective effect against to stroke according to these analyses.

Despite the limited number of the patients, this study generates the hypothesis that the preventive effect of vitamin D in ischemic stroke can be affected by the genotypes of CY24A1 and CYP2R1. The study illustrate the biologic plausibility of a relationship between polymorphisms in CYP24A1 and CYP2R1 enzymes involved in vitamin D metabolism and ischemic stroke which is worth further investigation.

As a conclusion, in present study, CYP24A1 rs927650 and CYP2R1 rs10741657 polymorphisms were examined in terms of their effect on having ischemic stroke. There wasn't any direct association between the different genotype groups of CYP24A1 rs927650 and CYP2R1 rs10741657 polymorphisms and ischemic stroke. However, in subgroup analysis, some risk evaluation and relation was found when conventional risk factors and different genotypes are considered together. Increase in the number of studies related with this study is required to provide the utility of these SNPs in the fields of genetic testing and personalized medicine.

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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:

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 B

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 C

REAGENTS

The reagents used in human genomic DNA isolation from whole blood samples are,

1. TKME Buffer

Constituents of TKME buffer are 10 mM Tris-HCl at pH 7.6, 10 mM KCl, 4 mM $MgCl_2$, and 2 mM EDTA. 242.2 mg Tris, 149,1 mg KCl, 162,6 mg $MgCl_2$ and 148.9 mg EDTA were weighed and dissolved in 100 mL of dH_2O. pH was adjusted to 7.6 with HCl and volume was completed to 200 mL. After preparation, the solution is autoclaved for sterilization and stored at 4°C.

2. TE Buffer

100 mM Tris-HCl at pH 8.0 and 500 mM EDTA at pH 8.0 are used for solution. For preparation of 100 mL TE buffer,10 mL 100 mM Tris-HCl and 0.2 mL 500 mM EDTA was mixed and final volume was completed to 100 mL with dH2O.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 dH2O. 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 Na2EDTA.2H2O 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.

<u>Reagents used in PCR:</u>

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 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 used in agarose restriction endonuclease digestion:

1. **1X NE Buffer 3.1**

The buffer is obtained commercial with restriction enzyme Bg1II. The buffer includes 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$ and 100 µg/ml BSA at pH 7.9. The solution is stored at -20°C.

2. **1X CutSmart Buffer®**

The buffer is obtained commercial with restriction enzyme MnII. The buffer includes 10 Mm Magnesium Acetate, 20 mM Tris-acetate, 50 mM Potassium Acetate and 100 µg /mL BSA at pH 7.9. The solution is stored at -20°C.

APPENDIX D

LIST OF STUDY POPULATION

Table D.1 List of study population composed of 256 stroke patients and 132 controls including demographic characteristics, lipid parameters, CYP241 rs927650 and CYP2R1 rs10741657 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 rs927650 polymorphism; CT: Heterozygous for rs927650 polymorphism; TT: Homozygous mutant for rs927650 polymorphism; AA: Wild Type Homozygous for rs10742657 polymorphism; AG: Heterozygous for rs10741657 polymorphism; GG: Homozygous mutant for rs10741657 polymorphism

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
1.	C	76	M	Y	Y	N	N	3,43	1,05	1,9	1	CC	AG
2.	C	51	M	N	N	Y	N	3,69	1,29	2,13	0,9	CC	GG
3.	C	50	W	N	N	N	N	3,61	2,02	1,87	0,77	CT	AG
4.	C	42	W	N	N	N	N	2,7	1,27	1,46	0,62	CC	AG
5.	C	45	W	N	N	Y	N	3,67	0,6	1,87	1,46	CT	GG
6.	C	63	M	Y	Y	Y	N	3,04	1,12	1,54	0,95	CC	AG
7.	C	63	W	N	N	N	N	5,2	2,48	3,13	0,87	CC	AG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
8.	C	75	M	Y	Y	N	N	4,73	1,72	2,56	1,31	TT	GG
9.	C	58	W	Y	N	N	N	2,7	1,09	1,1	1,08	CT	AA
10.	C	78	W	Y	N	N	N	3,61	1,07	1,82	1,26	CT	GG
11.	C	74	M	N	N	N	N	4,34	0,92	2,56	1,31	CT	AA
12.	C	61	M	N	N	N	N	5,93	1,87	3,97	1,03	CC	AG
13.	C	85	M	Y	N	N	N	3,09	1,8	0,62	1,62	CT	GG
14.	C	65	W	N	Y	N	N	6,11	1,42	3,23	2,15	CT	AA
15.	C	65	M	N	N	N	N	4,97	0,64	3,28	1,33	CT	GG
16.	C	58	M	N	Y	Y	N	5,95	1,2	4,33	1	CT	AA
17.	C	61	W	N	N	N	Y	6,81	1,83	4,36	1,51	CT	AG
18.	C	80	W	Y	N	N	N	6,06	2,27	2,13	0,62	CT	AA
19.	C	80	W	N	N	N	N	3,35	0,71	1,18	1,82	CT	AA
20.	C	67	W	Y	Y	N	N	3,93	1,43	2,18	1,05	TT	AG
21.	C	76	M	N	N	N	N	6,01	2,32	3,67	1,21	CT	AG
22.	C	66	M	Y	Y	N	N	4,11	1,43	2,51	0,9	CT	GG
23.	C	69	W	Y	N	N	N	3,93	1,76	1,9	1,18	CC	GG
24.	C	66	M	N	Y	Y	N	6,97	3,92	4,1	0,97	CT	GG
25.	C	60	W	Y	N	N	N	4,97	1,38	3,1	1,15	CT	GG
26.	C	71	M	N	N	N	N	4,29	1,57	2,05	1,41	CT	GG
27.	C	68	M	N	N	N	N	5,46	1,29	3,15	1,64	CT	GG
28.	C	65	W	N	N	N	N	5,2	1,76	3,15	1,18	CT	AA
29.	C	72	W	Y	N	N	N	6,08	1,67	4,05	1,18	CC	AG
30.	C	65	M	N	N	N	N	3,04	0,9	1,64	0,95	CT	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
31.	C	63	W	Y	Y	N	Y	5,02	3,69	1,69	1,1	CC	GG
32.	C	70	W	N	N	N	N	4,86	1,48	3,03	1,1	CC	AA
33.	C	70	W	Y	N	N	N	5,25	1,18	3,33	1,31	CT	AG
34.	C	65	W	Y	N	N	N	4,52	1,27	2,18	1,69	CT	GG
35.	C	78	W	Y	N	N	N	4,39	1,25	2,39	1,39	CC	AA
36.	C	47	M	N	N	N	N	6,27	3,06	3,33	1,46	CT	AA
37.	C	77	W	Y	Y	N	N	4,06	0,98	2,64	0,92	CT	AA
38.	C	71	M	N	N	N	N	4,37	1,47	2,31	1,33	CC	AG
39.	C	73	M	Y	N	N	N	3,64	2,47	1,95	0,51	CT	AG
40.	C	61	M	N	N	Y	N	4,68	0,39	2,92	1,51	CC	GG
41.	C	52	M	N	N	N	N	6,94	1,16	4,69	1,62	CT	AA
42.	C	65	M	N	N	N	Y	4,16	0,85	2,9	0,82	CT	AA
43.	C	50	W	Y	N	N	N	3,2	0,85	2,15	0,62	CT	AA
44.	C	87	W	Y	N	N	Y	2,76	1,02	1,39	0,87	CT	AG
45.	C	67	M	N	N	N	N	4,37	1,15	2,1	0,15	CT	AG
46.	C	38	M	N	N	Y	N	4,78	0,56	2,85	1,62	CT	GG
47.	C	50	M	N	N	N	N	3,82	0,62	2,03	1,46	TT	AG
48.	C	80	W	N	Y	N	N	3,98	1,38	1,97	1,31	CT	AA
49.	C	44	M	N	N	Y	N	5,2	2,38	2,46	1,59	CC	GG
50.	C	51	W	N	N	N	N	4,37	0,57	2,82	1,23	CC	AG
51.	C	67	M	Y	N	N	N	4,47	1,66	2,59	1,05	CC	GG
52.	C	73	W	Y	Y	N	N	5,9	2,32	3,67	1,1	CC	AG
53.	C	88	W	Y	N	N	N	4	0,8	2,56	1,03	CT	AG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
54.	C	59	M	N	N	N	N	4,08	1,24	2,31	1,15	CC	AG
55.	C	69	W	N	Y	N	N	6,27	1,34	4,03	1,54	CC	AA
56.	C	51	W	N	N	N	N	2,86	1,01	1,39	0,97	CC	AG
57.	C	77	M	N	N	N	N	4,65	1,88	2,05	1,18	CT	AG
58.	C	52	W	N	N	N	N	3,82	1,12	1,87	1,39	CT	AA
59.	C	77	W	Y	N	N	N	4,21	2	2,03	1,21	CT	GG
60.	C	79	M	N	N	N	N	5,25	0,87	3,85	0,95	CT	AG
61.	C	46	M	Y	N	Y	N	7,9	2,83	5,31	1,21	CT	GG
62.	C	38	M	N	N	Y	N	4,63	1,45	2,56	1,33	CT	AG
63.	C	41	W	N	N	N	N	3,9	1,46	2,15	1,03	CT	GG
64.	C	48	W	N	N	N	Y	2,6	0,97	1,41	0,72	CT	GG
65.	C	43	W	N	N	Y	N	5,77	1,84	3,51	1,33	CT	GG
66.	C	45	W	N	N	N	N	5,23	2,75	2,59	1,31	CC	GG
67.	C	38	W	N	N	N	N	5,72	1,29	3,56	1,49	CT	AG
68.	C	66	M	N	N	N	N	5,3	0,7	3,46	1,46	CT	GG
69.	C	69	W	Y	N	N	N	4,86	1,17	2	1,21	CT	GG
70.	C	42	M	N	N	Y	N	4,63	2,74	2,46	0,85	CT	GG
71.	C	65	M	Y	N	N	N	3,85	1,29	1,95	1,26	CT	AG
72.	C	87	M	Y	Y	N	N	2,7	1,24	0,97	1,13	CT	GG
73.	C	75	W	N	N	Y	Y	4,78	0,91	2,9	1,41	CT	AG
74.	C	77	M	Y	N	N	N	3,61	0,67	2,44	0,82	CT	GG
75.	C	69	W	N	N	N	N	4,63	1,29	2,72	1,26	CT	AG
76.	C	65	M	N	N	N	N	4,13	0,7	2,64	1,13	CC	AA

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
77.	C	78	M	N	N	N	N	4,11	0,92	2,23	1,41	CT	GG
78.	C	81	M	Y	N	N	N	2,81	0,61	1,23	1,26	TT	AG
79.	C	56	M	N	Y	N	N	4,6	1,37	2,82	1,1	CT	AA
80.	C	64	W	Y	Y	N	N	4,32	2,76	2,21	0,8	CC	AG
81.	C	79	W	Y	N	N	N	4,71	1,24	2,82	1,26	TT	GG
82.	C	67	W	N	N	N	N	7,75	1,15	5,85	1,28	CT	AA
83.	C	76	W	Y	N	N	N	4,32	1,24	2,18	1,51	CC	GG
84.	C	64	M	N	N	Y	N	3,38	1,02	2,21	0,67	CC	AG
85.	C	77	M	N	Y	N	N	8,79	1,78	6,23	1,62	TT	GG
86.	C	75	M	N	N	N	N	4,97	1,67	2,8	1,33	CT	AG
87.	C	68	W	Y	N	N	Y	4,84	1,91	2,49	1,41	CT	AG
88.	C	71	W	Y	N	N	N	3,87	1,09	2,31	1,03	CT	AG
89.	C	52	W	N	N	N	N	4	0,8	2,56	1,03	CC	AG
90.	C	78	M	Y	N	N	N	4,13	1,39	2,49	0,95	CT	GG
91.	C	80	M	Y	N	N	N	5,12	0,97	3,23	1,39	CT	AG
92.	C	78	W	N	N	N	N	4,08	0,94	2,51	1,05	CC	GG
93.	C	57	W	N	N	N	N	2,44	1,05	0,49	1,44	CC	GG
94.	C	60	W	Y	N	N	N	5,36	2,23	2,87	1,39	CT	AA
95.	C	63	W	Y	N	N	N	6,68	2,76	4,1	1,23	TT	GG
96.	C	58	M	Y	N	N	N	3,9	2,25	2,05	0,77	CT	GG
97.	C	54	M	Y	N	N	N	4,32	1,16	2,41	1,31	CT	GG
98.	C	78	M	N	N	N	N	3,69	0,67	2,05	1,28	TT	GG
99.	C	75	M	Y	Y	N	N	4,81	0,8	2,95	1,44	CT	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
100.	C	77	M	Y	N	N	N	3,07	0,99	1,64	0,92	CT	GG
101.	C	59	W	N	N	N	N	5,54	1,42	3,23	1,59	TT	GG
102.	C	57	W	N	N	N	N	4,37	1,87	2,56	0,9	TT	GG
103.	C	79	W	Y	Y	N	N	4,24	1,65	2,21	1,23	CT	AG
104.	C	52	W	N	N	N	N	6,06	1,28	4,18	1,21	CC	GG
105.	C	79	W	Y	N	N	N	1,53	1,11	0,59	0,41	CC	GG
106.	C	54	M	N	Y	N	N	7,33	2,47	4,9	1,21	CT	AG
107.	C	67	W	N	N	N	N	4,16	0,79	2,36	1,33	CT	GG
108.	C	50	W	N	N	N	N	3,41	2,76	1,74	0,36	CT	GG
109.	C	90	W	N	Y	N	N	5,56	1,64	3,41	1,33	CT	AG
110.	C	77	W	Y	N	N	N	8,16	1,94	5,18	1,97	CT	AA
111.	C	57	M	Y	Y	N	N	4,52	0,55	2,64	1,56	CC	AG
112.	C	68	M	N	N	N	N	3,54	0,58	1,85	1,39	CT	GG
113.	C	77	M	Y	Y	Y	N	5,23	0,91	3,26	1,49	CT	AG
114.	C	60	M	Y	N	N	N	4	1	2,36	1,13	CC	GG
115.	C	44	M	N	N	N	Y	4,45	1,23	2,82	1	TT	GG
116.	C	52	W	Y	N	N	N	5,2	1,94	2,85	1,39	CC	GG
117.	C	58	W	N	N	N	N	4,08	1,67	2,31	0,95	CT	AG
118.	C	79	M	Y	Y	N	N	5,17	1,94	3,31	0,9	CC	GG
119.	C	73	W	N	N	N	N	5,28	1,25	3,62	1,03	CC	GG
120.	C	55	W	N	N	N	N	4,5	1,16	2,54	1,36	CT	AG
121.	C	52	W	N	N	N	N	4,42	1,37	2,69	1,08	CT	AG
122.	C	51	M	N	N	N	N	3,95	1,18	2,36	1,03	CT	AG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
123.	C	66	M	N	N	N	N	5,04	0,85	3,28	1,31	CC	GG
124.	C	66	W	N	N	N	N	6,32	1,53	4,15	1,39	CC	AG
125.	C	54	M	N	N	N	N	5,46	1,88	3,26	1,36	CT	GG
126.	C	55	W	N	N	N	N	6,45	2,76	3,97	1,13	TT	GG
127.	C	61	M	N	N	Y	N	6,19	1,49	4,1	1,28	CC	GG
128.	C	74	M	N	N	N	N	6,45	2,01	4,21	1,23	CC	AG
129.	C	52	M	N	N	N	N	5,64	2,01	3,31	0,92	CT	GG
130.	C	53	M	N	N	N	N	6,47	2,2	3,64	1,74	CT	GG
131.	C	64	W	N	N	N	N	6,86	1,48	4,44	1,67	CC	AG
132.	C	62	W	N	N	N	N	5,59	1,88	3,31	1,36	CT	AG
133.	P	75	M	Y	Y	Y	N	3,09	1,28	1,85	0,62	CC	AA
134.	P	57	W	Y	Y	N	N	5,46	1,53	3,39	1,31	CT	GG
135.	P	41	M	N	N	Y	N	4,32	1,57	2,67	0,87	TT	AA
136.	P	73	M	Y	N	N	N	3,72	0,72	1,9	1,46	CC	GG
137.	P	53	M	Y	Y	N	N	10,43	2,6	7,59	1,51	TT	AG
138.	P	66	M	Y	Y	Y	N	3,38	1,42	2,18	0,51	CC	AG
139.	P	56	W	Y	Y	N	N	3,59	2,38	1,8	0,97	CT	GG
140.	P	54	M	Y	N	Y	N	5,2	2,44	2,95	1,08	CT	GG
141.	P	67	W	Y	N	N	N	5,43	1,19	3,56	1,26	TT	AG
142.	P	76	M	Y	N	Y	N	4,03	0,76	2,33	1,28	CC	AG
143.	P	78	W	Y	Y	N	N	3,69	1,54	2,03	0,92	CC	AG
144.	P	75	W	Y	N	N	N	4,73	1,17	3,26	0,87	CT	GG
145.	P	74	W	Y	Y	N	N	4,34	0,7	2,74	1,23	CT	AG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
146.	P	68	W	Y	N	Y	N	3,64	2,27	1,92	0,64	CT	AG
147.	P	72	W	N	N	N	N	6,66	2,84	4,23	1,03	CC	AG
148.	P	81	W	Y	N	N	N	4,89	1,75	2,95	1,08	CT	GG
149.	P	73	W	Y	Y	N	N	5,38	2,27	3,21	1,08	TT	AG
150.	P	73	W	Y	N	N	Y	4,68	0,99	2,74	1,41	CT	GG
151.	P	67	W	Y	N	N	N	5,41	1,39	3,67	1,03	CC	AA
152.	P	61	M	Y	Y	N	N	4,39	1,37	2,77	0,95	TT	GG
153.	P	40	M	N	N	Y	N	3,77	1,17	2	1,21	CT	GG
154.	P	60	W	Y	Y	N	Y	4,99	1,69	3,05	1,1	CT	GG
155.	P	75	M	N	N	N	N	4,55	1,08	1,82	2,18	CC	AG
156.	P	76	W	Y	N	N	N	3,22	1,52	1,28	1,18	TT	AG
157.	P	70	M	Y	N	N	N	3,02	0,82	1,51	1,08	CT	GG
158.	P	76	M	N	N	Y	N	3,33	1,39	1,87	0,77	CT	GG
159.	P	71	M	Y	N	N	N	5,38	2,61	3,23	0,9	CC	AG
160.	P	61	M	N	Y	N	N	3,85	1,62	1,95	1,1	CT	AG
161.	P	64	W	N	N	Y	Y	4,32	1,06	2,54	1,23	CC	GG
162.	P	58	W	Y	Y	N	N	9,1	4,05	5,85	1,28	CC	GG
163.	P	74	W	Y	N	N	Y	6,27	1,73	4,15	1,23	TT	GG
164.	P	80	W	Y	N	N	Y	3,77	0,97	2,31	0,97	CT	GG
165.	P	62	M	Y	Y	N	N	4,89	1,2	3,28	1	CC	AG
166.	P	63	M	Y	N	N	N	3,41	1	1,85	1,05	CT	GG
167.	P	68	M	Y	N	N	N	4,45	0,9	2,44	1,54	CT	GG
168.	P	77	W	Y	N	N	N	5,07	1,07	3,51	1	CC	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
169.	P	80	W	Y	N	N	N	5,23	1,06	3,08	1,59	CT	GG
170.	P	55	M	Y	N	N	N	4,08	1,39	2,44	0,95	CC	GG
171.	P	62	M	N	N	N	N	3,69	1,01	1,39	1,1	CT	GG
172.	P	77	M	N	N	N	N	3,82	0,61	1,74	1,74	CT	AG
173.	P	24	M	N	N	N	N	6,11	2,87	3,67	1,05	TT	AG
174.	P	53	W	N	N	Y	N	2,5	2,16	0,59	0,9	CT	AA
175.	P	61	M	N	N	N	N	4,84	1,6	3,1	0,95	CC	AA
176.	P	78	M	Y	N	Y	N	5,23	2,06	3,08	1,13	TT	GG
177.	P	81	M	Y	N	N	N	3,46	3,03	0,95	1,08	CT	GG
178.	P	80	W	Y	N	N	Y	2,73	1,27	1,46	0,64	CC	GG
179.	P	75	W	Y	N	N	N	2,86	0,65	1,33	1,18	CC	AA
180.	P	26	M	N	N	N	N	4,21	1,07	2,05	1,62	CC	AG
181.	P	55	M	Y	N	N	N	5,04	0,93	3,15	1,39	CC	AA
182.	P	26	M	N	N	N	N	4,06	0,72	2,92	0,74	CC	AG
183.	P	73	W	Y	N	N	N	4	1,14	2,54	0,9	CC	AG
184.	P	36	M	N	N	N	N	4,86	1,57	2,97	1,1	TT	AA
185.	P	56	M	N	N	N	N	4,34	1,23	2,56	1,15	CT	AA
186.	P	47	W	N	N	N	N	4,78	1,38	3,15	0,92	CT	AA
187.	P	81	W	Y	Y	N	N	3,61	2,17	1,77	0,8	CT	AG
188.	P	73	M	Y	Y	N	N	5,15	2,52	3,03	0,9	CT	AG
189.	P	73	W	Y	N	N	N	4	1,14	2,54	0,9	CC	AG
190.	P	74	W	Y	N	N	N	5,98	1,43	3,95	1,31	CC	GG
191.	P	66	M	Y	Y	N	N	3,35	2,85	1,26	0,74	CC	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
192.	P	61	W	Y	Y	N	N	3,22	2	1,62	0,64	TT	AA
193.	P	78	M	N	N	Y	N	3,38	1,24	1,8	1,03	CC	AG
194.	P	80	M	Y	N	N	N	7,9	0,83	6,13	1,28	TT	AG
195.	P	76	W	Y	Y	N	N	4,11	1,24	2,51	0,97	CC	GG
196.	P	79	W	Y	N	N	N	5,25	1,53	3,31	1,18	CC	AG
197.	P	21	M	N	N	N	N	3,87	0,93	2,56	0,82	TT	GG
198.	P	76	M	N	N	N	Y	5,02	1,19	3,39	1,03	CC	AG
199.	P	28	M	N	N	Y	N	3,93	1,64	2	1,13	CC	GG
200.	P	20	M	N	N	Y	N	4,32	0,9	2,56	1,28	CT	AA
201.	P	80	W	Y	N	N	N	5,02	2,82	2,21	1,46	CT	GG
202.	P	64	W	Y	N	N	N	3,77	1,62	1,97	1	TT	GG
203.	P	71	M	N	N	Y	N	4,94	0,91	3,39	1,08	CT	AG
204.	P	67	W	Y	Y	N	Y	10,17	2,54	7,62	1,26	CT	AG
205.	P	58	W	Y	Y	N	N	7,12	2,88	4,33	1,39	CC	AG
206.	P	49	M	N	N	Y	N	5,88	2,29	3,56	1,18	CT	GG
207.	P	78	W	N	N	N	N	4,63	0,99	2,64	1,46	CT	AG
208.	P	65	M	N	N	N	N	4	1,84	2,03	1,08	CT	GG
209.	P	75	M	Y	N	N	N	5,07	1,6	3,33	0,95	CC	AG
210.	P	79	M	N	N	Y	N	2,73	0,9	1,39	0,9	CT	AA
211.	P	73	W	Y	Y	N	N	7,57	3,54	5,26	0,59	CT	GG
212.	P	25	M	N	N	N	N	4,6	1,57	2,74	1,1	CC	GG
213.	P	73	M	Y	N	N	N	5,07	0,89	3,05	1,54	CT	GG
214.	P	74	M	Y	N	N	N	3,33	1,33	1,8	0,87	TT	AG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
215.	P	78	W	Y	Y	N	Y	4,71	1,55	2,8	1,13	CT	AG
216.	P	56	M	Y	Y	N	N	4,6	1,76	2,69	1,05	CT	AG
217.	P	67	M	Y	N	N	N	4,29	1,73	2,28	1,1	CT	AA
218.	P	74	M	Y	N	N	N	4,39	2,26	2,03	1,28	CT	AG
219.	P	64	M	Y	Y	N	N	4,37	1,91	2,39	1,05	TT	AG
220.	P	73	M	Y	Y	N	N	4,63	1,94	2,56	1,1	CT	AA
221.	P	57	M	N	N	Y	N	4,86	1,67	2,97	1,05	TT	GG
222.	P	76	M	N	N	N	N	4,19	1,92	2,41	0,85	TT	AG
223.	P	61	M	N	N	Y	N	4,86	1,39	2,97	1,18	CT	GG
224.	P	62	W	N	Y	N	N	4,08	1,63	2,33	0,95	CC	GG
225.	P	73	M	N	N	Y	N	7,9	2,98	5,23	1,21	CT	AA
226.	P	63	M	N	N	Y	N	3,46	1,16	1,69	1,18	TT	AG
227.	P	52	M	N	N	Y	N	4,11	1,1	2,77	0,8	CT	GG
228.	P	61	W	Y	N	N	N	3,15	1,39	1,59	0,87	CT	GG
229.	P	45	M	N	N	Y	N	3,93	3,74	1,31	0,85	CC	GG
230.	P	64	M	N	N	Y	N	4,32	1,09	2,64	0,92	CT	GG
231.	P	56	W	N	Y	N	N	7,02	3,19	4,33	1,13	CC	AG
232.	P	67	M	Y	Y	N	N	6,92	2,84	4,39	1,13	CT	AA
233.	P	53	M	Y	N	N	N	7,38	3,39	4,51	1,23	CC	GG
234.	P	80	M	N	N	N	N	5,72	1,34	3,74	1,28	CT	GG
235.	P	62	M	Y	Y	N	N	5,56	1,24	3,67	1,18	CT	GG
236.	P	80	M	N	N	N	N	5,28	1,69	3,15	1,28	CT	GG
237.	P	61	M	N	N	Y	N	3,41	1,35	1,92	0,87	CT	AG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
238.	P	67	W	N	Y	N	N	4,94	1,52	3,21	0,97	TT	GG
239.	P	65	W	N	N	Y	N	5,77	1,18	3,82	1,33	CC	AG
240.	P	79	W	Y	Y	N	N	4,26	1,69	2,64	0,8	CT	AG
241.	P	61	M	Y	N	Y	N	4,71	1,2	3,05	1,05	CT	AG
242.	P	36	W	N	N	Y	N	6,4	1,94	3,8	1,62	CT	AG
243.	P	80	M	Y	Y	N	N	7,51	4,24	4,44	1,05	CC	GG
244.	P	61	M	N	Y	Y	N	3,72	1,15	2,15	1	CT	GG
245.	P	69	M	Y	N	N	N	4,06	0,87	2,69	0,92	TT	GG
246.	P	76	W	Y	Y	N	N	4,11	0,76	2,67	1,03	CT	AG
247.	P	69	W	N	N	N	N	3,2	1,11	1,8	0,85	CT	GG
248.	P	77	M	Y	Y	Y	N	3,85	0,82	2,62	0,8	CC	GG
249.	P	82	M	N	N	N	N	3,77	1,66	2,05	0,9	CC	AG
250.	P	78	W	Y	N	N	N	4,89	0,7	3,18	1,33	CC	AA
251.	P	58	W	N	N	N	N	4,32	1,1	2,41	1,33	CT	AG
252.	P	59	W	Y	Y	N	N	5,2	1,88	3,41	0,85	CT	AG
253.	P	53	M	N	N	Y	N	3,61	0,37	2,21	1,18	CT	GG
254.	P	54	M	N	N	N	N	4,42	0,66	3,1	0,95	CC	AG
255.	P	75	W	N	Y	N	N	4,91	0,98	3,03	1,39	CC	GG
256.	P	78	M	Y	Y	N	N	4,24	1,52	2,56	0,92	TT	AG
257.	P	40	W	N	N	Y	Y	4,99	1,06	3,26	1,18	CT	GG
258.	P	48	M	Y	N	N	N	5,49	0,81	3,77	1,28	CC	GG
259.	P	80	W	Y	Y	N	N	4,78	0,87	2,87	1,39	CC	AG
260.	P	41	M	N	N	N	N	3,77	1,35	2,26	0,85	CC	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
261.	P	77	W	Y	Y	N	N	4,99	1,45	3,15	1,1	TT	AG
262.	P	63	W	Y	Y	N	Y	3,93	0,78	2,56	0,95	CT	AA
263.	P	55	M	Y	N	Y	N	7,2	1,83	5,31	0,95	CC	GG
264.	P	79	M	Y	Y	N	N	3,12	0,76	1,56	1,15	CC	AA
265.	P	67	W	Y	Y	N	N	5,12	1,43	2,92	1,49	CC	GG
266.	P	63	M	Y	Y	N	N	3,77	1,76	2,13	0,8	CC	GG
267.	P	75	W	Y	N	N	N	5,93	1,9	3,9	1,08	CC	AG
268.	P	54	W	N	N	N	N	5,23	3,38	2,92	0,69	CC	GG
269.	P	61	W	Y	N	N	N	4,68	1,24	3,15	0,9	CC	GG
270.	P	71	M	Y	N	N	N	3,25	0,8	1,87	0,97	CC	GG
271.	P	74	W	Y	N	N	N	5,49	1,85	3,15	1,41	CC	AG
272.	P	59	M	N	N	N	N	3,33	1,43	1,54	0,59	TT	AG
273.	P	62	M	N	N	Y	N	4	0,79	2,64	0,95	CC	GG
274.	P	82	W	Y	Y	N	N	4,65	1,18	3,08	0,97	CC	AG
275.	P	70	M	Y	N	N	N	5,15	0,85	3,44	1,26	CT	AG
276.	P	69	M	Y	N	N	N	4,42	0,93	2,85	1,08	CT	AG
277.	P	57	W	N	Y	N	N	5,46	1,73	3,46	1,13	CT	AG
278.	P	80	M	Y	N	N	N	3,54	0,7	2	1,18	CT	AG
279.	P	74	M	N	Y	N	N	3,38	0,73	1,67	1,33	CT	AG
280.	P	50	M	Y	N	N	N	2	0,84	0,62	0,97	CT	AG
281.	P	57	M	N	Y	N	N	5,02	1,43	3,56	0,74	CT	GG
282.	P	62	M	Y	N	N	N	5,07	2,62	3,05	0,74	CT	AG
283.	P	81	W	Y	Y	N	N	4,19	0,96	2,46	1,23	CC	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
284.	P	77	W	Y	Y	N	N	3,02	2,1	1,56	0,46	TT	AA
285.	P	54	M	Y	Y	N	N	4,13	1,34	2,85	0,62	CT	GG
286.	P	82	W	Y	Y	N	N	4,34	1,01	2,62	1,21	CC	GG
287.	P	71	W	Y	Y	N	N	4,24	1,93	2,33	0,97	CT	GG
288.	P	80	W	Y	N	N	N	4,29	1,56	2,31	1,21	TT	AG
289.	P	69	M	Y	Y	N	N	5,38	2,51	2,8	1,36	CT	GG
290.	P	54	W	Y	Y	N	N	5,15	1,2	2,59	1,39	CT	GG
291.	P	70	W	Y	N	N	N	4,71	1,63	2,44	1,46	CC	GG
292.	P	31	W	N	N	N	N	6,4	3,01	3,62	1,31	CT	AG
293.	P	55	W	N	N	N	N	7,1	0,7	4,87	1,82	CT	AA
294.	P	71	M	Y	Y	N	N	5,62	2	3,74	0,87	CC	GG
295.	P	77	M	Y	Y	N	N	4,58	2,17	2,36	1,15	CT	GG
296.	P	74	W	Y	Y	N	N	7,41	3,11	4,44	1,46	CC	AG
297.	P	47	M	Y	Y	N	N	4,55	1,45	2,44	1,39	CT	GG
298.	P	67	M	Y	Y	N	N	5,51	0,98	3,87	1,13	CC	AG
299.	P	69	W	N	N	N	N	6,45	1,65	4,36	1,26	CT	GG
300.	P	74	M	N	N	Y	N	4	1,08	2,72	0,74	CC	GG
301.	P	71	M	Y	Y	Y	N	5,95	2,63	3,62	1,05	CC	GG
302.	P	74	M	Y	N	N	N	3,64	1,37	2,05	0,92	CC	GG
303.	P	76	W	Y	Y	N	N	4,42	1,96	2,21	1,26	CC	GG
304.	P	68	W	Y	Y	Y	N	3,04	1,23	1,41	1,03	CC	GG
305.	P	68	W	Y	N	N	N	5,33	1,03	3,62	1,18	CT	AG
306.	P	74	W	Y	N	N	Y	3,9	0,79	2,49	1	CC	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
307.	P	57	M	Y	Y	Y	N	6,16	2,84	3,9	0,87	CT	AA
308.	P	34	W	N	N	N	N	5,8	1,96	2,64	0,62	CT	GG
309.	P	61	M	Y	N	N	N	4,58	1,48	2,92	0,92	CT	AG
310.	P	62	M	N	N	N	N	4,71	2,11	2,69	0,97	TT	AA
311.	P	62	M	Y	N	N	Y	5,69	1,52	3,8	1,13	CT	GG
312.	P	63	W	Y	Y	N	N	6,4	1,84	4,51	0,95	CT	AG
313.	P	68	M	Y	N	Y	Y	4,86	1,06	3,59	0,72	CT	AA
314.	P	71	W	N	N	N	N	5,95	1,1	4,44	0,92	CT	AG
315.	P	70	M	Y	Y	Y	N	5,59	1,01	3,82	1,23	CT	AG
316.	P	69	W	Y	N	N	N	5,36	0,52	3,82	1,23	CC	AG
317.	P	71	M	Y	N	N	N	5,67	1,38	4,08	0,87	CC	GG
318.	P	79	M	Y	Y	Y	Y	4,37	1,43	3,05	0,62	CT	GG
319.	P	38	M	Y	N	Y	N	6,63	1,69	4,72	1,05	CT	AA
320.	P	71	W	Y	N	N	Y	5,25	1,42	3,21	1,33	CT	AG
321.	P	73	M	Y	N	Y	Y	6,37	1,28	4,54	1,15	CT	GG
322.	P	63	M	N	N	Y	Y	4,47	0,78	2,59	1,46	CT	GG
323.	P	30	M	N	N	N	Y	4,03	1,33	2,49	0,87	CT	GG
324.	P	68	M	Y	N	Y	Y	4,55	1,12	3,03	0,95	CT	AG
325.	P	61	W	Y	N	N	Y	4,94	1,29	2,56	1,64	TT	GG
326.	P	75	M	N	N	N	N	3,64	0,69	2,26	1,03	CC	GG
327.	P	42	M	N	N	N	N	5,98	0,88	4,28	1,21	CT	GG
328.	P	59	W	Y	N	N	Y	6,81	1,82	4,05	1,85	CT	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
329.	P	73	M	Y	N	Y	Y	6,19	1,97	3,9	1,31	CT	GG
330.	P	78	M	N	N	N	Y	4,71	1,33	3,1	0,92	CT	GG
331.	P	71	M	Y	Y	Y	Y	5,72	3,78	3,69	1	CT	AG
332.	P	76	W	Y	N	Y	Y	5,12	2,07	3,33	0,77	CT	GG
333.	P	74	W	Y	Y	N	Y	5,62	3,34	3,41	0,62	CC	AG
334.	P	79	M	Y	Y	N	Y	5,07	1,08	3,77	0,74	CT	GG
335.	P	59	M	Y	N	N	Y	4,39	1,28	2,8	0,95	TT	GG
336.	P	70	W	Y	Y	N	Y	5,95	1,8	4	1,05	TT	AG
337.	P	21	M	N	N	N	N	4,13	1,02	2,51	1,1	CT	AG
338.	P	52	M	N	N	N	N	5,28	0,69	3,33	1,31	TT	AA
339.	P	77	W	Y	Y	Y	Y	6,79	1,64	4,74	1,21	CT	AG
340.	P	77	M	Y	N	Y	N	3,33	1,61	1,9	1,05	CT	GG
341.	P	79	W	Y	N	N	N	4,55	0,81	3,05	1,08	CT	AG
342.	P	55	M	Y	N	Y	Y	5,82	3,83	3,15	0,85	CT	AG
343.	P	68	M	Y	N	Y	Y	6,34	1,18	4,64	1,08	CT	AG
344.	P	79	W	Y	Y	N	Y	6,42	1,71	4,62	0,95	TT	GG
345.	P	73	M	Y	N	Y	Y	4,37	1,61	2,59	1	TT	AG
346.	P	70	M	Y	Y	N	Y	6,6	2,18	4,51	1	CT	AG
347.	P	73	W	Y	Y	N	N	3,35	1,08	2,18	0,64	CC	AA
348.	P	79	W	Y	N	Y	Y	6,63	1,03	3,97	2,1	CT	AA
349.	P	81	W	N	N	N	N	5,64	2,35	3,54	0,95	CC	GG
350.	P	79	M	N	Y	N	Y	7,88	0,66	6,1	1,36	CT	GG
351.	P	66	M	N	Y	Y	N	3,95	4,45	1,31	0,56	TT	GG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
352.	P	75	M	N	N	Y	Y	6,47	2,78	4	1,13	TT	AG
353.	P	64	M	Y	N	Y	Y	2,81	0,87	1,77	0,62	CT	AA
354.	P	81	M	N	N	Y	Y	2,52	0,83	1,46	0,64	CT	GG
355.	P	72	W	Y	N	Y	N	5,07	1,08	3,18	1,39	CT	GG
356.	P	64	W	Y	Y	N	Y	4,55	0,92	3,1	0,97	CT	GG
357.	P	71	M	Y	Y	Y	Y	4,32	1,16	3,03	0,69	CT	GG
358.	P	64	W	N	N	N	Y	5,95	1,26	4,18	1,13	CT	AG
359.	P	51	M	N	N	Y	Y	4,52	1,92	2,92	0,67	CT	AG
360.	P	65	M	Y	Y	Y	Y	5,38	3,75	2,49	1,1	CT	GG
361.	P	60	W	N	N	Y	N	5,88	1,2	4	1,26	CT	AG
362.	P	53	M	N	N	Y	Y	5,33	1,82	3,31	1,13	CT	AG
363.	P	63	W	N	N	Y	Y	6,21	2,11	3,8	1,36	CT	AG
364.	P	77	W	N	N	N	N	5,07	1,25	3,28	1,15	CT	AG
365.	P	81	W	Y	N	N	Y	7,51	0,75	5,51	1,56	CT	AG
366.	P	71	W	N	Y	N	Y	3,51	1,08	2,03	0,95	CT	AG
367.	P	75	M	Y	N	Y	Y	4,97	1,11	2,92	0,92	CT	AG
368.	P	65	M	N	Y	Y	Y	5,41	2,1	3,33	1,13	CT	AG
369.	P	72	W	Y	Y	N	N	4,26	1,58	2,39	1,1	CT	AG
370.	P	70	W	Y	N	N	Y	5,54	0,96	3,39	1,64	CC	AG
371.	P	57	M	N	N	N	Y	6,66	1,17	4,62	1,41	CT	AG
372.	P	70	M	Y	Y	N	Y	3,77	1,28	2,36	0,77	CT	GG
373.	P	67	W	N	Y	N	N	4,86	1,57	3,36	0,72	CT	AG
374.	P	82	W	N	N	Y	N	4,97	1,29	3,21	1,1	CT	AG

Table D.1 (Continued)

Sample No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total cholesterol	Triglyceride	LDL	HDL	rs927650 SNP	rs10741657 SNP
375.	P	76	W	N	N	N	Y	5,98	1,96	4,03	0,97	CT	GG
376.	P	32	M	Y	N	Y	N	3,8	1,24	2,28	0,9	CT	AG
377.	P	52	W	N	N	N	N	5,64	1,62	3,64	1,18	CC	GG
378.	P	80	M	Y	N	N	N	3,2	0,53	1,69	1,23	CT	AG
379.	P	80	W	N	N	N	Y	5,02	1,29	3,13	1,33	CT	AG
380.	P	71	W	Y	Y	N	Y	7,57	1,47	0	0	CC	GG
381.	P	66	M	Y	Y	N	N	5,15	1,2	2,97	1,41	CT	AG
382.	P	56	W	Y	N	N	N	4,94	1,17	3,28	1,05	CT	AG
383.	P	46	M	N	Y	N	N	4,37	1,32	2,74	0,95	CT	AG
384.	P	51	M	Y	N	Y	Y	3,77	1,15	2,18	1	CC	AA
385.	P	72	M	N	N	N	N	6,84	0,72	4,08	2,33	TT	AA
386.	P	81	W	Y	N	N	N	4,42	1,85	2,18	1,54	CT	AA
387.	P	77	M	N	N	N	N	4,94	1,06	3,33	1,05	CT	GG
388.	P	78	W	N	N	N	Y	4,37	1,08	2,82	0,97	CC	AG