APOLIPOPROTEIN A5 GENETIC POLYMORPHISMS IN TURKISH POPULATION AND THE RISK OF ISCHEMIC STROKE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ESRA ŞAHİN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOCHEMISTRY

SEPTEMBER 2008

Approval of the thesis:

APOLIPOPROTEIN A5 GENETIC POLYMORPHISMS IN TURKISH POPULATION AND THE RISK OF ISCHEMIC STROKE

submitted by **ESRA ŞAHİN** in partial fulfillment of the requirements for the degree of **Master of Science in Biochemistry Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Mesude İşcan _____
Head of the Department, **Biochemistry**

Prof. Dr. Orhan Adalı _____
Supervisor, **Biology Dept., METU**

Dr. Birsen Can Demirdöğen _____
Co-Supervisor, **Food Safety and Nutrition Dept., Refik Saydam Hygiene Centre**

Examining Committee Members:

Prof. Dr. Tülin Güray _____
Biology Dept., METU

Prof. Dr. Orhan Adalı _____
Biology Dept., METU

Assoc. Prof. Dr. Ayşegül Gözen _____
Biology Dept., METU

Assoc. Prof. Dr. İrfan Kandemir _____
Biology Dept., Ankara University

Dr. Birsen Can Demirdöğen _____
Food Safety and Nutrition Dept., Refik Saydam Hygiene Centre

Date: 02.09.2008

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Lastname: Esra Şahin

Signature :

ABSTRACT

APOLIPOPROTEIN A5 GENETIC POLYMORPHISMS IN TURKISH POPULATION AND THE RISK OF ISCHEMIC STROKE

Şahin, Esra

M.S., Department of Biochemistry

Supervisor : Prof. Dr. Orhan Adalı

Co-Supervisor: Dr. Birsen Can Demirdöğen

September 2008, 130 pages

Stroke is the third leading cause of death and the most common cause of disabilities worldwide. Apolipoprotein A5 gene (APO A5), which encodes a 369 amino acid protein called Apolipoprotein AV (apo AV), has several single nucleotide polymorphisms (SNPs) found to be associated with altered triglyceride (TG) levels. Atherosclerosis is a major cause of ischemic stroke and this pathology may be associated with variability of TG levels. The main objective of this study was to investigate the coding region (c.553G>T) and promoter region (-1131T/C) polymorphisms of the APO A5 gene as a risk factor for ischemic stroke.

The study group in Turkish population consisted of 198 unrelated ischemic stroke patients and 130 control subjects. There was no statistically significant difference between the groups with respect to age and gender. Total blood samples were obtained from Gülhane Military Medical Academy

Hospital, Neurology Department, Ankara. In stroke patients, hypertension and diabetes were 2.5 times more common and high-density lipoprotein cholesterol (HDL-C) was significantly lower than controls. Logistic regression analysis showed that hypertension, diabetes and smoking were significant predictors of stroke. The frequency of risky alleles c.553T and -1131C were 0.003 and 0.098, respectively, in patients and were nearly the same with controls. The risk of hypertensive and diabetic individuals having ischemic stroke was higher in -1131C allele carriers (Odds ratio; OR= 3.4 and 6.4, respectively) than -1131TT individuals (OR= 2.3 and 1.9, respectively). Stroke patients with -1131C allele had significantly higher TG levels (1.70 mmol/L) and lower HDL-C levels (1.05 mmol/L) when compared to controls (1.35 mmol/L and 1.20 mmol/L, respectively) with the same genotype. Logistic regression analysis revealed elevated TG level to be associated with 2.2-fold and low levels of HDL-C to be associated with 1.8-fold increase in the risk of ischemic stroke versus control status.

This is the first study investigating the relation between APO A5 c.553G>T polymorphism and stroke risk. Additionally, in Turkish population -1131T/C polymorphism was analyzed for the first time in terms of its relation to ischemic stroke. The present study demonstrated that the frequency of risky alleles c.553T and-1131C were nearly the same in stroke patients and control subjects. Consequently, we decided that carrying minor alleles of c.553G>T and-1131T/C polymorphisms do not constitute a risk for ischemic stroke.

Key words: Apolipoprotein A5, Ischemic Stroke, Genetic Polymorphism, c.553G>T, -1131T/C, Turkish Population.

ÖZ

TÜRKİYE POPÜLASYONUNDA APOLİPOPROTEİN A5 GENETİK POLİMORFİZMLERİ VE İSKEMİK İNME RİSKİ İLE İLİŞKİSİNİN İNCELENMESİ

Şahin, Esra
Yüksek Lisans, Biyokimya Bölümü
Tez Yöneticisi: Prof. Dr. Orhan Adalı
Ortak Tez Yöneticisi: Dr. Birsen Can Demirdöğen

Eylül 2008, 130 sayfa

İnme tüm dünyada üçüncü ölüm sebebi ve en yaygın sakatlık nedenidir. 369 amino asit içeren apolipoprotein AV (apo AV) proteinini kodlayan Apolipoprotein A5 geni (APO A5), değişmiş trigliserit (TG) düzeyleri ile ilişkili bir çok tek nükleotid polimorfizmi (SNP) içermektedir. Ateroskleroz iskemik inmenin başlıca sebebidir ve bu patoloji TG düzeyindeki değişiklikler ile ilişkili olabilir. Bu çalışmanın ana amacı APO A5 geninin kodlayan (c.553G>T) ve promotor bölgesindeki (-1131T/C) polimorfizmlerini iskemik inme için risk faktörü olarak incelemektir.

Türk popülasyonundaki çalışma grubu, aralarında akrabalık olmayan 198 iskemik inme hastası ve 130 kontrolden oluşmuştur. Gruplar arasında yaş ve cinsiyet açısından istatistiksel olarak anlamlı fark görülmemiştir. Tam kan

örnekleri Ankara Gülhane Askeri Tıp Akademisi Hastanesi Nöroloji Bölümünce temin edilmiştir. Hipertansiyon ve diyabet, inme hastalarında kontrollerden 2,5 kat fazla ve yüksek dansiteli lipoprotein kolesterol (HDL-C) hastalarda anlamlı bir biçimde düşük bulunmuştur. Lojistik regresyon analizi, hipertansiyon, diyabet ve sigaranın inme için önemli tahmin unsurları olduğunu göstermiştir. c.553T ve -1131C riskli alellerin frekansları hastalarda sırasıyla 0,003 ve 0,098 idi ve kontrollerle neredeyse aynıydı. Hipertansiyonlu ve diyabetli insanlarda iskemik inme riski -1131C aleli taşıyanlarda (eşitsizlik oranı [Odds ratio; OR] sırasıyla= 3,4 ve 6,4) -1131TT genotipli bireylere göre daha yüksekti (sırasıyla TG OR= 2,3 ve HDL-C 1,9). Aynı genotipteki kontrollerinkiler ile kıyaslandığında (sırasıyla 1,35 mmol/L ve 1,20 mmol/L), -1131C aleli taşıyan inme hastalarının TG düzeyleri (1,70 mmol/L) anlamlı biçimde yüksek ve HDL-C düzeyleri (1,05 mmol/L) anlamlı şekilde düşüktü. Lojistik regresyon analizi, iskemik inme için yüksek TG düzeyinin 2,2 kat ve düşük HDL-C düzeyinin 1,8 kat risk oluşturduğunu ortaya koymuştur.

Bu çalışma APO A5 c.553G>T polimorfizmi ile inme riski arasındaki ilişkiyi inceleyen ilk çalışmadır. Ayrıca, -1131T/C polimorfizminin iskemik inme ile ilişkisi Türk popülasyonunda ilk kez çalışılmıştır. Bu çalışma, c.553T ve -1131C riskli alel frekanslarının inmeli hastalarda ve kontrol grubunda neredeyse aynı olduğunu göstermiştir. Bu sonuçlara göre, c.553G>T ve -1131T/C polimorfizmleri minor aleli taşımanın iskemik inme için risk faktörü oluşturmadığına karar verilmiştir.

Anahtar kelimeler: Apolipoprotein A5, İskemik İnme, Genetik Polimorfizm, c.553G>T, -1131T/C, Türk Popülasyonu.

dedicated to my family,

AKNOWLEDGEMENTS

I am deeply grateful to my supervisor Prof. Dr. Orhan ADALI and co-supervisor Dr. Birsen CAN DEMİRDÖĞEN for their invaluable guidance, continued advice and critical discussions throughout this study.

I am thankful to Prof. Dr. Emel ARINÇ for her guidance, suggestions and comments that were not restricted to this thesis study.

I would like to thank to Assoc. Prof. Dr. Şeref DEMİRKAYA and Dr. Semai BEK from Gülhane Military Medical Academy Hospital Neurology Department for providing the blood samples for my study. I also thank all volunteers who gave blood to be used in this study.

I wish to thank my examining committee members Prof. Dr. Tülin GÜRAY, Assoc. Prof. Dr. Ayşegül GÖZEN and Assoc. Prof. Dr. İrfan KANDEMİR for their valuable suggestions and criticism.

I am also thankful to my labmates Haydar ÇELİK, Şevki ARSLAN, Gülen ULUSOY, Tuğba BOYUNEĞMEZ TÜMER, Aysun TÜRKANOĞLU, Çiğdem KALIN, Mine NUYAN, Serdar KARAKURT, Duygu YILMAZ and Cihan TANRIKUT for their support and friendship.

I would like to express my sincere gratitude to my parents Mualla and Ali Adnan ŞAHİN and my sister Ece ŞAHİN for their help, permanent support and encouragement throughout this study. I have special thanks to my fiance Altuğ KANKAL for his encouragement, motivation, support and love.

Finally, I wish to thank TÜBİTAK (The Scientific and Technological Research Council of Turkey) for their support and scholarship.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

APO	Apolipoprotein
CI	Confidence intrval
CT	Computed tomography
CAD	Coronary artery disease
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
HDL	High density lipoprotein
LDL	Low density lipoprotein
PCR	Polymerase chain reaction
RE	Restriction endonuclease
OR	Odds ratio
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TC	Total cholesterol
TG	Triglyceride
TIA	Transient ischemic attack

CHAPTER 1

INTRODUCTION

1.1 Stroke

A stroke is caused by the interruption of the blood supply to the brain, usually because a blood vessel bursts or is blocked by a clot. When this happens, the supply of oxygen and nutrients cut off, leading to damage of the brain tissue.

The most common symptom of a stroke is sudden weakness or numbness of the face, arm or leg, most often on one side of the body. Other symptoms include: confusion, difficulty speaking or understanding speech; difficulty seeing with one or both eyes; difficulty walking, dizziness, loss of balance or coordination; severe headache with no known cause; fainting or unconsciousness (http://www.who.int).

Stroke is the third leading cause of death in most countries behind the diseases of heart and cancer, causing more serious chronic disabilities than any other diseases (Elkind and Sacco, 1998). Stroke is frequent, recurring, and is more often disabling than fatal and for this reason has immense financial and social implications (Sacco, 1995; Ali *et al.,* 2007). Between $30 and $40 billion per year was spent in the United States alone for stroke management (Ali *et al.,* 2007).

1.1.1 Classification of Stroke

Stroke kills more than a 150.000 people a year and more than 700.000 new stroke cases occur in each year in United States (Hinkle and Guanci, 2007) and has three pathologic subtypes: ischemic stroke (about 80% of all strokes in white populations), primary intracerebral hemorrhage (about 15%), and subarachnoid hemorrhage (about 5%) (Warlow *et al.*, 2003).

The American Stroke Association estimates that in the United States of America, ischemic stroke accounts for about 83 percent and hemorrhagic stroke accounts for about 17 percent of all stroke cases. Among the stroke patients in Turkish population, the prevalence of ischemic stroke was found 77%, primary intracerebral hemorrhage was 19% and subarachnoid hemorrhage was 4% (Kumral *et al.*, 1998).

1.1.1.1 Ischemic Stroke

Ischemic stroke is the lack of blood supply to the brain due to the obstruction within a carotid artery (Ustrell and Serena, 2007) (Figure 1.1). Blockage of the blood vessels that causes ischemic stroke occurs as a result of three conditions:

- the formation of a clot within a blood vessel of the brain or neck, called thrombosis
- the movement of a clot from another part of the body, such as from the heart to the neck or brain, called an embolism
- a severe narrowing of an artery in or leading to the brain, called stenosis (http://www.nihseniorhealth.gov).

Therefore; an ischemic stroke can be either "thrombotic" or "embolic". Thrombotic strokes occur when the clogging is in the arteries supplying oxygen and nutrients to the brain. Embolic stroke is the travel of blood clot to the brain formed somewhere in the body especially in the heart. The blood clot can be occurred by injuries of the blood vessels, disorders of blood clotting or inflammatory diseases of blood vessels. The most common cause of narrowing in the arteries in the head or neck is atherosclerosis which is fatty deposits (http://www.heartandstroke.com, http://www.stroke.org).

Transient ischemic attack (TIA) is a sudden blockage of blood vessels in the brain for a short period of time and lasts for less than 24 hours. TIAs are often called "mini-strokes" or "warning strokes" and occur most often during the hours and days immediately preceding the stroke (Albers *et al.,* 2002; Rothwell and Warlow, 2005). 17% of the patients who presented with ischemic stroke have had a TIA on the same day, 9% on the previous day and 43% during the preceding seven days (Rothwell and Warlow, 2005).

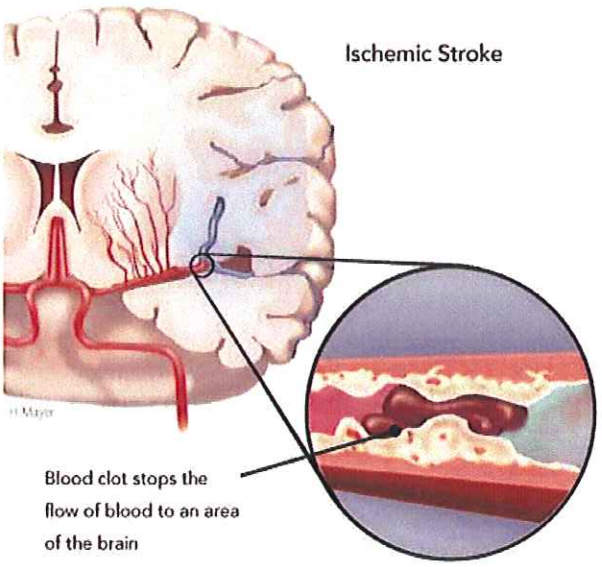


Figure 1.1 Ischemic stroke (taken from http://www.heartandstroke.ca).

1.1.1.2 Hemorrhagic Stroke

A hemorrhagic stroke is caused by a blood vessel that breaks and bleeds into the brain (Figure 1.2). The blood accumulates and compresses the surrounding brain tissue. There are two types of hemorrhagic strokes: intracerebral hemorrhage; uncontrolled bleeding on the surface of the brain, in the area between the brain and the skull and subarachnoid hemorrhage; bleeding within the deep artery of the brain (http://www.strokeassociation.org, http://www.heartandstroke.ca).

Hemorrhagic stroke occurs most frequently in patients under 40 years of age, and it is 2 to 3 times more common among blacks and Asians (Foulkes *et al.,* 1988).

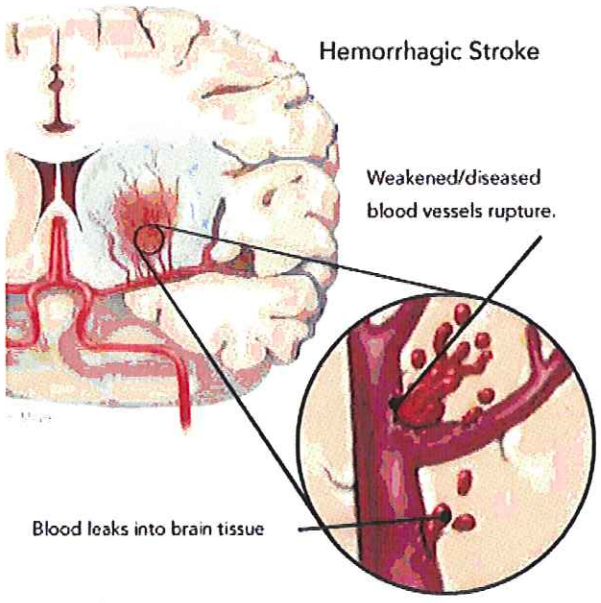


Figure 1.2 Hemorrhagic stroke (taken from http://www.heartandstroke.ca).

1.1.2 Risk Factors of Stroke

The risk factors of stroke can be modifiable that results from a lifestyle of a person or nonmodifiable meaning that a person cannot change the factors occurring as a heredity or natural process.

In a study showing the stroke profile of Turkey, hypertension was found as the major risk factor of ischemic stroke (63%), followed by hypercholesterolemia (37%), diabetes mellitus (35%), ischemic heart disease (23%), atrial fibrillation (20%) and smoking (17%) (Kumral *et al.,* 1998).

1.1.2.1 Nonmodifiable Risk Factors

Age, heredity (family history) and race, sex (gender), prior stroke, transient ischemic attack or heart attack are nonmodifiable risk factors of stroke. Stroke is common among elderly and incidence is higher among blacks and Hispanics compared with whites (White *et al.,* 2005). African-Americans have a higher stroke risk than Caucasians (Sacco, 1997). Being male and having transient ischemic attacks also increase the stroke prevalence (Kumral *et al.,* 1998; Elkind and Sacco, 1998).

1.1.2.2 Modifiable Risk Factors

Although a few of the major risk factors for stroke are nonmodifiable, many can be modified with proper education and medical attention (Benson and Sacco, 2000). High blood pressure, cigarette smoking, diabetes mellitus, carotid or other artery disease, atrial fibrillation, other heart diseases, sickle cell disease, high blood cholesterol, poor diet, physical inactivity and obesity are all modifiable risk factors of stroke.

Hypertension is the strongest modifiable and independent risk factor for both ischemic and hemorrhagic strokes. Cigarette smoking doubles, atrial fibrillation increases five-fold and diabetes is associated with a two to four-fold increased risk of stroke (http://www.eusi-stroke.com; Benson and Sacco, 2000; Chobaniann *et al.,* 2003; Turek, 2004).

1.1.2.3 Uncertain Risk Factors

The geographic location, socioeconomic factors, alcohol and drug abuse are less well documented risk factors of stroke.

1.1.3 Atherosclerosis

Atherosclerosis is a chronic and systemic disease that affects arteries and causes thickening and hardening of the vascular wall. Fatty material called plaque deposits in the inner lining of the arteries cause them to narrow and make them less flexible. When the plaque grows large enough to narrow the artery, the flow of oxygen-rich blood to vital organs is reduced and this leads to serious problems including heart attack, stroke or even death. But most of the damage occurs when plaques become fragile and rupture (http://medlineplus.gov, http://www.americanheart.org).

The plaque is made up of fatty substances, cholesterol, cellular waste products, calcium and other substances found in the blood as shown in Figure 1.3. When the plaque ruptures, blood clots (thrombus) are formed that may block the blood flow through an artery or break off and travel to another part of the body (embolus). If either happens and blokes the artery that feeds the heart (coronary artery), heart attack may occur and if affected artery feeds the brain (carotid artery), transient ischemic attack or stroke may occur (American Heart Association). In the early 1950s, Harvard neurologist C.

Miller Fisher stressed the importance of carotid artery atherosclerosis as a major cause of stroke (Fisher, 1951).

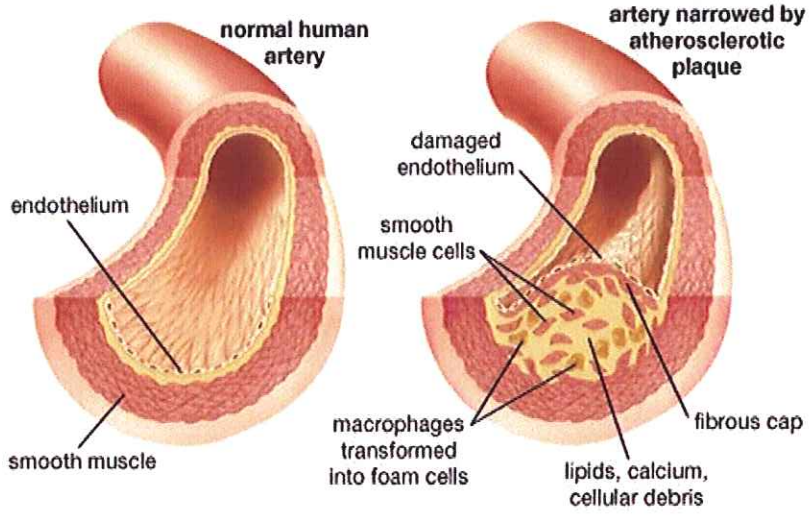


Figure 1.3 A normal artery and artery containing plaque build up (taken from http://www.britannica.com).

Severe atherosclerotic plaques in the aortic arch were found to be a third leading cause of embolic stroke in 1990 (Tunick and Kronzon, 1990). The prevalence of severe aortic plaque in stroke patients (14% to 21%) is nearly same with other two important causes of embolic stroke; carotid artery disease (10% to 13%) and atrial fibrillation (18% to 30%) (Amarenco *et al.,* 1994; Jones *et al.,* 1995; Kronzon and Tunick, 2006). Increased plaque thickness is related with increased risk of stroke and the odds ratio (OR) for stroke in patients with severe plaques was found to be 13.8 (Amarenco *et al.,* 1994).

1.1.4 Prevention of Stroke

Although the treatment of acute ischemic stroke has improved, the greatest reductions in stroke mortality and morbidity may possibly be achieved through more effective prevention strategies (Sacco 1998). Therapies to prevent a first or recurrent stroke are based on treatment of modifiable risk factors for stroke, such as hypertension, atrial fibrillation, diabetes and modification of individual's lifestyle behaviors (http://www.ninds.nih.gov).

Blood pressure control, smoking cessation, lipid-lowering agents, warfarin treatment for atrial fibrillation and antiplatelet agents can reduce the stroke risk (Elkind and Sacco, 1998; Hankey, 1999). One other stroke prevention strategy is surgery for carotid stenosis (carotid endarterectomy) in patients with asymptomatic carotid stenosis. Although quite expensive, it is reasonable option for patients with stenosis of more than 70% (Hankey, 1999; Rothwell *et al.,* 2003).

Reduction in the risk of first stroke by lowering the blood pressure has been known for years (Collins and MacMahon, 1994). Keeping the usual diastolic blood pressure 5 to 6 mm Hg below usual for a prolonged period of time could lower the risk of a first stroke by 35% to 40% (MacMahon *et al.,* 1990; Collins *et al.,* 1990). Aspirin reduces stroke risk by reducing the risk of venous thromboembolism (Gubitz *et al.,* 2003). Decreasing the blood cholesterol with statin therapy reduces the risk of stroke although higher levels of cholesterol were not seem to be associated with stroke risk (Heart Protection Study Collaborative Group, 2002; Warlow *et al.,* 2003). An anticoagulant agent warfarin prevents the embolization in patients with severe aortic plaque, treats the atrial fibrillation and is used as another method for stroke prevention (Tunick and Kronzon, 1990). The future burden of stroke can reduce if the understanding of stroke risk factors improved.

1.2 Apolipoproteins

Apolipoproteins are lipid-binding proteins in the blood that transport triacylglycerols, phospholipids, cholesterol and cholesteryl esters between organs. Apolipoproteins ("apo" means "detached" or "separate", designating the proteins in its lipid free form) are the constituents of the plasma lipoproteins, sub-microscopic spherical particles with hydrophobic lipids at the core and hydrophilic protein side chains and lipid head groups at the surface (Nelson and Cox, 2005; http://www.en.wikipedia.org). Figure 1.4 shows the molecular structure of a lipoprotein.

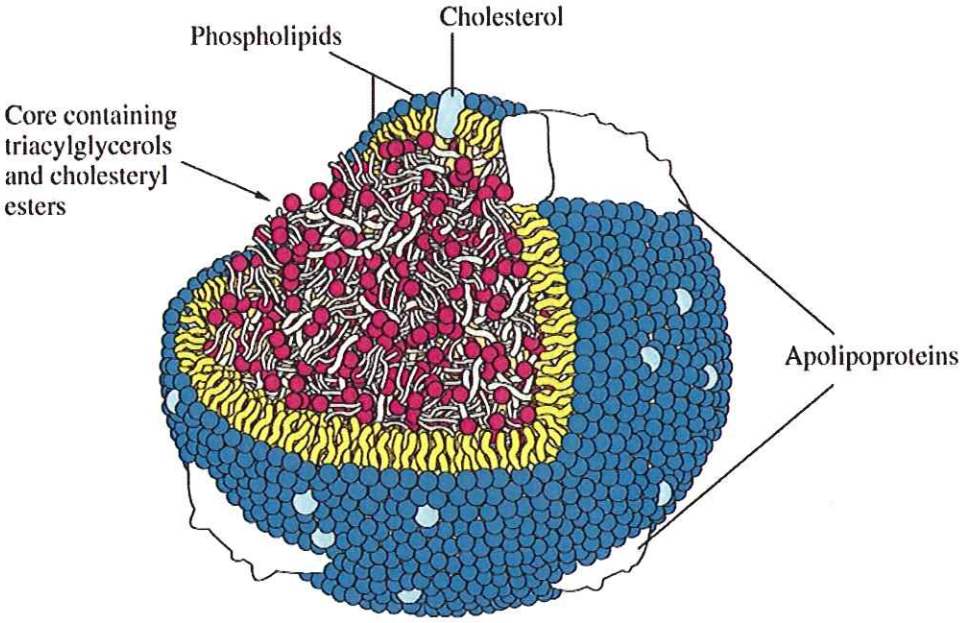


Figure 1.4 Molecular structure of a lipoprotein containing apolipoproteins, cholesterol, phospholipids, triacylglycerols and cholesteryl esters (taken from http://www.uwsp.edu).

There are six major classes of apolipoproteins and several subclasses: A (apo A-I, apo A-II, apo A-IV, and apo A-V), B (apo B48 and apo B100), C (apo C-I, apo C-II, apo C-III, and apo C-IV), D, E and H. A total of nine major apolipoproteins are found in human lipoproteins and their properties are given in Table 1.1 (Mathews and Van Holde, 1995; http://www.en.wikipedia.org).

Table 1.1 Apolipoproteins of the human plasma lipoproteins (taken from Dennis E. Vance and Jean E. Vance, 1985).

Apolipoprotein	Molecular Weight	Characteristics
apoA-I	28,331	Major protein in HDL
apoA-II	17,380	Major protein in HDL
apoB-48	241,000	Found exclusively in chylomicrons
apoB-100	500,000	Major protein in VLDL and LDL
apoC-I	7,000	Found in HDL and VLDL
apoC-II	8,837	Found in chylomicrons, VLDL, HDL
apoC-III	8,750	Found in chylomicrons, VLDL, HDL
apoD	33,000	HDL protein
apoE	34,145	Found in chylomicrons, VLDL, HDL

Although all lipoproteins share common structural features (spherical shape), they differ in lipid and protein composition producing particles of different densities. Lipids have a much lower density than proteins, so the lipid content of a lipoprotein class is inversely related to its density. The standard lipoprotein classification includes, in increasing order of density: chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), high-density lipoprotein

(HDL) and very high-density lipoprotein (VHDL) (Mathews and Van Holde, 1995; Nelson and Cox, 2005).

The regulation of apolipoprotein synthesis in the intestine is controlled by the fat content of the diet and in the liver it is controlled by dietary composition, hormones (insulin, glucagon, thyroxin, estrogens, androgens), alcohol intake and various drugs (statins, niacin, and fibric acids) (http://www.en.wikipedia.org).

1.2.1 Functions of Apolipoproteins

The apolipoproteins of the various lipoproteins regulate lipoprotein metabolism and determine the unique roles of these lipoproteins in lipid metabolism (Mahley *et al.,* 1984).

One important function of specific apolipoproteins in the regulation of lipoprotein metabolism is the transport and redistribution of lipids among various tissues. The cell surface lipoprotein receptors recognize the specific apolipoproteins for the delivery of lipids to specific cells (Mahley *et al.,* 1984). Apo B-100 and apo E receptors mediate the uptake of lipoproteins containing these apolipoproteins and regulate their levels in the plasma. In addition, apolipoproteins B-100 and E are responsible for the redistribution of cholesterol among cells (Mahley and Innerarity, 1983).

Some apolipoproteins have specific roles as cofactors for enzymes of lipid metabolism (Mahley *et al.,* 1984; Mathews and Van Holde, 1995). For instance, apo C-II is an activator of the hydrolysis of chylomicrons and VLDL triglycerides catalyzed by lipoprotein lipase. The LCAT (lecithin: cholesterol acyltransferase) reaction is also activated by an apolipoprotein (apo A-I) (Havel *et al.,* 1980).

The maintenance of the structure of the lipoproteins is another function of specific apolipoproteins. apo B, apo A-I and apo E stabilize the micellar structure of the lipoproteins and provide a hydrophilic surface (Mahley *et al.*, 1984).

1.2.2 Apolipoprotein A5

Apolipoprotein A5 (APO A5), also known as apo AV, is a new member of apolipoprotein gene family. In 2001, human APO A5 gene was discovered by Pennacchio *et al.* and this gene was located approximately 30 kb downstream from the *ApoAI-ApoCIII-ApoAIV* gene cluster on chromosome 11q23 (Pennacchio *et al.*, 2001) (Figure 1.5).

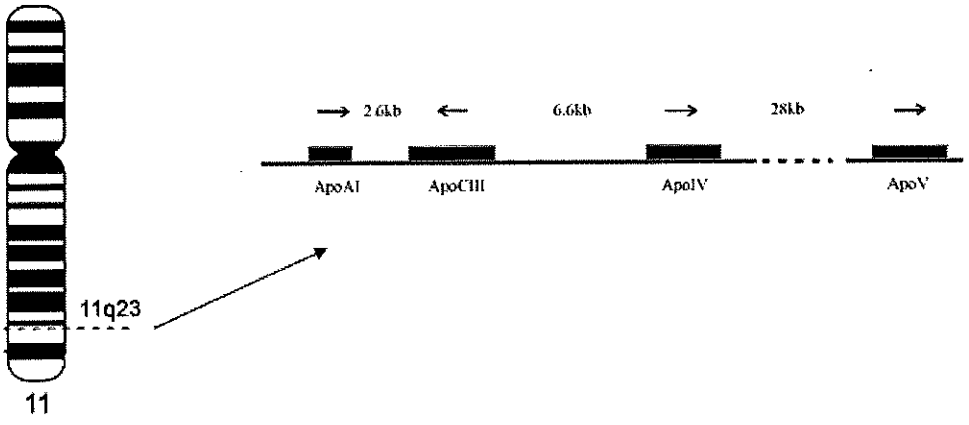


Figure 1.5 The human ApoAI/CIII/AIV/AV gene cluster on chromosome 11q23 (adapted from Gao *et al.*, 2005 and http://www.nature.com).

APO A5 gene has been independently identified in human and mouse by a combined approach of comparative sequence analysis and differential gene expression as a gene highly up-regulated in the early phase of liver regeneration (Pennacchio *et al.,* 2001; van der Vliet *et al.,* 2001). The human APOA5 gene consists of four exons and three introns and codes for a 369 amino acid protein, named as apo AV (Pennacchio *et al.,* 2001). Interestingly, 2 initiation codons have been reported, the first was identified by Pennachio *et al.,* (2001), followed by the second ATG codon, 9 nucleotides downstream, as reported by van der Vliet *et al.* (2001).

Although the exact function of APO A5 is not known, studies in animals and humans have demonstrated that APO A5 gene may play an important role in triglyceride (TG) metabolism. Functional studies in mice have shown that altering the level of APO A5 substantially affected plasma TG levels. Transgenic mice overexpressing the human APO A5 gene exhibit one-third lower plasma TG levels than controls, whereas APO A5 knockout mice have four times as much plasma TG levels, with no effect on plasma cholesterol levels (Pennacchio *et al.,* 2001). Recombinant adenoviral vector-mediated transfer of APO A5 into mice was associated with markedly decreased (−70%) serum TG levels caused primarily by the reduced triglyceride content of the very low density lipoprotein (VLDL) fraction (van der Vliet *et al.,* 2002).

The precise mechanism of the role of APO A5 on TG metabolism is still unclear. APO A5 protein is synthesized predominantly in the liver and is secreted into the plasma as a regulator of TG level. APO A5 is a component of several lipoprotein fractions, including very low density lipoprotein (VLDL), high density lipoprotein (HDL) and chylomicrons. Both HDL and VLDL may be involved in APO A5 mechanism of action (Merkel and Heeren, 2005).

Surface chemistry techniques have demonstrated that APO A5 displays high affinity, low elasticity and slow binding kinetics at hydrophobic interfaces which possibly retards triglyceride-rich particle assembly (Weinberg *et al.*, 2003). Najib *et al.* (2004) showed that recombinant APO A5 could interact with lipoprotein lipase and significantly increase its activity. These results suggest that APO A5 might induce a decrease in VLDL-TG levels by both decreasing hepatic VLDL synthesis and increasing VLDL clearance. (Najib *et al.*, 2004).

1.3 Polymorphism

Genetic polymorphism ("poly" means many, and "morph" means form) is the presence of two or more variants (alleles or haplotypes) in a population. The term is usually qualified to mean that the rarer genetic variant that appears in at least 1% of a population (Futuyma, 2005).

Polymorphisms are a type of genetic diversity within a gene pool of the population. Depending on its exact nature, a polymorphism may or may not affect biological function of the organism. Polymorphisms arise through mutation, which may be due to a change from one type of nucleotide to another, an insertion or deletion, or a rearrangement of nucleotides. Once formed, a polymorphism can be inherited like any other DNA sequence from parent to child (Weaver and Philip, 1992).

1.3.1 Single Nucleotide Polymorphism (SNP)

A single nucleotide polymorphism (SNP) is a variation in DNA sequence of a single base pair (Figure 1.6) occurring with a frequency of at least 1% in a population. By February 2001, 1.42 million SNPs had been identified and placed in a public data base. SNPs are distributed throughout the human genome with a frequency of one SNP in every 1900 bp and with one SNP in per 1080 bp in exons (Int. SNP Map Work Group, 2001).

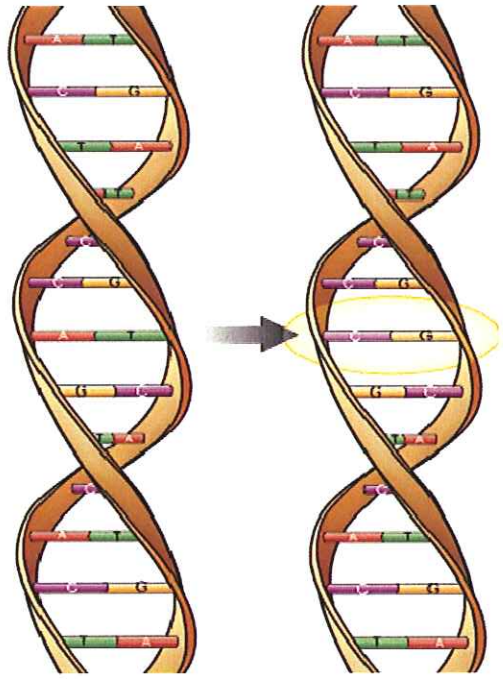


Figure 1.6 A schematic representation of variation in single base pair: single nucleotide polymorphism (taken from http://www.scq.ubc.ca).

SNPs may be placed within coding sequences, non-coding regions of genes or intergenic regions between the genes. If SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein and

lead to same polypeptide due to degeneracy of the genetic code, it is termed as the synonymous or silent mutation. If amino acid sequence change produces a different polypeptide, the non-synonymous mutation occurs. SNPs that are in non-coding regions may still have consequences for gene splicing, transcription factor binding or the sequence of non-coding RNA (http://www.wikipedia.org).

Apart from being genetic markers, SNPs play important roles in our susceptibility to complex diseases that involve the activities of a number of different genes. In addition, SNPs may determine how a person reacts to a particular drug, whether they have beneficial or serious side effects to patient (Karp, 2002).

1.3.2 Polymorphisms of APO A5

The *ApoAI-ApoCIII-ApoAIV* gene cluster on human chromosome 11q23 was known to influence plasma lipid parameters and defined mutations in this cluster dramatically affect plasma lipid profiles in both humans and mice (Pennacchio *et al.,* 2001). Human APO A5 gene is fairly polymorphic and several SNPs of APO A5 associated with increased TG concentrations have been identified. When Pennacchio *et al.* (2001) identified the APO A5; they also found three SNPs (c.1259T>C, IVS3+476G>A, -1131T/C) across the APO A5 locus and the minor allele of each of these SNPs was associated with higher TG levels independent of diet. Figure 1.7 shows a schematic representation of the gene and some associated polymorphisms.

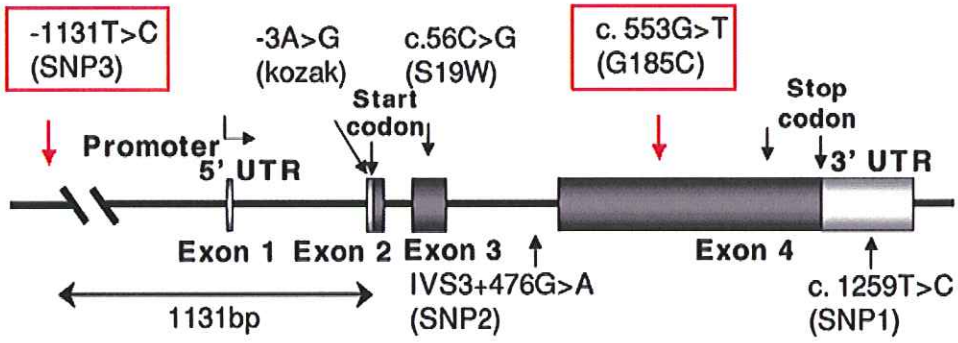


Figure 1.7 Schematic representation of human APO A5 gene with relative positions of single nucleotide polymorphisms. APO A5 exons are shown in solid boxes. The predicted transcription start side is depicted by a bent arrow, and the position of promoter and the start and stop codons are also shown. The polymorphisms investigated in this study are indicated in red boxes and red arrows (adapted from Matsunaga *et al.,* 2007).

1.3.2.1 APO A5 c.553G>T Polymorphism

A novel genetic variant of the APO A5 gene, c.553G>T, which occurs within the coding region causes a change in the amino acid sequence. This polymorphism causes a substitution of a cysteine for a glycine residue at amino acid residue 185 (G185C).

APO A5 c.553G>T polymorphism has been found to correlate strongly with increased TG levels in Chinese (Kao *et al.,* 2003) but not in Caucasians (Hubacek *et al.,* 2004). The impact of the c.553G>T polymorphism on plasma lipid profile was also shown with the significantly higher plasma TG levels in minor allele carriers in both CAD and control groups in Chinese population (Tang *et al.,* 2006). This may be caused by the change in function of apo AV which may lead to increased TG concentration, due to change in amino acid residue (Kao *et al.,* 2003). Since this variation introduces a cysteine which contains sulfur atom, in place of glycine at residue 185 and another cysteine is present at residue 204, a disulphide bridge may be created between these two cysteines. Weinberg *et al* (2003), also suggested that the binding of apo AV to lipid interfaces may be determined by only a few discrete domains, one candidate being residues 171–188, a predicted α-helical segment that is both hydrophobic and highly amphipathic which can rapidly bind and penetrate the phospholipid interface. Therefore, the possible disulphide bond formation between the residues 185 and 204 may change the primary structure of the protein, perhaps influencing the α-helix formation and/or stability, thereby altering the binding capacity of apo AV to lipid interfaces, and weakening the role of apo AV in retarding VLDL assembly and nascent VLDL movement (Tang *et al.,* 2006).

Kao *et al.* (2003), suggested that c.553G>T polymorphism in APO A5 gene can be used as a prognostic indicator of hypertriglyceridemia

susceptibility in Chinese. The minor allele (c.553T) frequencies were found to be 0.042 for control group and 0.27 for hypertriglyceridemic patients. Carriers of c.553T allele had an odds ratio of 11.73 for developing hypertriglyceridemia in comparison to individuals without that allele in Chinese population (Kao *et al.*, 2003).

In a study of Chinese population, it was demonstrated that the allelic frequency of c.553T allele was approximately doubled in patients with cardiovascular diseases (CAD) when compared with control subjects (Tang *et al.*, 2006). The individuals having c.553T allele were found to be at a significantly higher frequency within the CAD patients than in controls in Taiwanese Chinese population (Hsu *et al.*, 2006). However, the frequency of c.553T allele is extremely low in Caucasians and also in Turkish population (Hodoglugil *et al.*, 2006). Hubacek *et al.*, did not detect c.553T allele in more than 500 Caucasians (Hubacek *et al.*, 2004). Therefore, this variant may vary between populations of different ethnicity.

1.3.2.2 APO A5 -1131T/C Polymorphism

When Pennacchio *et al.* identified a new apolipoprotein, APO A5 that might play an important role in TG metabolism; they also found three SNPs (SNP1-3) across the APO A5 locus to be significantly associated with plasma TG concentration (Pennacchio *et al.*, 2001). Several groups have confirmed the association between a T/C polymorphism in the promoter region of the APO A5 gene at position 1131 (SNP3) and elevated TG levels in different populations, including Caucasians (Pennacchio *et al.*, 2001 and 2002), Turks (Hodoglugil *et al.*, 2006; Komurcu-Bayrak *et al.*, 2008), African Americans and Hispanics (Pennacchio *et al.*, 2002), Japanese (Nabika *et al.*, 2002; Endo *et al.*, 2002) as well as Chinese (Baum *et al.*, 2003). The rare allele of -1131T/C polymorphism may predispose to cardiovascular diseases (Szalai *et al.*, 2004;

Bi *et al.*, 2004; Hsu *et al.*, 2006), stroke (Havasi *et al.*, 2006) and insulin resistance (Martin *et al.*, 2003).

The -1131T/C polymorphism in the promoter region of the APO A5 gene was associated with 20-30% elevation in plasma TG levels of the 500 unrelated Caucasian subjects (Pennacchio *et al.*, 2001 and 2002). The carriers of the APO A5 -1131C allele in Taiwanese Chinese (Hsu *et al.*, 2006), Hungarian (Szalai *et al.*, 2004), Japanese American (Austin *et al.*, 2004) and Chinese (Bi *et al.*, 2004; Li *et al.*, 2004) populations also had significantly elevated TG levels. In addition to elevated TG levels, this polymorphism was also associated with reduced HDL-C levels in both Asian and Caucasian populations (Pennacchio *et al.*, 2001 and 2002; Aouizerat *et al.*, 2003). The frequency of the rare allele was less than 10% in the Caucasian population (Pennacchio *et al.*, 2001). In contrast, the frequency of this allele was 38% in Japanese Americans (Austin *et al.*, 2004) and was over 30% in Japanese (Endo *et al.*, 2002) and Chinese (Baum *et al.*, 2003; Li *et al.*, 2004).

The precise mechanism behind the association of the APO A5-1131T/C polymorphism with the elevated plasma TG level is not certain. Since this polymorphism is located in the promoter region of the APO A5 gene, one possibility is that it might affect the transcriptional activity of the APO A5 gene and thus, affect plasma TG levels. Another possibility is that an unknown functional mutation may be present at, or adjacent to the APO A5 gene locus which is in strong linkage disequilibrium (LD) with this polymorphism, giving rise to the inter-individual triglyceride and HDL-C variation. The -3A > G SNP which is the critical nucleotide of the Kozak sequence preceding the translation start codon, was found to be in complete LD with -1131T/C SNP. Therefore, the -3A > G SNP could be a candidate to affect potentially the rate of APO A5 translation (Pennacchio *et al.*, 2002). Talmud *et al.* (2002) demonstrated that

the -1131T/C SNP was in a LD with APO C3 -482C > T SNP which was strongly associated with plasma TG level.

1.4 The Aim of This Study

Stroke is the third leading cause of death behind the diseases of heart and cancer and causes more serious chronic disabilities than any other diseases. Atherosclerosis which causes thickening and hardening of the vascular wall due to plaque deposition in the inner lining of the arteries is a major cause of stroke. Recently, a new apolipoprotein, APO A5 that might play an important role in TG metabolism was identified. Genetic polymorphisms in APO A5 gene are associated with variability in plasma TG levels and these polymorphisms may increase the tendency to ischemic stroke.

It is well established that APO A5 polymorphisms differ in frequency among populations of different ethnicity. Therefore, identification of APO A5 polymorphisms in different populations, as well Turkish population becomes important. Since a limited number of studies on APO A5 553G>T and -1131T/C polymorphisms were carried out in Turkish population, the present study investigates these polymorphisms in Turkish population. Additionally, no study is available in the literature showing the relationship between stroke and these two polymorphisms at the same time.

The aim of this study is to investigate the two important APO A5 polymorphisms which are c.553G>T polymorphism in coding region and -1131T/C polymorphism in promoter region, in both ischemic stroke patients and healthy volunteers in order to determine susceptibility to ischemic stroke in Turkish population. To accomplish this aim, this study was designed to follow the steps given below:

- obtaining total blood samples from ischemic stroke patients and healthy controls,
- isolation of genomic DNA in intact form from blood samples,
- amplification of two regions in APO A5 gene; one in coding region (c.553G>T) and one in promoter region (-1131T/C) by using polymerase chain reaction (PCR),
- digestion of amplified fragments with restriction endonucleases to determine the genotype of each individual for c.553G>T and -1131T/C single nucleotide polymorphisms,
- determination of the genotype and allele frequencies of c.553G>T and -1131T/C single nucleotide polymorphisms for Turkish population
- comparison of genotype and allele frequencies between ischemic stroke and control groups,
- comparison of the results of this study with other studies performed on different ethnicities.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Population and Blood Sampling

The study population was comprised of 198 consecutive unrelated adult Caucasian patients with acute hemispheric ischemic stroke and 130 symptom-free Caucasian controls from the same geographic region (central Anatolia, Turkey). Total blood samples of the participants were obtained from Gülhane Military Medical Academy Hospital Neurology Department, Ankara. Informed consent was obtained from all participants before study entry and a copy of the informed consent form is given in Appendix A. The study was approved by the Ethical Committee of the Gülhane Military Medical Academy (see Appendix B) and was carried out according to the principles of the Declaration of Helsinki.

Cases were selected among patients suffering atherothrombotic ischemic stroke admitted to the neurology services of Gülhane Medical Faculty, Ankara, within 24 hours after onset, from October 2005 to August 2007. Recruitment of the patients was performed consecutively. Stroke was defined as clinical designation for a rapidly developing loss of brain functions that lasted at least 24 hours and had no apparent cause other than that of vascular origin. The cerebral infarction was initially diagnosed on the basis of neurological examination and brain computer tomography (CT) scan and then

transthoracic echocardiographic examination, Holter study and Transcranial Doppler emboli detection procedure to rule out emboli source. In order to be considered eligible the patients should meet following criteria: having anterior circulation stroke, no other major illnesses, including autoimmune diseases, neoplasms, coagulopathies, hepatic or renal failure, no known embolic source (aortic arch, cardiac or carotid), no family history of hematological, autoimmune or chronic inflammatory diseases, no history of myocardial infarction within 3 weeks or of transient ischemic attack or stroke at any time. The control group was selected randomly from the neurology outpatient clinics. All exclusion criteria were applied to the controls exactly plus not having carotid stenosis (lumen narrowing) > 50% or ulcerated carotid plaque. All subjects underwent bilateral carotid Doppler ultrasound (CUSG) and transthoracic echocardiographic studies.

A detailed history of conventional vascular risk factors and conditions from each participant was obtained from Gülhane Medical Faculty Neurology Department by our colleagues. Hypertension was defined as systolic blood pressure > 140 mm Hg and/or diastolic blood pressure > 90 mm Hg and/or use of antihypertensive drugs. Diabetes was defined as fasting glucose $\geq$ 6.99 mmol/L and/or use of pharmacological treatment. Obesity was assigned when body mass index was 30 or higher. Smoking status of an individual was assigned "yes" if the individual is currently smoking or have quitted less than 3 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, HDL, VLDL-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.

The study population (198 patients and 130 controls) were selected from a larger population of 217 patients and 153 controls. The rationale for selection was as follows:

- 14 controls were removed from the group since they had either ischemic heart disease or carotid stenosis > %50, or both.
- In order to maintain mean age of patient and control groups close, 19 patients whose age was 85 years and older and 9 controls aged 32 years and younger were removed from the group.

2.1.2 Chemicals

Agarose (A-9539), bromophenol blue (B-5525), ethidium bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), sodium chloride (NaCl; S-3014), sodium dodecyl sulfate (SDS; L-4390), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T-1503) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

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

Taq DNA polymerase –supplied together with $MgCl_2$ and amplification buffer– (#EP0407), dNTP mix (#R0191), Gene Ruler^{TM} 50bp DNA Ladder (#SM0371) and restriction enzymes *BsuRI (HaeIII;* #ER0151) and *TruI (MseI;* #ER0981) –supplied with their buffers– were purchased from MBI Fermentas, USA.

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

2.1.3 Primers

Two sets of oligonucleotide primers were used for the amplification of polymorphic regions of *APOA5* gene from genomic DNA. They were derived from known sequences of human *APOA5* gene (Tang *et al.*, 2006; Pennacchio *et al.*, 2001).

c.553G>T Single Nucleotide Polymorphism was analyzed using;

Forward primer: 5'-AGA CAC CAA GGC CCA GTT GCT GGG-3'

Reverse primer: 5'-ATG CCG CTC ACC AGG CTC TCG GCG-3'

-1131T/C Single Nucleotide Polymorphism was analyzed using;

Forward primer: 5'-GAT TGA TTC AAG ATG CAT TTA GGA C-3'

Reverse primer: 5'-CCC CAG GAA CTG GAG CGA AAT T-3'

Primer pairs were purchased from Iontek (Iontek, Istanbul, Turkey) and Alpha DNA (Alpha DNA, Canada, USA) to analyze c.553G>T and -1131T/C SNPs respectively. Primers were stored at -20°C.

2.2 Methods

2.2.1 Preparation of Genomic DNA for PCR

2.2.1.1 Isolation of Genomic DNA from Human Whole Blood Samples

Principle:

Isolation of genomic DNA were performed using a salting-out method according to the method described by Lahiri and Schnabel (1993), with slight modifications. Whole blood samples which were used as DNA source were collected to EDTA containing tubes.

Reagents:

1. TKM Buffer: 10 mM Tris-HCl at pH 7.6
   10 mM KCl
   4 mM $MgCl_2$
   2 mM EDTA

To prepare 200 mL TKM buffer; 242.2 mg Tris, 149.1 mg KCl, 162.6 mg $MgCl_2$ and 148.9 mg EDTA were weighed and dissolved in 100 mL of dH_2O. pH was adjusted to 7.6 with HCl and volume was completed to 200 mL. The solution was autoclaved for sterilization and stored at 4°C.

2. 10% SDS: 0.1 g molecular grade SDS is dissolved in 1 mL dH_2O. This solution did not require sterilization and stored at 4°C.

3. Saturated NaCl (~6 M): 3.51 g NaCl was weighed and dissolved in 10 mL dH_2O. The solution was autoclaved for sterilization and stored at 4°C.

4. TE Buffer: 10 mM Tris-HCl at pH 8.0
1 mM EDTA at pH 8.0

For the preparation of 100 mL TE buffer; 10 mL 100 mM Tris-HCl and 0.2 mL 500 mM EDTA was taken and final volume was completed to 100 mL with dH_2O.

- 100 mM Tris-HCl (pH 8.0): 1.21 g Tris was weighed and dissolved in 100 mL dH_2O. The pH of this solution was adjusted to 8.0 with HCl and autoclaved for sterilization.

- 500 mM EDTA (pH 8.0): 18.61 g EDTA was weighed and dissolved in 100 mL dH_2O. The pH of this solution was adjusted to 8.0 with NaCl and autoclaved for sterilization.

Procedure:

750 µL of whole blood was transferred into eppendorf tube and was treated with equal volume of low-salt TKM buffer. 20 µL of Triton X-100 was added and tube was mixed by inversions several times. By this way the cells were lysed. The suspension was centrifuged at 1,000g for 10 minutes at room temperature by using Sigma 1-15 benchtop microfuge (Sigma, Postfach 1713-D-37507, Osterode). After centrifugation, two layers were formed in the tube; the top layer was the supernatant and the bottom layer was the pellet containing the genomic DNA. The pellet of mostly leukocytes was saved and washed two more times with 750 µL TKM buffer. The final pellet was resuspended in 200 µL of TKM buffer by tapping. 10 µL of 10 % SDS (sodium dodecyl sulfate) was added and the whole suspension was mixed thoroughly and incubated at 58°C for 10 minutes. After adding 75 µL of cold saturated NaCl (~6 M), the tube was mixed well and centrifuged at 14,000g for 10 minutes at 4°C. The supernatant contained DNA and was taken to another eppendorf tube. The

DNA was precipitated using 2x volume ice-cold ethanol. The tubes were stored at -20°C for at least 30 minutes and centrifuged at 10,000g for 10 minutes at 4°C to precipitate DNA. Supernatant was removed and DNA containing pellet was solubilized with 100 µL of TE buffer. Tubes were incubated at 37°C for at least 2 hours for complete dissolvation.

2.2.1.2 Quantification of Genomic DNA Concentration by Spectrophotometry

In order to determine the concentration of DNA in the sample, absorbance values at 260 nm and 280 nm were measured in quartz cuvettes using Shimadzu UV160-A double-beam spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). The nucleic acids gave maximum absorbance at 260 nm therefore reading at this wavelength was used to calculate the concentration of DNA in the sample. Based on the knowledge that an absorbance value of 1.0 corresponds approximately to 50 µg/mL for double-stranded DNA, the concentration of DNA was calculated using the following formula:

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

2.2.1.3 Qualification of Genomic DNA by Spectrophotometry

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

2.2.1.4 Qualification of Genomic DNA by Agarose Gel Electrophoresis

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

Reagents:

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

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

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

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

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

Procedure:

0.5% of agarose gel was prepared by adding 0.15 g agarose to 30 mL of 0.5x TBE buffer, pH 8.3 containing 450 mM Tris, 450 mM Borate and 10 mM EDTA, in an erlenmeyer flask, so that the buffer did not occupy more than half of the flask. The slurry in the erlenmeyer flask was heated in microwave oven till all of the grains of the agarose dissolved.

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

The mold, plastic tray and comb were cleaned with 70% ethanol while the agarose gel is being heated. The plastic tray was settled in the mold and the comb was placed 0.5-1.0 mm above the plate. The warm agarose solution was then poured into the mold and any air bubbles -if present-, especially under or between the teeth of the comb were removed with the help of a pipette tip. The gel was allowed to solidify completely for approximately 20-40 minutes at room temperature.

The gel tank was filled with approximately 300 mL of 0.5x TBE buffer. The comb was carefully removed from the gel and the gel in the plastic tray was mounted in the electrophoresis tank so that the slots of the gel faced towards the negative pole-cathode. 0.5x TBE buffer was added to the tank until it covered the gel to a depth of about 1 mm. Any air bubbles in the wells, if present, were removed with the help of a pipette tip.

5 µL (0.25-0.5 ng) of DNA sample was mixed with 1 µL of gel loading buffer by sucking in and out of a micropipette. The mixture was slowly loaded

into the slots of the gel. The lid of the tank was closed and the electrical leads were attached to the power supply. The power supply was set to the constant voltage of 100 volts so that a voltage of 5 V/cm (measured as the distance between the electrodes) was applied. The gel was run for 45 minutes and then examined under UV light and the photograph was taken by using Vilber Lourmat Gel Imaging System (Marre La Vallee, Cedex, France) and Bio-Capture (Version 99.03) computer software.

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

2.2.2 Genotyping for c.553G>T and -1131T/C Single Nucleotide Polymorphisms of APO A5

Standard PCR protocols, followed by restriction enzyme digestions were used to genotype the APO A5 for c.553G>T and -1131T/C polymorphisms (see Table 2.1).

Thermocycler used in PCR was Techne Progene (Techne (Cambridge) Ltd., Duxford, Cambridge). *HaeIII* and *MseI* restriction endonucleases were used in RFLP analysis of APO A5 polymorphisms, c.553G>T and -1131T/C, respectively.

Table 2.1 Genotyping of APO A5; table showing the regions of amplification, SNP position, primer pairs used for amplification, size of the PCR products, and size of digestion products and their interpretation.

Region of amplification	SNP position	Primers	PCR product size	RE	Size of digestion products and interpretation
Coding region	c.553G>T	5'-AGA CAC CAA GGC CCA GTT GCT GGG-3' 5'-ATG CCG CTC ACC AGG CTC TCG GCG-3'	138bp	*BsuRI (HaeIII)*	**553GG:** 87, 51bp **553GT:** 138, 87, 51bp **553TT:** 138bp
Promoter region	-1131T/C	5'-GAT TGA TTC AAG ATG CAT TTA GGA C-3' 5'-CCC CAG GAA CTG GAG CGA AAT T-3'	187bp	*TruI I (MseI)*	**-1131TT:** 167, 20bp **-1131TC:** 187, 167, 20bp **-1131CC:** 187bp

2.2.2.1 c.553G>T Single Nucleotide Polymorphism

2.2.2.1.1 Polymerase Chain Reaction for c.553G>T SNP

APO A5 c.553G>T SNP region was amplified using primer sequences given by Tang *et al.*, 2006 (Table 2.1). Sequence of the amplified fragment in coding region of APO A5 gene that includes c.553G>T single nucleotide polymorphism is given in Figure 2.1.

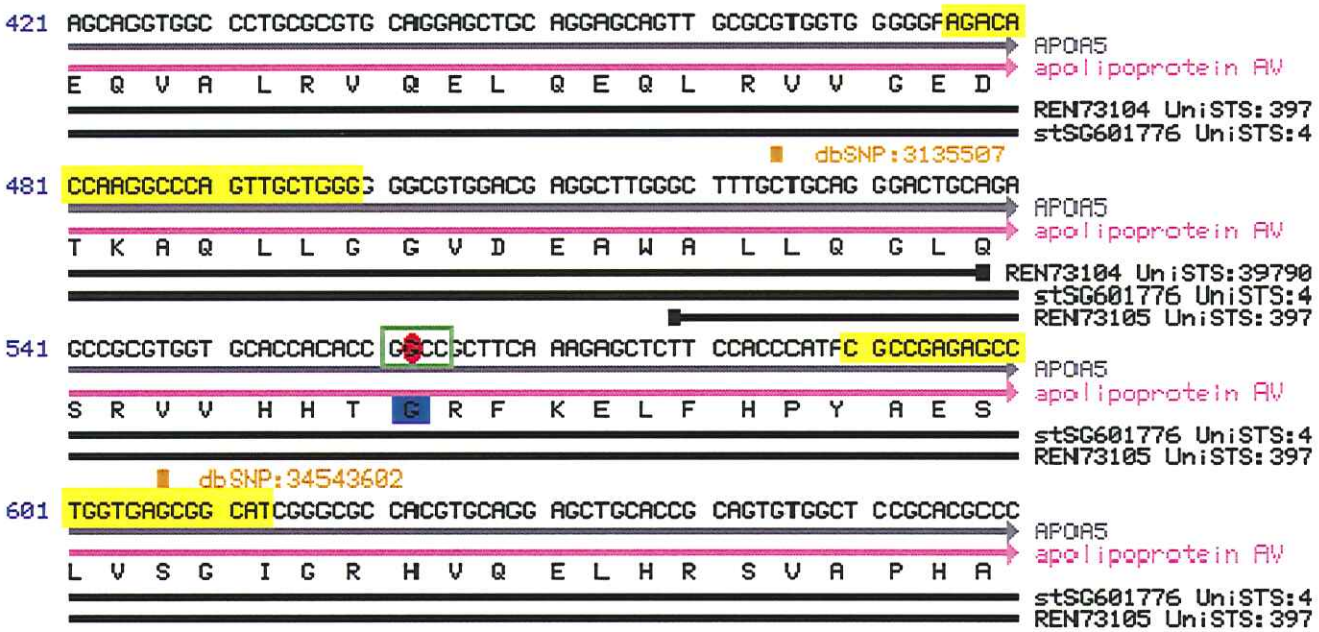


Figure 2.1 Sequence of amplified fragment in coding region of APO A5 gene that includes c.553G>T single nucleotide polymorphism. The yellow highlighted sequences are forward and reverse primers, red highlighted nucleotide G shows the location of SNP and the green box shows the recognition site for restriction enzyme. The polymorphic amino acid (G) is highlighted blue (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Reagents:

1. Taq DNA Polymerase.

2. PCR Amplification Buffer with KCl: 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% Nonidet P40.

This buffer and 25 mM $MgCl_2$ solution were supplied together with Taq DNA Polymerase. Taq DNA Polymerase, amplification buffer and $MgCl_2$ solutions were stored at -20°C.

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

4. Forward and reverse primers.

Procedure:

In order to obtain a single band belonging to the c.553G>T SNP region of APO A5 gene, devoid of non-specific bands, different parameters like $MgCl_2$ concentration, primer and template DNA amount and amplification program were tested in PCR. The optimized PCR mixture for the amplification of c.553G>T SNP is given in Table 2.2. Approximately 400 ng genomic DNA, 200 µM dNTPs, 20 pmol of each primer, 2.0 mM $MgCl_2$ and 2.5 Unit of Taq Polymerase were included in PCR reaction (Table 2.2).

Table 2.2 Components of PCR mixture for APO A5 c.553G>T SNP.

Constituent	Stock concentration	Volume added	Final concentration in 50 µL reaction mixture
Sterile apyrogen H_2O		up to 50 µL	
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	4 µL	2.0 mM
dNTP mixture	10 mM	1 µL	200 µM
Forward primer	10 pmol/ µL	2 µL	20 pmol
Reverse primer	10 pmol/ µL	2 µL	20 pmol
Template DNA	varies	varies	~400 ng
Taq DNA Polymerase	5 U/ µL	0.5 µL	2.5 U

The optimized program of thermalcycler used for the amplification of c.553G>T SNP region of APO A5 is given in Table 2.3 (Tang *et al.*, 2006).

Table 2.3 PCR program used for the amplification of c.553G>T SNP region of APO A5.

Initial denaturation	96°C	5 min	
Denaturation	96°C	30 sec	35 cycles
Annealing	58°C	30 sec	
Extension	72°C	30 sec	
Final Extention	72°C	7 min	

PCR products were analyzed on 2.5 % agarose gel prepared by adding 1.25 g agarose to 50 mL of 0.5x TBE buffer as described in section 2.2.1.4. 9 µL of PCR product was mixed with 2 µL of gel loading buffer and applied to the wells of the gel. 6 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V.

2.2.2.2.1 Restriction Endonuclease Digestion of PCR Products for the Determination of c.553G>T SNP

Principle:

It is possible to detect the c.553G>T single nucleotide polymorphism by RFLP technique if the polymorphic nucleotide is part of a restriction endonuclease recognition site, so that with the base substitution from wild type to mutated form, a restriction site is lost. As a result, the genotype of the individual was determined by the digestion of the PCR product with corresponding restriction enzyme. Schematic representation of the protocol for the determination of c.553G>T genotypes is given in Figure 2.2.

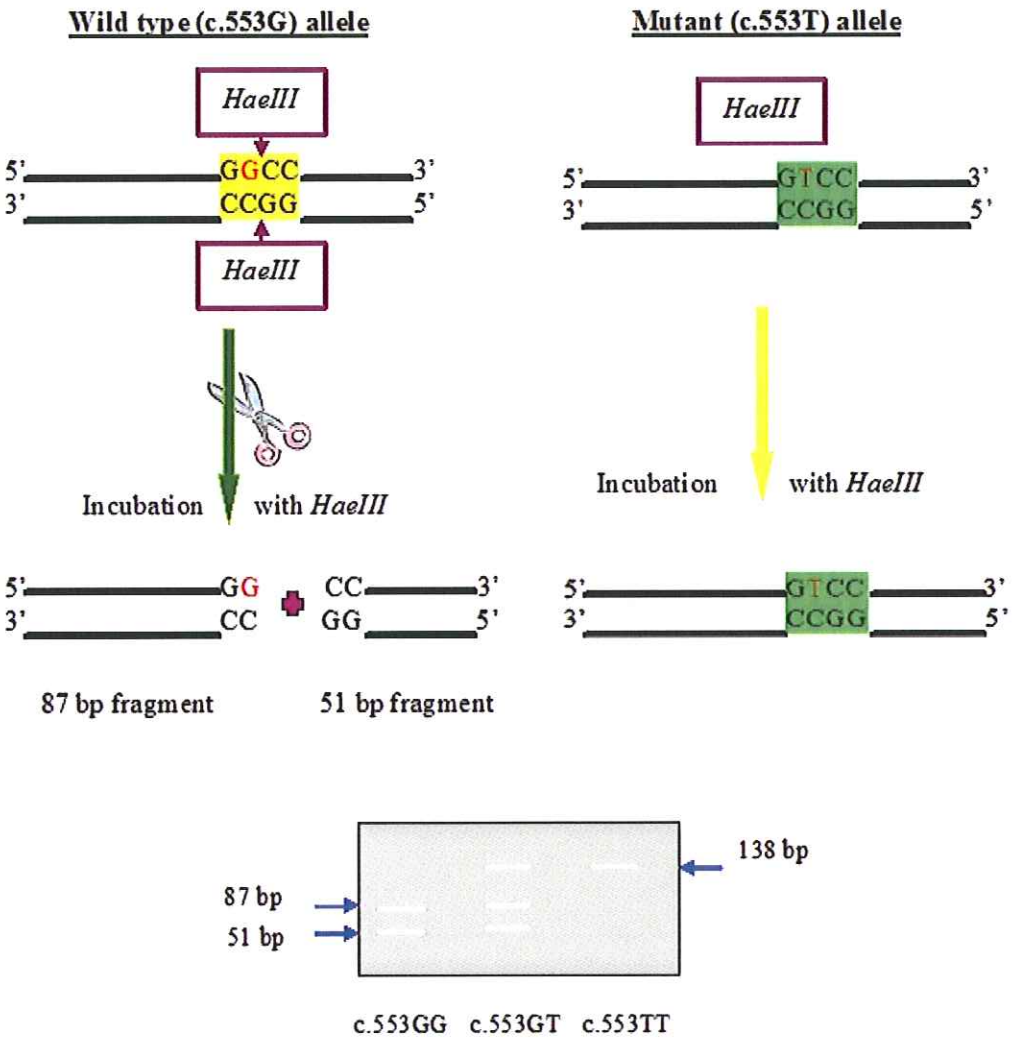


Figure 2.2 Schematic representation of c.553G>T genotype determination. In the left panel, the codon (GGC) for the 185th amino acid glycine in wild type allele (c.553G) can be seen. A recognition site for *HaeIII* is created within the wild type allele and restriction sites are shown with purple arrows. *HaeIII* cuts the 138 bp PCR product containing the recognition site and results in two fragments of 87 bp and 51 bp. In the right panel (polymorphic allele c.553T), G is replaced with T and codes for cysteine (GTC). *HaeIII* does not recognize the restriction site, thus an undigested 138 bp fragment implies c.553T allele. In heterozygotes, two alleles exist together. At the bottom a representative agarose gel photograph of wild type, heterozygote and mutant alleles are given.

Molecular basis of the c.553G>T polymorphism is a single nucleotide change in DNA from guanine (G) to thymine (T). The codon formed in the wild type allele, GGC, codes for glycine, thus the PCR product bears the recognition site for restriction endonuclease *HaeIII* (c.553G allele). The codon formed in the polymorphic allele, GTC, codes for cysteine, and there is no suitable sequence that *HaeIII* can recognize and cut the PCR product (c.553T allele). Recognition site for *HaeIII* is given below:

5'...G G ↓ C C...3'
3'...C C ↑ G G...5'

In the wild type allele, digestion with *HaeIII* cuts the PCR product into two fragments of 87 bp and 51 bp which indicate c.553GG genotype. A single undigested 138 bp band in the agarose gel indicates a c.553TT genotype. Three bands in agarose gel show heterozygote c.553GT genotype which contains two alleles together (Figure 2.2).

Reagents:

1. *BsuRI (HaeIII)* restriction enzyme.

2. Buffer R: 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA.

This buffer was supplied together with the restriction enzyme *HaeIII.* The restriction enzyme and buffer were stored at -20°C.

Procedure:

Incubation of 10 µL of 138 bp PCR product with 5U *HaeIII* restriction enzyme, at 37°C for at least 20 hours, is the first step of the procedure to determine c.553G>T single nucleotide polymorphism of APO A5 gene. Constituents of reaction mixture are given in Table 2.4.

Table 2.4 Constituents of reaction mixture for restriction endonuclease (*HaeIII*) digestion of PCR products for the determination of APO A5 c.553G>T SNP.

Constituent	**Stock Concentration**	**Volume added**	**Final concentration in 30 µL reaction mixture**
Sterile apyrogen H_2O		up to 30 µL	
Buffer R	10x	3 µL	1x
BsuRI (HaeIII)	10 U/µL	0.5 µL	5 U
PCR product		10 µL	

At the end of the incubation period, digestion products were analyzed on 2.5% agarose gel (1.25 g agarose dissolved in 50 mL 0.5x TBE buffer). 30 µL of digestion product was mixed with 3 µL of gel loading buffer and applied to the wells of the gel. 6 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1.5 hours at 100 V.

2.2.2.2 -1131T/C Single Nucleotide Polymorphism

2.2.2.2.1 Polymerase Chain Reaction for -1131T/C SNP

APO A5 -1131T/C SNP region was amplified using primer sequences given by Pennacchio *et al.*, 2001 (Table 2.1). Sequence of the amplified fragment in promoter region of APO A5 gene that includes -1131T/C single nucleotide polymorphism is given in Figure 2.3. Note that the nucleotide sequence of the reverse primer is different from that of DNA.

Primer's sequence: CCC CAG GAA CTG GAG CGA AAT T

DNA's sequence: GGG GTC CTT GAC CTC GCT TTC A

The reason for such a modification is to create a recognition site for *MseI* restriction endonuclease in the PCR product. Recognition site for *MseI* is given in next section.

Reagents:

1. Taq DNA Polymerase.

2. PCR Amplification Buffer with $(NH_4)_2SO_4$: 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20.

This buffer and 25 mM $MgCl_2$ solution were supplied together with Taq DNA Polymerase. Taq DNA Polymerase, amplification buffer and $MgCl_2$ solutions were stored at -20°C.

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

4. Forward and reverse primers.

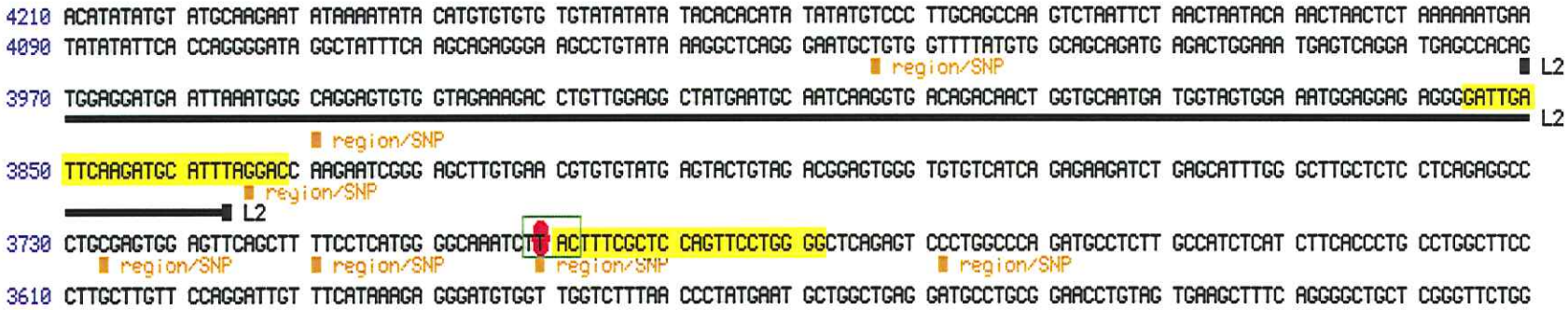


Figure 2.3 Sequence of amplified fragment in promoter region of APO A5 gene that includes -1131T/C single nucleotide polymorphism. The yellow highlighted sequences are forward and reverse primers, red highlighted nucleotide T shows the location of SNP and the green box shows the recognition site for restriction enzyme (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Procedure:

In order to obtain a single band belonging to the -1131T/C SNP region of APO A5 gene, devoid of non-specific bands, different parameters like $MgCl_2$ concentration, primer and template DNA amount and amplification program were tested in PCR. The optimized PCR mixture for the amplification of -1131T/C SNP is given in Table 2.5. Approximately 400 ng genomic DNA, 200 µM dNTPs, 20 pmol of each primer, 1.25 mM $MgCl_2$ and 2.5 Unit of Taq Polymerase were included in PCR reaction (Table 2.5)

Table 2.5 Components of PCR mixture for APO A5 -1131T/C SNP.

Constituent	Stock concentration	Volume added	Final concentration in 50 µL reaction mixture
Sterile apyrogen H_2O		up to 50 µL	
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	2.5 µL	1.25 mM
dNTP mixture	10 mM	1 µL	200 µM
Forward primer	10 pmol/ µL	2 µL	20 pmol
Reverse primer	10 pmol/ µL	2 µL	20 pmol
Template DNA	varies	varies	~400 ng
Taq DNA Polymerase	5 U/ µL	0.5 µL	2.5 U

The optimized program of thermalcycler used for the amplification of -1131T/C SNP region of APO A5 is given in Table 2.6 (Pennacchio *et al.*, 2001).

Table 2.6 PCR program used for the amplification of -1131T/C SNP region of APO A5.

Initial denaturation	96°C	2 min	
Denaturation	94°C	15 sec	32 cycles
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extention	72°C	3 min	

PCR products were analyzed on 2.5 % agarose gel prepared by adding 1.25 g agarose to 50 mL of 0.5x TBE buffer as described in section 2.2.1.4. 9 µL of PCR product was mixed with 2 µL of gel loading buffer and applied to the wells of the gel. 6 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V.

2.2.2.2.2 Restriction Endonuclease Digestion of PCR Products for the Determination of -1131T/C SNP

Principle:

It is possible to detect the -1131T/C single nucleotide polymorphism by RFLP technique if the polymorphic nucleotide is part of a restriction endonuclease recognition site, so that with the base substitution from wild type to mutated form, a restriction site is lost. As a result, the genotype of the individual was determined by the digestion of the PCR product with the corresponding restriction enzyme. Schematic representation of the protocol for the determination of -1131T/C genotypes is given in Figure 2.4.

Molecular basis of the -1131T/C polymorphism is a single nucleotide change in DNA from thymine (T) to cytosine (C). The sequence around thymine is a recognition site for *MseI* restriction enzyme, which is given below:

5'...T ↓ T A A...3'
3'...A A T ↑ T...5'

In the wild type allele, digestion with *MseI* cuts the 187 bp PCR product into two fragments of 167 bp and 20 bp which indicate -1131TT genotype. A single undigested 187 bp band in the agarose gel indicates a -1131CC genotype. Three bands in agarose gel show heterozygote -1131TC genotype which contains two alleles together (Figure 2.4).

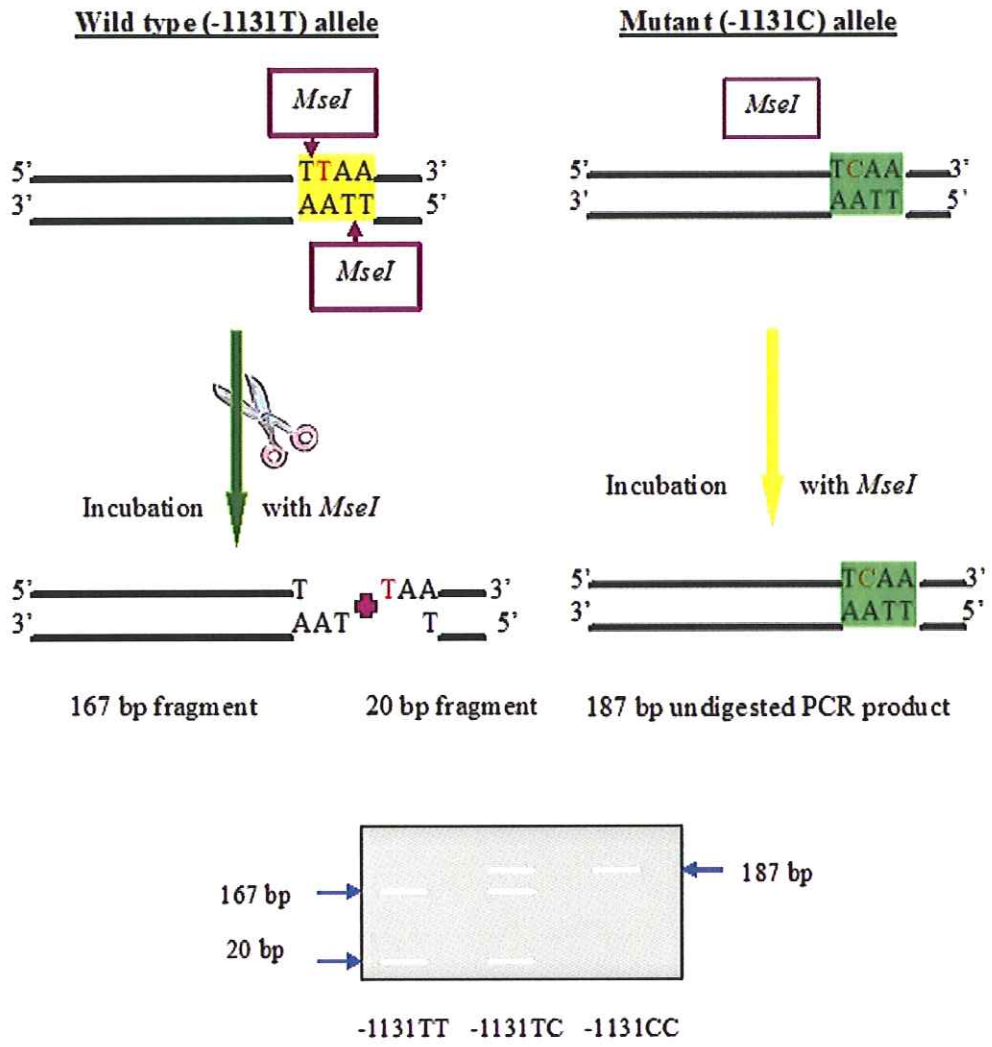


Figure 2.4 Schematic representation of -1131T/C genotype determination. In the left panel, the wild type allele (-1131T) can be seen. A recognition site for *MseI* is created within the wild type allele and restriction sites are shown with purple arrows. *MseI* cuts the 187 bp PCR product containing the recognition site and results in two fragments of 167 bp and 20 bp. In the right panel, the polymorphic allele (-1131C), can be seen. *MseI* does not recognize the site around the C nucleotide as a restriction site, thus an undigested 187 bp fragment implies -1131C allele. In heterozygotes, two alleles exist together. At the bottom a representative agarose gel photograph of wild type, heterozygote and mutant alleles are given.

Reagents:

1. *TruII (MseI)* restriction enzyme.

2. Buffer R: 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA.

This buffer was supplied together with the restriction enzyme *MseI*. The restriction enzyme and buffer were stored at -20°C.

Procedure:

Incubation of 10 µL of 187 bp PCR product with 5U *MseI* restriction enzyme, at 65°C for at least 20 hours, is the first step of the procedure to determine -1131T/C single nucleotide polymorphism of APO A5 gene. Constituents of reaction mixture are given in Table 2.7.

Table 2.7 Constituents of reaction mixture for restriction endonuclease (*MseI*) digestion of PCR products for the determination of APO A5 -1131T/C SNP.

Constituent	Stock Concentration	Volume added	Final concentration in 30 µL reaction mixture
Sterile apyrogen H_2O		up to 30 µL	
Buffer R	10x	3 µL	1x
TruII (MseI)	10 U/µL	0.5 µL	5 U
PCR product		10 µL	

At the end of the incubation period, digestion products were analyzed on 2.5% agarose gel (1.25 g agarose dissolved in 50 mL 0.5x TBE buffer). 30 μL of digestion product was mixed with 3 μL of gel loading buffer and applied to the wells of the gel. 6 μL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1.5 hours at 100 V.

2.2.3 Statistical Analysis

Statistical analyses were performed by using SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± SD. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov-Smirnov test. Differences of continuous variables were evaluated by the Student's t-test or Mann-Whitney U test, depending on the shape of the distribution curves. Categorical variables were expressed as proportions and compared using χ^2 test.

In order to determine the effects of vascular risk factors, lipid parameters and APO A5 genotypes in the prediction of ischemic stroke, logistic regression analyses with backward selection method was used. Age and sex were also included as covariates. 2-tailed probability values with 95% confidence intervals were estimated for each odds ratio. The Hosmer-Lemeshow goodness of fit test was used for calibration. A *P* value of less than 0.05 was evaluated as statistically significant.

CHAPTER 3

RESULTS

3.1 Study Population

The study population consisted of 198 ischemic stroke patients and 130 healthy controls. The two important single nucleotide polymorphisms in APO A5 gene were determined in blood samples obtained from subjects by PCR and RFLP techniques, using the corresponding restriction enzymes. Clinical characteristics like blood serum total cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol levels were measured. Besides, conventional risk factors including hypertension, diabetes, obesity and cigarette smoking were recorded for ischemic stroke patients and controls. Raw data including these parameters in addition to APO A5 genotypes of study participants are listed in Appendix C.

Demographic features, the results of clinical laboratory tests and the prevalence of conventional risk factors of patients and control subjects are given in Table 3.1. The age of study population varied between 20 to 84 years in stroke patients and 34 to 90 years in controls. There was no statistically significant difference in mean age of the patient (65.8 ± 14.3 years) and the control groups (63.5 ± 13.7 years; P=0.059). There were 111 males and 87 females among stroke patients. Patient group contained more males (56.1%) than the control group (47.7%, P=0.138). The prevalence of conventional risk factors, hypertension, diabetes and smoking in the patient group were found to be higher than the control group. Hypertension was significantly higher in the

patient group (63.6%) when compared to control group (41.5%, P=0.000). Out of 180 hypertensive people, 126 (70%) were stroke patients. Diabetes mellitus was observed in 34.3% of stroke patients, while 18.5% of control subjects were diabetic (P=0.002). Among 92 diabetic subjects, 68 (73.9%) had ischemic stroke. Smokers were found to be insignificantly higher in the patient group (21.2%) than the control group (13.8%, P=0.091). Out of 60 smokers, 42 (70%) were stroke patients.

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

Parameter	Stroke (n = 198)	Controls (n = 130)	*P*
Age (years)[a]	65.8 ± 14.3	63.5 ± 13.7	0.059
Male, n (%)[b]	111 (56.1)	62 (47.7)	0.138
Hypertension, n (%)[b]	126 (63.6)	54 (41.5)	0.000
Diabetes mellitus, n (%)[b]	68 (34.3)	24 (18.5)	0.002
Smokers, n (%)[b]	42 (21.2)	18 (13.8)	0.091
Obesity, n (%)[b]	12 (6.1)	9 (6.9)	0.755
Statin, n (%)[b]	17 (8.6)	4 (3.1)	0.048
Stenosis, %[a]	40 ± 0.4	15 ± 0.2	0.000
Total cholesterol (mmol/L)[c]	4.8 ± 1.3	4.8 ± 1.3	0.964
Triglycerides (mmol/L)[c]	1.6 ± 0.7	1.4 ± 0.7	0.098
HDL-cholesterol (mmol/L)[c]	1.1 ± 0.3	1.2 ± 0.3	0.004
LDL-cholesterol (mmol/L)[c]	2.8 ± 1.1	2.6 ± 1.0	0.166
VLDL-cholesterol (mmol/L)[c]	0.7 ± 0.4	0.7 ± 0.4	0.965

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

The frequency of obese individuals in stroke patients (6.1%) was slightly lower than the controls (6.9%, P=0.755). The prevalence of statin drug users was 8.6% in stroke patients and 3.1% in controls (P=0.048). Stroke patients had 40% stenosis of their carotid artery and controls had 15% blockage (P=0.000).

Estimates of some conventional risk factors are given in Table 3.2. Hypertension and diabetes mellitus have significant effect and exhibit more than 2-fold relative risk for ischemic stroke. Gender, smoking and obesity did not have significant effect on ischemic stroke risk.

Table 3.2 Effects of conventional risk factors on ischemic stroke.

Parameter	OR (95% CI)	*P*
Gender (male)	1.399 (0.898–2.182)	0.138
Hypertension	2.463 (1.565–3.876)	0.000
Diabetes mellitus	2.310 (1.358–3.930)	0.002
Smoking	1.675 (0.916–3.062)	0.091
Obesity	0.867 (0.355–2.121)	0.755

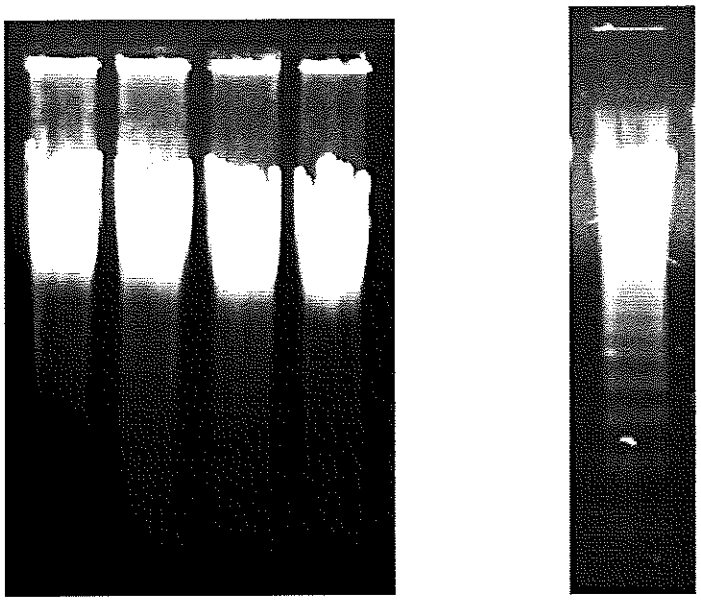
According to clinical laboratory tests given in Table 3.1, the level of HDL-cholesterol was significantly lower in ischemic stroke patients (1.1 ± 0.3 mmol/L) when compared to controls (1.2 ± 0.3 mmol/L, P=0.004), while the level of LDL-cholesterol was insignificantly higher in patients (2.8 ± 1.1 mmol/L) than controls (2.6 ± 1.0 mmol/L, P=0.166). In addition, total cholesterol levels were almost the same in patients and controls (4.8 ± 1.3

mmol/L, P=0.964). Triglyceride level of patients (1.6 ± 0.7 mmol/L) was insignificantly higher than controls (1.4 ± 0.7 mmol/L, P=0.098).

3.2 Genomic DNA Isolation from Human Whole Blood Samples

The purity of the isolated genomic DNA is important and was tested spectrophotometrically. DNA samples having A_{260} / A_{280} ratio below 1.6 or above 2.0 were discarded and the isolation procedure was repeated for these samples. Also the quantity of DNA was determined by spectrophotometry and the amount of DNA sample to be added to PCR mixture was calculated from this information.

Obtaining the DNA in intact form during genomic DNA isolation is another important point to be considered. The quality of genomic DNA isolated from whole blood was tested by agarose gel electrophoresis. Intact genomic DNA should be observed as an intense single band (Figure 3.1.a). However, a smear on the gel shows that DNA is degraded and cannot be used in further studies (Figure 3.1.b). Figure 3.1 shows a representative agarose gel photograph.



a b

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

3.3 Determination of APO A5 c.553G>T and -1131T/C Genotypes by Polymerase Chain Reaction Followed by Restriction Enzyme Digestion

Two regions of APO A5 gene; one in coding region and one in promoter region were amplified by PCR. To determine the genotypes of APO A5 c.553G>T and -1131T/C single nucleotide polymorphisms, amplification procedure was followed by restriction enzyme digestion with *HaeIII* and *MseI* restriction endonucleases, respectively.

3.3.1 APO A5 c.553G>T Single Nucleotide Polymorphism

3.3.1.1 Polymerase Chain Reaction Results for c.553G>T SNP

In order to obtain a single band belonging to the c.553G>T SNP region of APO A5 gene, devoid of non-specific bands, different parameters like $MgCl_2$ concentration, primer and template DNA amount and amplification program were tested in PCR. Approximately 400 ng genomic DNA, 200 µM dNTPs, 20 pmol of each primer, 2.0 mM $MgCl_2$ and 2.5 Unit of Taq DNA polymerase were included in PCR reaction.

PCR products were analyzed on 2.5 % agarose gel prepared by adding 1.25 g agarose to 50 mL of 0.5x TBE buffer as described in section 2.2.1.4. 9 µL of PCR product was mixed with 2 µL of gel loading buffer and applied to the wells of the gel. 6 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V. Figure 3.2 shows a photograph of a representative agarose gel electrophoresis pattern of PCR results for c.553G>T SNP.

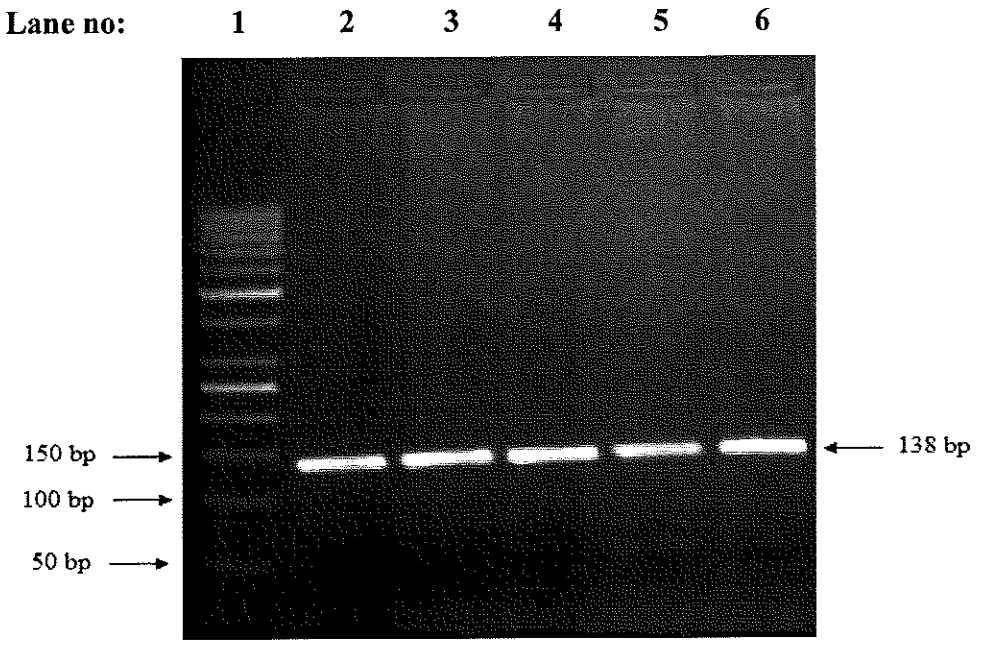


Figure 3.2 2.5% Agarose gel electrophoresis showing PCR products of c.553G>T SNP region of APO A5 gene. As expected, a single band of 138 bp PCR product was obtained. Lane 1 contained DNA ladder (50-1000 bp) and lanes 2-6 contained PCR products of subjects 183, 184, 185, 186 and 187, respectively.

3.3.1.2 Restriction Endonuclease Digestion Results for c.553G>T SNP

In c.553G>T single nucleotide polymorphism, 553rd nucleotide of APO A5 gene is occupied with G in wild type allele, while the same location is T in mutated allele. In wild type allele, with G in position 553, the PCR product bears a recognition site for *BsuRI (HaeIII)* restriction endonuclease. Digestion of 10 µL of 138 bp PCR product with 5U *HaeIII* restriction enzyme, at 37°C for at least 20 hours, efficiently cuts the PCR product, producing two fragments of 87 bp and 51 bp. However in mutated allele, with T in position 553, there is no suitable recognition sequence that *HaeIII* can recognize and cut the PCR

product. In this case, digestion with *HaeIII* yields one fragment of 138 bp undigested PCR product itself.

If the individual is homozygous wild type (c.553GG), the presence of recognition site for *HaeIII* would cause effective digestion of PCR product and two bands of 87 bp and 51 bp would be obtained. While in a homozygous mutated individual (c.553TT), *HaeIII* restriction endonuclease would fail to cut the PCR product and a single 138 bp fragment would be the result. Throughout the study, a homozygous mutated individual was not encountered. In heterozygote (c.553GT), both undigested 138 bp band and digested fragments of 87 bp and 51 bp would be observed.

Digestion products were analyzed on 2.5 % agarose gel (1.25 g agarose to 50 mL of 0.5x TBE buffer). 30 µL of digestion product was mixed with 3 µL of gel loading buffer and applied to the wells of the gel. 6 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1.5 hours at 100 V. Figure 3.3 shows a representative agarose gel photograph of restriction endonuclease digestion results for c.553G>T SNP. *HaeIII* cuts the 138 bp PCR product resulting two fragments of 87 bp and 51 bp fragments for the c.553G allele and an undigested 138 bp fragment implies c.553T allele.

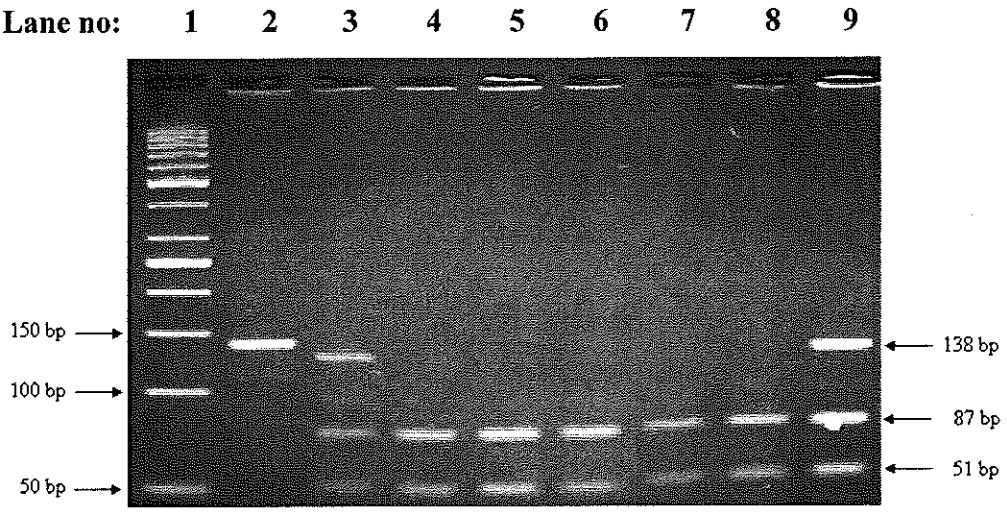


Figure 3.3 2.5% Agarose gel electrophoresis showing restriction endonuclease digestion products of c.553G>T SNP region of APO A5 gene. Lane 1 contained DNA ladder (50-1000 bp), lane 2 contained PCR product which was not subjected to digestion and lanes 3-9 contained digestion products of subjects 97, 98, 99, 100, 101, 105 and 107, respectively.

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

Lane 3 (Subject 97): c.553GT
Lane 4 (Subject 98): c.553GG
Lane 5 (Subject 99): c.553GG
Lane 6 (Subject 100): c.553GG
Lane 7 (Subject 101): c.553GG
Lane 8 (Subject 105): c.553GG
Lane 9 (Subject 107): c.553GT

3.3.2 APO A5 -1131T/C Single Nucleotide Polymorphism

3.3.2.1 Polymerase Chain Reaction Results for -1131T/C SNP

In order to obtain a single band belonging to the -1131T/C SNP region of APO A5 gene, devoid of non-specific bands, different parameters like $MgCl_2$ concentration, primer and template DNA amount and amplification program were tested in PCR. Approximately 400 ng genomic DNA, 200 μM dNTPs, 20 pmol of each primer, 1.25 mM $MgCl_2$ and 2.5 Unit of Taq Polymerase contained in PCR reaction.

PCR products were analyzed on 2.5 % agarose gel prepared as described in section 2.2.1.4. 9 μL of PCR product was mixed with 2 μL of gel loading buffer and applied to the wells of the gel. 6 μL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100 V. Figure 3.4 shows a photograph of a representative agarose gel electrophoresis pattern of PCR results for -1131T/C SNP.

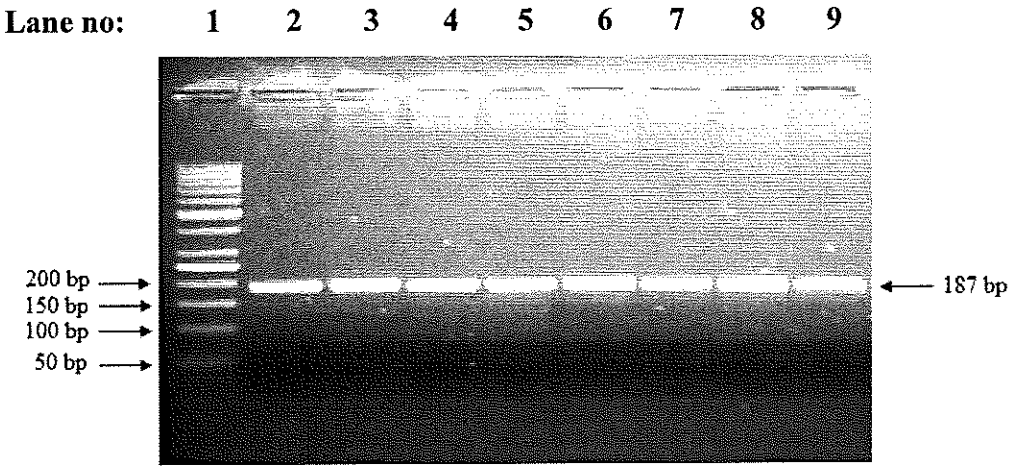


Figure 3.4 2.5% Agarose gel electrophoresis showing PCR products of -1131T/C SNP region of APO A5 gene. As expected, a single band of 187 bp PCR product was obtained. Lane 1 contained DNA ladder (50-1000 bp) and lanes 2-9 contained PCR products of subjects 1, 2, 3, 4, 5, 6, 7 and 8, respectively.

3.3.2.2 Restriction Endonuclease Digestion Results for -1131T/C SNP

In -1131T/C SNP, -1131st nucleotide of APO A5 gene is occupied by T in wild type allele, while it is substituted to C in the mutated allele. In wild type allele, with T in position -1131, the PCR product bears a recognition site for *TruII (MseI)* restriction endonuclease. Digestion of 10 µL of 187 bp PCR product with 5U *MseI* restriction enzyme, at 65°C for at least 20 hours, efficiently cuts the PCR product, producing two fragments of 167 bp and 20 bp. However in mutated allele, with C in position -1131, there is no suitable recognition sequence that *MseI* can recognize and cut the PCR product. In this case, digestion with *MseI* yields one fragment of 187 bp undigested PCR product itself.

When analyzing the *MseI* restriction endonuclease digestion results in agarose gel, a homozygous wild type individual (-1131TT) would yield two bands of 167 bp and 20 bp as the enzyme can cut the PCR product. While, a homozygous mutated individual (-1131CC) would yield a single band of 187 bp as the *MseI* restriction endonuclease fails to cut the PCR product. In heterozygote (-1131TC), both undigested 187 bp band and digested fragments of 167 bp and 20 bp would be observed.

Digestion products were analyzed on 2.5 % agarose gel. 30 µL of digestion product was mixed with 3 µL of gel loading buffer and applied to the wells of the gel. 6 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1.5 hours at 100 V. Figure 3.5 shows a representative agarose gel photograph of restriction endonuclease digestion results for -1131T/C SNP. *MseI* cuts the 187 bp PCR product resulting two fragments of 167 bp and 20 bp fragments for the -1131T allele and an undigested 187 bp fragment implies -1131C allele.

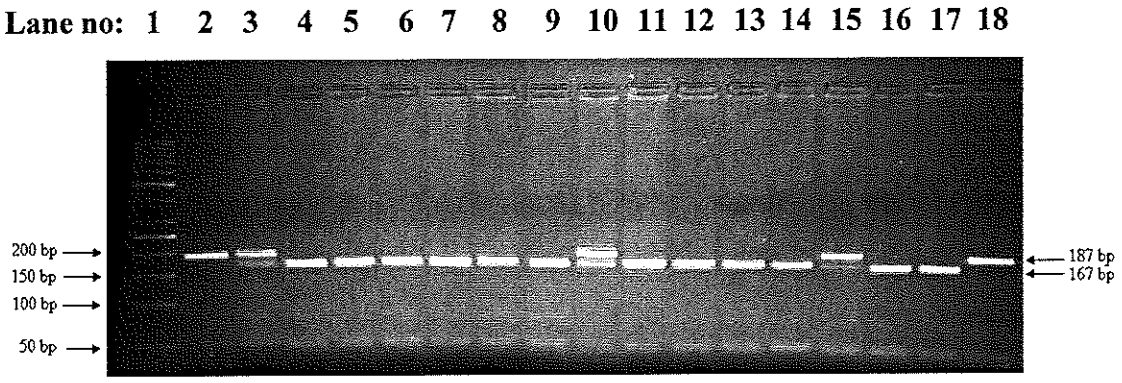


Figure 3.5 2.5% Agarose gel electrophoresis showing restriction endonuclease digestion products of -1131T/C SNP region of APO A5 gene. Lane 1 contained DNA ladder (50-1000 bp), lane 2 contained PCR product which was not subjected to digestion and lanes 3-18 contained digestion products of subjects 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 19, 22, 23, 24, 25 and 319, respectively.

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

Lane 3 (Subject 5): -1131TC
Lane 4 (Subject 6): -1131TT
Lane 5 (Subject 7): -1131TT
Lane 6 (Subject 8): -1131TT
Lane 7 (Subject 9): -1131TT
Lane 8 (Subject 10): -1131TT
Lane 9 (Subject 15): -1131TT
Lane 10 (Subject 16): -1131TC
Lane 11 (Subject 17): -1131TT
Lane 12 (Subject 18): -1131TT
Lane 13 (Subject 19): -1131TT
Lane 14 (Subject 22): -1131TT
Lane 15 (Subject 23): -1131TC
Lane 16 (Subject 24): -1131TT
Lane 17 (Subject 25): -1131TT
Lane 18 (Subject 319): -1131CC

3.4 APO A5 Genotypes and Allele Frequencies

The distribution of APO A5 genotypes and allele frequencies of the stroke patient and control groups for the c.553G>T and -1131T/C polymorphisms are presented in Table 3.3.

Table 3.3 Distribution of genotypes and allele frequencies for APO A5 c.553G>T and -1131T/C single nucleotide polymorphisms in stroke patients and controls.

	Stroke (n=198)	**Controls (n=130)**	**OR (95% CI)**	***P***
APO A5 c.553				
Genotypes, n(%)				
GG	197 (99.5)	129 (99.2)		
GT	1 (0.5)	1 (0.8)	0.655[a] (0.041-10.563)	0.764
TT	0 (0)	0 (0)		
Alleles				
G	0.997	0.996		
T	0.003	0.004	0.747[b] (0.047-11.989)	0.836
APO A5 -1131				
Genotypes, n(%)				
TT	159 (80.3)	105 (80.8)		
TC	39 (19.7)	24 (18.5)	1.030[c] (0.589-1.802)	0.917
CC	0 (0)	1 (0.8)		
Alleles				
T	0.902	0.900		
C	0.098	0.100	0.983[d] (0.583-1.659)	0.949

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

In this study, a total of 198 ischemic stroke patients and 130 control subjects were investigated for c.553G>T polymorphism. Among them, 197 patients and 129 controls were homozygous wild type (c.553GG) while, only 2 individuals were heterozygote (c.553GT), one is patient and one is control. A homozygous mutated individual (c.553TT) was not encountered throughout this study. The c.553GG genotype frequency was nearly the same in patients (99.5%) and controls (99.2%). Since no TT homozygote was seen, the GT and TT genotypes were ascribed as a single group and compared to the wild type GG genotype, the T allele carriers were almost the same in patients and controls (P=0.764). There was also no significant difference between T allele frequencies of patients (0.003) and controls (0.004, P=0.836).

A total of 198 ischemic stroke patients and 130 control subjects were also investigated for -1131T/C polymorphism. Since only one CC homozygote mutant was seen in the control group and none in stroke patients, the TC and CC genotypes were ascribed to a single group and these genotypes are so-called risky genotypes. Among 198 stroke patients 159 were homozygous wild type (-1131TT) and 39 were heterozygote (-1131TC) while, among 130 controls 105 were homozygous wild type (-1131TT) and 25 were so-called risky genotypes (-1131TC and -1131CC). The -1131TT genotype frequency was nearly the same in patients (80.3%) and controls (80.8%). When compared to the wild type TT genotype, the C allele carriers were almost the same in patients and controls (P=0.917). There was also no significant difference between C allele frequencies of patients (0.098) and controls (0.100, P=0.949).

3.5 APO A5 Genotypes in Different Subgroups of Ischemic Stroke Patients and Controls

The APO A5 genotypes were analyzed in three subgroups having different vascular risk factors. Since there is only two heterozygote individual (one is patient and one is control) and no homozygote mutant, the genotypes of c.553G>T polymorphism were not analyzed in these subgroups. APO A5 -1131 genotypes were compared between ischemic stroke patients and controls in subgroups of hypertensive versus normotensive, diabetic versus non-diabetic and smoker versus non-smoker.

While making comparison between the genotypes of -1131T/C polymorphism, the TC and CC genotypes were ascribed to a single group because only one CC homozygote was seen in the control group and none in stroke patients. Table 3.4 shows the distribution of the -1131 genotypes between patients and controls in hypertensive and normotensive subgroups. Among 180 hypertensive individuals, 126 were stroke patients and 54 were controls. The proportion of so-called risky genotypes (TC+CC) was higher in patients (19%) than controls (14.8%). When compared to the wild type TT genotype, the C allele carriers were higher in patients than controls (P=0.496) in hypertensive subgroup. However between normotensive individuals, the C allele carriers were almost the same in patients and controls (P=0.821) when compared to the wild type genotype.

Table 3.4 Distribution of -1131 genotypes in hypertensive/normotensive subgroups of stroke patients and controls.

Genotypes n (%)	Hypertensive (n=180)			Normotensive (n=148)		
	Stroke (n=126)	Controls (n=54)	*P*	Stroke (n=72)	Controls (n=76)	*P*
-1131 TT	102 (81)	46 (85.2)		57 (79.2)	59 (77.6)	
-1131 TC	24 (19)	8 (14.8)	0.496*	15 (20.8)	16 (21.1)	0.821*
-1131 CC	0 (0)	0 (0)		0 (0)	1 (1.3)	

*TC+CC vs. TT

Table 3.5 shows that the higher proportion of diabetic individuals was also ischemic stroke patients, 68 of the 92 diabetic individuals suffered from stroke. Among the diabetics, proportion of C allele carriers was higher in stroke patients (20.6%) than controls (8.3%). When compared to the wild type TT genotype, stroke patients were more susceptible to carry C allele (P=0.173) in diabetics while among non-diabetics, the C allele carriers were almost the same in patients and controls (P=0.640).

Table 3.5 Distribution of -1131 genotypes in diabetic/non-diabetic subgroups of stroke patients and controls.

Genotypes n (%)	Diabetic (n=92)			Non-diabetic (n=236)		
	Stroke (n=68)	Control (n=24)	*P*	Stroke (n=130)	Control (n=106)	*P*
-1131 TT	54 (79.4)	22 (91.7)		105 (80.8)	83 (78.3)	
-1131 TC	14 (20.6)	2 (8.3)	0.173*	25 (19.2)	22 (20.8)	0.640*
-1131 CC	0 (0)	0 (0)		0 (0)	1 (0.9)	

*TC+CC vs. TT

Table 3.5 also demonstrates that when the diabetic people have risky genotype (TC+CC), the possibility of having stroke was higher than the non-diabetic people with the same genotype (OR=2.800; 95% CI=2.036-3.864, *P*=0.000).

As given in Table 3.6, among smokers there was no difference between the stroke patients and controls in terms of -1131 genotypes. Also the distribution of -1131 genotypes was nearly the same in patients and controls in non-smokers.

Table 3.6 Distribution of -1131 genotypes in smoker/non-smoker subgroups of stroke patients and controls.

Genotypes n (%)	Smokers (n=60)			Non-smokers (n=268)		
	Stroke (n=42)	Control (n=18)	*P*	Stroke (n=156)	Control (n=112)	*P*
-1131 TT	35 (83.3)	15 (83.3)		124 (79.5)	90 (80.3)	
-1131 TC	7 (16.7)	3 (16.7)	1*	32 (20.5)	21 (18.8)	0.861*
-1131 CC	0 (0)	0 (0)		0 (0)	1 (0.9)	

*TC+CC vs. TT

3.6 Effects of Conventional Vascular Risk Factors in Different APO A5 Genotypes of Ischemic Stroke Patients and Controls

The conventional vascular risk factors, hypertension, diabetes and smoking were analyzed in terms of proportion of stroke patients to controls within the so-called risky genotype group and the non-risky genotype group. Since there are only two heterozygote individuals (one is patient and one is control) and no homozygote mutant, the genotypes of c.553G>T polymorphism were not analyzed with these vascular risk factors. The results were only stratified by the APO A5 -1131 genotypes. While making comparison between the genotypes of -1131T/C polymorphism, the TC and CC genotypes were ascribed to a single group because only one CC homozygote was seen in the control group and none in stroke patients (Table 3.7).

Table 3.7 shows that, in each genotype group of -1131T/C polymorphism, the proportion of stroke patients to controls was increased in hypertensive, diabetic or smoker group when compared to normotensive, non-diabetic or non-smoker group.

Table 3.7 Stratification of hypertensive-normotensive, diabetic-non-diabetic, smoker-non-smoker individuals according to -1131 genotypes and stroke-control status.

	-1131TT (n=264) Stroke / Control	OR (95% CI)	*P*	-1131TC+CC (n=64) Stroke / Control	OR (95% CI)	*P*
Hypertensive (n=180)	102 / 46	2.295[a]	0.001	24 / 8	3.400[a]	0.021
Normotensive (n=148)	57 / 59	(1.387-3.798)		15 / 17	(1.179-9.808)	
Diabetic (n=92)	54 / 22	1.940[b]	0.022	14 / 2	6.440[b]	0.012
Non-diabetic (n=236)	105 / 83	(1.094-3.442)		25 / 23	(1.318-31.459)	
Smokers (n=60)	35 / 15	1.694	0.117	7 / 3	1.604[c]	0.523
Non-smokers (n=268)	124 / 90	(0.873-3.286)		32 / 22	(0.374-6.890)	

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

The risk of having stroke was higher in the -1131TC+CC group when compared to -1131TT in hypertensives and diabetics (Table 3.7). However, the -1131TT genotype group also had an increased proportion of stroke patients when compared to controls with C allele carriers. For hypertension, the proportion of hypertensive stroke patients to hypertensive controls compared to the proportion of normotensive stroke patients to normotensive controls was highest in the -1131TC+CC group (OR=3.400; 95% CI=1.179-9.808, *P*=0.021). Therefore, in hypertensive individuals with -1131TC or -1131CC genotype the risk of having stroke is 3.4 times higher than the normotensives with the same genotype. In -1131TT genotype group, risk of ischemic stroke in hypertensives is nearly 2.3 times higher than the normotensives (OR=2.295; 95% CI=1.387-3.798, *P*=0.001).

As given in Table 3.7, among diabetics, the highest approximate relative risk for stroke was found for -1131TC+CC group (OR against non-diabetics is 6.440; 95% CI=1.318-31.459, *P*=0.012). Similarly, among -1131TT genotype, a significant risk for ischemic stroke was found in diabetics compared to non-diabetics (OR=1.940; 95% CI=1.094-3.442, *P*=0.022).

In each genotype group of -1131T/C polymorphism, the proportion of stroke patients to controls was increased in smoker group when compared to non-smoker group. However, risk associated with ischemic stroke in smokers was not significant in any of the -1131T/C genotypes (Table 3.7).

3.6.1 Effect of APO A5 -1131C Allele on Stroke Risk in Risk Groups

The effect of APO A5 -1131C allele on stroke risk in hypertensives and diabetics is given in Table 3.8. Among hypertensives -1131C allele carriers slightly higher risk for ischemic stroke (OR=1.316; 95% CI=0.571-3.030, *P*=0.518). This allele was also associated with an insignificantly increased risk

of stroke in diabetics (OR=2.639; 95% CI=0.577-12.067, *P*=0.195). There is no difference between stroke patients and controls according to -1131C allele frequency in normotensive and non-diabetic individuals. The -1131C allele of APO A5 was not significantly associated with stroke in neither hypertensives nor normotensives. This allele was also associated with an insignificant 2.639-fold increased risk of stroke in diabetics. However, this allele was not associated with stroke risk in non-diabetic individuals.

Table 3.8 APO A5 -1131C allele frequency in risk groups.

Group	-1131C allele frequency		OR (95% CI)	*P*
	Stroke	Control		
Hypertensive	0.095	0.074	1.316 (0.571-3.030)	0.518
Normotensive	0.104	0.118	0.826 (0.403-1.695)	0.602
Diabetic	0.103	0.042	2.639 (0.577-12.067)	0.195
Non-diabetic	0.096	0.113	0.804 (0.447-1.446)	0.804

3.7 APO A5 -1131T/C Polymorphism and Serum Lipid Parameters

In order to estimate the impact of the APO A5 -1131T/C polymorphism on lipid metabolism, the mean serum lipid levels of stroke patients and controls were compared according to the -1131 genotypes (Table 3.9). Because there is only one individual with CC genotype, the C allele carriers (TC+CC) were ascribed to a single group.

Table 3.9 Effect of lipid parameters on stroke risk according to APO A5 -1131T/C genotypes.

Variables (mmol/L)	TC+CC (n=64)			TT (n=264)		
	Stroke (n=39)	Controls (n=25)	*P*	Stroke (n=159)	Controls (n= 105)	*P*
TC	5.20±1.69	4.84±1.23	0.362	4.73±1.20	4.85±1.31	0.580
TG	1.70±0.72	1.35±0.51	0.038	1.53±0.74	1.45±0.68	0.367
HDL-C	1.05±0.24	1.20±0.30	0.028	1.11±0.27	1.19±0.34	0.030
LDL-C	3.12±1.44	2.52±0.89	0.066	2.72±0.95	2.66±1.06	0.636

Data are mean ± SD, comparisons are by Independent Samples T-test

According to Table 3.9, stroke patients having APO A5 -1131 TC or CC genotypes, had significantly higher TG levels than controls with same genotypes (P=0.038). Also, stroke patients with these genotypes had significantly lower HDL cholesterol when compared to control groups (P=0.028).

This table also represents that in stroke patient group, the C allele carriers had higher total cholesterol, higher triglyceride, lower HDL cholesterol and higher LDL cholesterol levels. The APO A5 -1131 genotypes influenced the serum lipid parameters of stroke patients. However in control group, all serum lipid parameters were nearly same in APO A5 -1131 genotypes.

3.8 Logistic Regression Analysis

Logistic regression analyses with backward selection method (backward likelihood ratio) were used to determine the effects of vascular risk factors, lipid parameters and APO A5 genotypes in the prediction of ischemic stroke. Different binary logistic regression models were established with different combinations of parameters in the overall population or in different subgroups.

Model 1

In model 1 age, sex, hypertension, smoking status, diabetes, APO A5 c.553G>T and -1131T/C genotypes were added as covariates and logistic regression revealed hypertension (OR=2.658, 95% CI=1.563-4.521, *P*=0.000), diabetes (OR=1.847, 95% CI=1.051-3.244, *P*=0.033) and smoking status (OR=2.386, 95% CI=1.276-4.462, *P*=0.006) to be the strongest determinants of ischemic stroke (Table 3.10). 65.9% of cases were predicted correctly by the model and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=5.5; 8 degrees of freedom; *P*=0.707).

Table 3.10 Logistic regression analysis of vascular risk factors (age, sex, hypertension, smoking and diabetes) and APO A5 c.553G>T and -1131T/C genotypes (Model 1).

Parameters	OR	95% CI	*P*
Hypertension	2.658	1.563-4.521	0.000
Diabetes	1.847	1.051-3.244	0.033
Smoking	2.386	1.276-4.462	0.006

Model 2

In addition to vascular risk factors (age, sex, hypertension, smoking status, diabetes) and APO A5 genotypes (c.553G>T and -1131T/C), the lipid parameters (total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol and VLDL-cholesterol) were also included in model 2. Hypertension and smoking were still associated with stroke but diabetes was not a statistically significant predictor of ischemic stroke anymore. In this analysis triglyceride (OR=2.214, 95% CI=1.188-4.126, *P*=0.012) and low HDL-cholesterol (OR=0.540, 95% CI=0.352-0.829, *P*=0.005) were found to be a risk factor for stroke. VLDL-cholesterol was also significant predictor of control versus stroke status (Table 3.11). 66.8% of cases were predicted correctly by the model and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=4.3; 8 degrees of freedom; *P*=0.833).

Table 3.11 Logistic regression analysis of vascular risk factors (age, sex, hypertension, smoking and diabetes), APO A5 genotypes (c.553G>T and -1131T/C) and lipid parameters (total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol and VLDL-cholesterol) (Model 2).

Parameters	OR	95% CI	*P*
Hypertension	2.651	1.621-4.335	0.000
Diabetes	1.720	0.963-3.073	0.067
Smoking	2.931	1.496-5.743	0.002
TG	2.214	1.188-4.126	0.012
HDL-C	0.540	0.352-0.829	0.005
VLDL-C	0.281	0.088-0.899	0.032

Model 3

In this model the elderly subjects (age > 59 years) were analyzed. In model 2 vascular risk factors (sex, hypertension, smoking status, diabetes), APO A5 genotypes (c.553G>T and -1131T/C) and lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) were added as covariates and logistic regression revealed hypertension, smoking, total cholesterol and LDL-cholesterol as significant predictors of ischemic stroke (Table 3.12). The APO A5 -1131C allele carriers were found to be insignificantly associated with a 1.938 times increased risk for stroke (95% CI=0.868-4.325, *P*=0.106). The triglyceride was also insignificantly related with a 1.548 times increased risk for stroke (95% CI=0.933-2.569, *P*=0.091).

Table 3.12 Logistic regression analysis of vascular risk factors (sex, hypertension, smoking and diabetes), APO A5 genotypes (c.553G>T and -1131T/C) and lipid parameters (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) in elderly population (Model 3).

Parameters	OR	95% CI	*P*
Hypertension	2.943	1.647-5.259	0.000
Smoking	3.922	1.465-10.499	0.007
Total cholesterol	0.580	0.415-0.813	0.002
TG	1.548	0.933-2.569	0.091
LDL-C	1.761	1.114-2.783	0.015
-1131TC+CC	1.938	0.868-4.325	0.106

It was remarkable that the risk associated with smoking was increased (OR=3.922, 95% CI=1.465-10.499, *P*=0.007) in the elderly group, compared to the risk of smoking in all age groups containing population (OR=2.386 for model 1, OR=2.684 for model 3). 68.8% of cases were predicted correctly by the model and the calibration of the model was satisfactory (chi-square=3.7; 8 degrees of freedom; *P*=0.879).

CHAPTER 4

DISCUSSION

Stroke is a sudden death of the brain cells due to disruption in the blood supply. It is the third leading cause of death after heart disease and cancer and the most common cause of disabilities worldwide. The risk of stroke is determined by both genetic and environmental influences. A number of specific risk factors include: a family history of stroke in first-degree relatives, older age, male sex, hypertension, smoking, diabetes and heart disease. Stroke exhibits a major burden to individuals, families, communities, and society as a whole because of the disability associated with it and the possibility of exhausting resources. It is hoped that an improved understanding of stroke risk factors will reduce the future burden of stroke.

Apolipoproteins are lipid-binding proteins and constituents of chylomicrons, HDL-C, LDL-C and VLDL-C. The apolipoproteins regulate lipoprotein metabolism and determine the unique roles of these lipoproteins in lipid metabolism (Mahley *et al.*, 1984). Recently discovered new member of apolipoprotein is APO A5 which may play an important role in TG metabolism (Pennacchio *et al.*, 2001). Human APO A5 gene is fairly polymorphic and several SNPs of APO A5 are associated with increased TG concentrations.

In this study we aimed to investigate two important APO A5 polymorphisms (c.553G>T and -1131T/C) as risk factors for ischemic stroke in Turkish population. Conventional risk factors and lipid parameters were also evaluated in stroke patients and healthy control groups.

The study population consisted of 198 ischemic stroke patients and 130 healthy controls. While creating this study group, lots of criteria were evaluated in order to minimize the differences between patient and control groups. All exclusion criteria were applied to the controls exactly plus not having carotid stenosis (lumen narrowing) > 50% or ulcerated carotid plaque

One of the most significant nonmodifiable risk factors of stroke is age. The age of study population varied between 20 to 90 years, with a mean age of 65.8 in patient group and 63.5 in control group. There was no statistically significant difference in mean age of the groups (P=0.059). Therefore, the results concerning the APO A5 genotypes would not be influenced by age.

Previous studies showed that men are 1.25 times more likely to suffer strokes than women (NINDS, 1999; Kumral *et al.,* 1998; Elkind and Sacco, 1998). In the present study, out of 198 ischemic stroke patients, 111 were male and 87 were female, showing that males have 1.4 times greater risk for ischemic stroke than females. The number of males in control group (47.7%) was similar with the patient group (56.1%). There was no statistically significant difference between the groups with respect to gender (P=0.138).

The prevalence of conventional risk factors, hypertension, diabetes and smoking in the patient group were found to be higher than the control group (Table 3.1). Hypertension and diabetes mellitus have significant effect and exhibit approximately 2.5-fold relative risk for ischemic stroke (Table 3.2). Smokers were found to be insignificantly higher in the patient group (21.2%) than the control group (13.8%, P=0.091). Hypertension, atrial fibrillation, diabetes, hyperlipidemia, cigarette smoking, alcohol abuse and physical inactivity are all modifiable risk factors for ischemic stroke (Sacco, 1995; Sacco, 1998; Elkind and Sacco, 1998) and current stroke prevention focuses on optimizing the treatment of these modifiable risk factors (Sacco and Liao,

2005). The modifiable risk factors increase the stroke susceptibility 2 to 4 fold; such as hypertension itself increases the population attributable risk by 50% to 60% (Benson and Sacco, 2000). A study with subjects from Turkey showed that hypertension increases the stroke risk by 63%, diabetes increases the stroke risk by 35%, and smoking increases the stroke risk by 17% (Kumral *et al.*, 1998).

The relation between serum total cholesterol and coronary heart disease is well established, but the relation with stroke is controversial. Prospective population-based studies have yielded doubtful results. Some have reported a direct association with total cholesterol and stroke (Harmesen *et al.*, 1990), others showed little or no association (Prospective Studies Collaboration, 1995) and also some demonstrated an inverse relation (Iso *et al.*, 1989; Gatchev *et al.*, 1993) between total cholesterol and stroke. A prospective study with middle-aged British men showed a weak positive association between elevated total cholesterol and nonfatal strokes (Wannamethee *et al.*, 2000). The EUROSTROKE project does not support the presence of an association between total cholesterol and fatal, non-fatal, hemorrhagic and ischemic stroke (Bots *et al.*, 2002). The association between serum total cholesterol and risk of mortality from ischemic stroke has been inconsistent. In Japan collaborative cohort study (JACC study), persons with serum total cholesterol levels <4.14 mmol/l (160 mg/dl) tended to have the increased risk of mortality from ischemic stroke (Cui *et al.*, 2007). In the present study total cholesterol levels were almost the same in patients and controls (4.8 ± 1.3 mmol/L, P=0.964) and it can be concluded that total cholesterol is not associated with stroke risk. This result was also observed in logistic regression analysis which demonstrated total cholesterol to be associated with a 0.580 times increased risk for stroke (Table 3.12). In other words, low levels of total cholesterol is 1.72 (1/0.580) times risky in our population (P=0.002).

The level of HDL-C was significantly lower in ischemic stroke patients (1.1 ± 0.3 mmol/L) when compared to controls (1.2 ± 0.3 mmol/L, P=0.004), while the level of LDL-C was insignificantly higher in patients (2.8 ± 1.1 mmol/L) than controls (2.6 ± 1.0 mmol/L, P=0.166). Keeping in mind that atherosclerosis, which is defined as accumulation in the arterial intima of mainly LDL-derived lipids, is a main risk factor for ischemic stroke and that HDL-C has a well recognized antioxidant activity, our results for LDL-C and HDL-C seem reasonable. Moreover, elevated LDL-C and low HDL-C were previously determined to be risk factors for atherosclerotic ischemic stroke (Sanossian and Tarlov, 2008; Bang *et al.,* 2008). In addition, VLDL-C levels were found almost the same in patients and controls (0.7 ± 0.4 mmol/L, P=0.965).

APO A5 polymorphisms differ in frequency among populations of different ethnicity. Therefore identification of APO A5 polymorphisms even in healthy subjects is very important. The frequency of the minor allele (c.553T) of c.553G>T polymorphism of APO A5 in healthy subjects in different populations are given in Table 4.1. In the present study, the frequency of c.553T allele in 130 healthy subjects was found as 0.004. Similarly, Hoduglugil *et al.* also found the frequency of this allele to be vey low in Turkish population (Hodoglugil *et al.,* 2006). In addition, Hubacek *et al.* supposed that this variant is probably absent in Caucasian population since they did not detect c.553T allele in 420 healthy subjects (Hubacek *et al.,* 2004). On the contrary, the frequency of this allele was higher in Chinese (Kao *et al.,* 2003; Hsu *et al.,* 2006; Tang *et al.,* 2006; Zhai *et al.,* 2006) and Japanese populations (Matsunaga *et al.,* 2007). Our result is consistent with the studies performed on Turks and Caucasians. In the literature the studies are predominantly done with Chinese and Japanese populations. Variations in c.553T allele frequencies can be observed even in the same country (Table 4.1).

The APO A5 c. 553G>T genotype frequencies of control subjects found in the present study were also different from Chinese and Japanese populations. There was no subject having c. 553TT genotype in the present study, while 3.2% of the subjects in Japan (Matsunaga *et al.*, 2007) and 8.7% in China (Kao *et al.*, 2003) had this genotype.

Table 4.1 Comparison of APO A5 c.553T allele frequency in different populations in healthy subjects.

Ethnicity	Reference	c.553T allele frequency
Turkish	**present study**	**0.004**
	Hodoglugil *et al.*, 2006	0.006
Chinese	Kao *et al.*, 2003	0.042
	Hsu *et al.*, 2006	0.065
	Tang *et al.*, 2006	0.040
	Zhai *et al.*, 2006	0.049
Japanese	Matsunaga *et al.*, 2007	0.105
Caucasian	Hubacek *et al.*, 2004	0.000

The frequency of APO A5 -1131T/C polymorphism also shows great differences between populations. The minor allele frequencies (-1131C) of APO A5 -1131T/C polymorphism in different populations of healthy subjects are given in Table 4.2.

Table 4.2 Comparison of APO A5 -1131C allele frequency in different populations in healthy subjects.

Ethnicity	Reference	-1131C allele frequency
Turkish	**present study**	**0.100**
	Hodoglugil *et al.*, 2006	0.128
Chinese	Hsu *et al.*, 2006	0.297
	Bi *et al.*, 2004	0.333
	Baum *et al.*, 2003	0.330
Chinese in Singapore	Lai *et al.*, 2003	0.294
Taiwanese Aborigines	Huang *et al.*, 2008	0.350
Japanese	Matsunaga *et al.*, 2007	0.315
	Nabika *et al.*, 2002	0.340
	Endo *et al.*, 2002	0.350
Japanese American	Austin *et al.*, 2004	0.380
Caucasian	Maasz *et al.*, 2007	0.062
	Talmud *et al.*, 2002	0.060
	Pennacchio *et al.*, 2002	0.060
	Pennacchio *et al.*, 2001	0.092
Hungarian	Havasi *et al.*, 2006	0.050
	Szalai *et al.*, 2004	0.057
Hispanic	Pennacchio *et al.*, 2002	0.160
Spanish	Ribalto *et al.*, 2002	0.070
African Americans	Pennacchio *et al.*, 2002	0.120
Americans in Framingham	Lai *et al.*, 2008	0.069
Malay in Singapore	Lai *et al.*, 2003	0.269
Pune Indians	Chandak *et al.*, 2006	0.200
Asian Indians in Singapore	Lai *et al.*, 2003	0.232
UK whites	Chandak *et al.*, 2006	0.040

The -1131C allele frequency of 130 healthy subjects was found to be 0.100 in the present study. In general, the frequency of -1131C allele was less than 10% in Caucasians (Pennacchio *et al.,* 2001 and 2002; Talmud *et al.,* 2002; Maasz *et al.,* 2007). Recently, the rare allele frequency was found as 12.8% in Turkish population (Hodoglugil *et al.,* 2006). In contrast, the frequency of this allele was over 30% in the Japanese (Endo *et al.,* 2002; Nabika *et al.,* 2002; Austin *et al.,* 2004; Matsunaga *et al.,* 2007) and Chinese populations (Baum *et al.,* 2003; Bi *et al.,* 2004; Huang *et al.,* 2008).

Our result is similar to the result of a study in Caucasian population (Pennacchio *et al.,* 2001) and Turkish population (Hodoglugil *et al.,* 2006), however; -1131C allele frequency found in other studies in Caucasians (Pennacchio *et al.,* 2002; Talmud *et al.,* 2002; Maasz *et al.,* 2007) was lower than our finding.

In the present study, the genotype frequencies of APO A5 -1131T/C polymorphism -1131TT, -1131TC and -1131CC genotypes for control subjects were 80.8%, 18.5% and 0.8%, respectively which were consistent with the genotype frequencies (88.3%, 11.1% and 0.6%) found in a study with Caucasians (Talmud *et al.,* 2002). The genotype frequencies of -1131T/C polymorphism in Chinese (Bi *et al.,* 2004; Hsu *et al.,* 2006) and Japanese populations (Matsunaga *et al.,* 2007) were considerably higher.

When we compared the APO A5 genotypes of stroke patients and controls, we could not observe significant difference between the groups. Only 2 individuals were heterozygote for APO A5 c.553G>T polymorphism (c.553GT), one is patient and one is control, others had homozygous wild type c.553GG genotype. Therefore, the c.553GG genotype frequency was nearly the same in patients (99.5%) and controls (99.2%). There was also no significant difference between minor allele (c.553T) frequencies of patients (0.003) and

controls (0.004, P=0.836). Most of the studies about c.553G>T polymorphism were accomplished with CAD patients; there is no study in the literature demonstrating the relation between c.553G>T polymorphism and stroke. Thus, this is the first study testing the APO A5 c.553G>T polymorphism as a risk factor for ischemic stroke.

The genotype distribution of APO A5 -1131T/C polymorphism in stroke patients and controls were nearly the same. The wild type -1131TT genotype was observed in 80.3% of the patients and 80.8% of the controls. There was also no significant difference between minor allele (-1131C) frequencies of patients (0.098) and controls (0.100, P=0.949). Therefore, APO A5 -1131T/C polymorphism could not be considered as a risk factor for ischemic stroke. In a study performed with stroke patients and controls, the -1131C allele frequency was observed approximately twofold in patients compared with the controls (Havasi *et al.,* 2006). Since APO A5 -1131T/C polymorphism shows great variations between populations, the difference between our results and this study performed on Hungarians may be due to the population difference. The importance of the present study is to show the relationship between ischemic stroke and these two polymorphisms at the same time.

The APO A5 genotypes were analyzed in different subgroups of ischemic stroke patients and controls. Since there are only two heterozygote individuals (one is patient and one is control) and no homozygote mutant, the genotypes of c.553G>T polymorphism were not analyzed in these subgroups. APO A5 -1131 genotypes were compared between ischemic stroke patients and controls with respect to hypertension (Table 3.4), diabetes (Table 3.5) and smoking status (Table 3.6). While making comparison between the genotypes of -1131T/C polymorphism, the TC and CC genotypes were ascribed to a single group because only one CC homozygote was seen in the control group

and none in stroke patients. In a subgroup composed of hypertensive individuals, the proportion of so-called risky genotypes (TC+CC) was higher in patients (19%) than controls (14.8%). When compared to the wild type TT genotype, the C allele carriers were insignificantly higher in patients than controls (P=0.496) in hypertensive subgroup. Among normotensives, the distribution of stroke patients and control subjects carrying -1131C allele were nearly same (P=0.821).

In diabetics subgroup, the proportion of C allele carriers was higher in stroke patients (20.6%) than controls (8.3%). When compared to the wild type TT genotype, stroke patients were insignificantly more susceptible to carry C allele (P=0.173). If people with risky genotypes (TC+CC) also have diabetes, the risk of having stroke was 2.8-fold higher than the non-diabetic people with the same genotype. Among non-diabetics, C allele carriers were almost same in stroke patients and control groups (P=0.640).

Among smokers and non-smokers there was no difference between stroke patients and control subjects in terms of -1131T/C polymorphism of APO A5 gene.

The proportion of stroke patients to control subjects was increased in hypertensives, diabetics and smokers compared to normotensives, non-diabetics and non-smokers, irrespective of genotype group (Table 3.7). We also analyzed the effects of these conventional vascular risk factors on ischemic stroke with respect to -1131T/C genotypes. The TC and CC genotypes of -1131T/C polymorphism were ascribed to a single group since only one CC homozygote was seen in the control group and none in stroke patients. The effects of conventional vascular risk factors were not analyzed in c.553G>T polymorphism, since there are only two heterozygote individuals (one is

patient and one is control) and no homozygote mutant of the c.553G>T polymorphism.

Within the -1131TT genotype group, the risk of having stroke in hypertensive individuals was 2.3-fold (P=0.001) higher than normotensives. If hypertensive individuals have -1131C allele, the risk of having stroke increases to 3.4 (P=0.021). These results showed that not only hypertension, but also APO A5 -1131T/C polymorphism is a risk factor for ischemic stroke.

Diabetes is another vascular risk factor and increases the risk of ischemic stroke 1.9 times (P=0.022) compared to non-diabetics with -1131TT genotype. Ischemic stroke risk increases 6.4-fold (P=0.012) in diabetics with -1131TC or CC genotype compared to non-diabetics with same genotype. The -1131C allele of APO A5 was also associated with an insignificant 2.6-fold (P=0.195) increased risk of stroke in diabetics (Table 3.8).

The smoking status of the individual insignificantly increased the risk of stroke approximately 1.6 times in each genotype group of -1131T/C polymorphism when compared to non-smokers. The studies in the literature did not compare their results with respect to conventional vascular risk factors and -1131T/C genotypes.

Studies in animals and humans have demonstrated that APO A5 gene may play an important role in triglyceride (TG) metabolism although the exact function of APO A5 is not known. Several polymorphisms of human APO A5 gene associated with increased TG concentrations have been identified.

APO A5 c.553G>T polymorphism in Chinese population has been found to correlate strongly with increased TG levels (Kao *et al.*, 2003). Tang *et al.* reported that the minor c.553T allele carriers had significantly higher TG

levels (2.31 mmol/L) when compared to the wild type c.553GG genotype (1.68 mmol/L, P=0.002) in control group of a study with Chinese population (Tang *et al.,* 2006). Similarly, Hsu *et al.* (2006) obtained higher TG level in control group of minor c.553T allele carriers (1.91 mmol/L) than individuals with c.553GG genotype (1.59 mmol/L, P=0.014) in a study with Chinese in Twain. However, in Caucasians (Hubacek *et al.,* 2004) and Turks (Hodoglugil *et al.,* 2006) a correlation between c.553G>T polymorphism and TG level was not found. In the present study, serum lipid parameters were not analyzed with respect to c.553G>T polymorphism, since there are only two heterozygote individuals (one is patient and one is control) and no homozygote mutant in our population.

The increase in TG level may be caused by the change in function of apo AV due to change in cysteine in place of glycine at amino acid residue 185 (Kao *et al.,* 2003). Since this variation introduces a cysteine which contains sulfur atom and another cysteine is present at residue 204, a disulphide bridge may be created between these two cysteines. The binding of apo AV to lipid interfaces may be determined by only a few discrete domains, one candidate being residues 171–188, a predicted α-helical segment that is both hydrophobic and highly amphipathic which can rapidly bind and penetrate the phospholipid interface (Weinberg *et al.,* 2003). Therefore, disulphide bond between the residues of 185 and 204 possibly formed and this disulfide bond may change the primary structure of the apo AV protein, perhaps influencing the α-helix formation and/or stability, thereby altering the binding capacity of apo AV to lipid interfaces (Tang *et al.,* 2006).

The mean serum lipid levels of stroke patients and healthy controls were compared according to the APO A5 -1131 genotypes in order to estimate the impact of APO A5 -1131T/C polymorphism on lipid metabolism (Table 3.9). Because there is only one individual with CC genotype, the C allele

carriers (TC+CC) were ascribed to a single group. TG level of stroke patients having -1131C allele was significantly higher (1.70 mmol/L) than controls with the same genotype (1.35 mmol/L, P=0.038) and this level was also higher than TG level of stroke patients with -1131TT genotype (1.53 mmol/L). Stroke patients with -1131TC or CC genotype had significantly lower HDL-C levels (1.05 mmol/L) when compared to controls with the same genotype (1.20 mmol/L, P=0.028). Similarly, HDL-C levels of stroke patients with -1131TT genotype (1.11 mmol/L) were significantly lower than those of the healthy controls (1.19 mmol/L, P=0.030) with the same genotype. In stroke patients, the -1131C allele carriers had higher TC, higher TG, lower HDL-C and higher LDL-C levels when compared to patients with -1131TT genotype. These results demonstrated that the APO A5 -1131 genotypes influenced the serum lipid parameters of stroke patients. In a study with ischemic stroke patients in Hungary, it was found that the-1131C allele carriers had higher TG levels than patients with wild type genotype (Havasi *et al.,* 2006). APO A5 -1131T/C polymorphism was found to be associated with significantly elevated TG levels in Caucasians (Pennacchio *et al.,* 2001 and 2002), Turks (Hodoglugil *et al.,* 2006; Komurcu-Bayrak *et al.,* 2008), African Americans and Hispanics (Pennacchio *et al.,* 2002), Hungarian (Szalai *et al.,* 2004; Havasi *et al.,* 2006), Japanese (Nabika *et al.,* 2002; Endo *et al.,* 2002; Austin *et al.,* 2004) as well as Chinese (Baum *et al.,* 2003; Bi *et al.,* 2004; Li *et al.,* 2004; Hsu *et al.,* 2006). In addition to elevated TG levels, this polymorphism was also associated with reduced HDL-C levels in both Asian and Caucasian populations (Pennacchio *et al.,* 2001 and 2002; Aouizerat *et al.,* 2003). The -1131C allele was found to be associated with lower HDL-C levels in Turkish women (Komurcu-Bayrak *et al.,* 2008).

The precise mechanism behind the association of the APO A5-1131T/C polymorphism with the elevated plasma TG level is not certain. The promoter region polymorphism of APO A5 gene might affect the transcriptional activity

of the gene and thus, affect plasma TG levels. An unknown functional mutation may be present at, or adjacent to the APO A5 gene locus which is in strong linkage disequilibrium (LD) with this polymorphism, giving rise to the inter-individual triglyceride and HDL-C variation. The -3A > G SNP which was found to be in complete LD with -1131T/C SNP, is the critical nucleotide of the Kozak sequence preceding the translation start codon. Therefore, the -3A > G SNP could be a candidate to affect potentially the rate of APO A5 translation (Pennacchio *et al.,* 2002). It was demonstrated that the -1131T/C SNP was in a LD with APO C3 -482C > T SNP which was strongly associated with plasma TG level (Talmud *et al.,* 2002).

Binary logistic regression analysis in a model containing vascular risk factors (age, sex, hypertension, smoking status, diabetes) and APO A5 c.553G>T and -1131T/C genotypes showed that hypertension, diabetes and smoking are significant and the strongest determinants of ischemic stroke. When we added lipid parameters to this model (TC, TG, LDL-C, HDL-C and VLDL-C), in addition to hypertension, diabetes and smoking, we found that elevated TG level was associated with a 2.2-fold increased risk of ischemic stroke. It was also found that low levels of HDL-C increased the stroke risk 1.8 times due to antioxidant effect of HDL-C. The vascular risk factors, APO A5 genotypes and lipid parameters were also analyzed in elderly subjects (Table 3.12). In this model the APO A5 -1131C allele carriers were found to be associated with a 1.9 times increased risk for stroke in elderly.

CHAPTER 5

CONCLUSION

Human APO A5 gene which is thought to play important role in TG metabolism is fairly polymorphic. Several single nucleotide polymorphisms (SNPs) of APO A5 might associate with increased TG concentrations. Atherosclerosis, which is a major cause of ischemic stroke, may be associated with variability of TG levels due to APO A5 polymorphisms. These polymorphisms show great variability in different populations, which were mostly studied in cardiovascular diseases. Therefore, in the present study APO A5 c.553G>T and -1131T/C polymorphisms were studied in Turkish population for the first time as a risk factor for ischemic stroke.

The study population consisted of 198 ischemic stroke patients and 130 healthy controls with no differences between the groups with respect to age and gender. Hypertensives, diabetics and smokers in patient group were found to be higher than control group. Hypertension and diabetes were also found as significant predictors for ischemic stroke. Logistic regression analysis revealed hypertension to be 2.7 times increased risk for ischemic stroke.

The minor allele frequency (c.553T) of APO A5 c.553G>T polymorphism was found to be 0.003 in stroke patients and 0.004 in control subjects. Therefore, we might say that carrying minor allele is not a risk factor for having stroke. The frequency of this allele in control subjects was higher in Chinese and Japanese populations while in Caucasian and Turkish population

allele frequency was nearly zero like in our population. This is the first study investigating the relation between c.553G>T polymorphism and stroke.

The allele frequency of the so-called risky allele of the promoter region polymorphism -1131T/C was found to be 0.098 in stroke patients and 0.100 in control subjects demonstrating that -1131C allele does not constitute a risk for ischemic stroke. While the frequency of -1131C allele in general was less than 10% in Caucasians, it was over 30% in Japanese and Chinese populations.

APO A5 -1131T/C polymorphism were also analyzed in hypertensives, diabetics and smokers. Hypertensives with -1131TT genotype had 2.3 times higher risk for ischemic stroke than normotensives. The risk of having stroke increases to 3.4-fold in hypertensives who carry the so-called risky allele (-1131C). Among diabetics, 6.4-fold relative risk for ischemic stroke was found in -1131TC+CC group when compared to non-diabetics with the same genotype. APO A5 -1131C allele was associated with 2.6-fold increased risk of stroke in diabetics. The proportion of stroke patients to controls was increased in smoker group when compared to non-smoker group and smoking increased the risk of stroke insignificantly approximately 1.6 times in each genotype group. The effects of conventional vascular risk factors were not analyzed in c.553G>T polymorphism since only one patient and one control had heterozygote genotype and no homozygote mutant subject were detected.

Although the exact function of APO A5 is not known, this gene was thought to have an important role in TG metabolism. c.553G>T polymorphism of APO A5 gene has been found to strongly correlate with increased TG levels in Chinese population, while in Caucasian and Turkish population there is no correlation between TG levels and this polymorphism. Since there are only two heterozygote individuals, serum lipid parameters were not analyzed with respect to c.553G>T polymorphism.

Logistic regression analysis revealed elevated TG levels and reduced HDL-C levels to be associated with 2.2 and 1.8-fold increase respectively in the risk of ischemic stroke. APO A5 -1131T/C polymorphism was found to be associated with elevated TG levels and reduced HDL-C levels in both Asian and Caucasian populations. In this study, stroke patients having -1131C allele had significantly higher TG levels and lower HDL-C levels when compared to controls with the same genotype. Therefore, it can be concluded that the APO A5 -1131 genotypes influenced the serum lipid parameters of stroke patients.

Genetic polymorphisms of APO A5 gene and effects of these polymorphisms on lipid metabolism and ischemic stroke risk were determined in the present study. The investigation of APO A5 polymorphisms came into prominence in Turkish population due to the variations of the allele frequencies in different populations and limited number of studies with APO A5 polymorphisms and ischemic stroke risk.

REFERENCES

Albers GW, Caplan LR, Easton JD, Fayad PB, Mohr JP, Saver JL, Sherman DG; TIA Working Group. Transient ischemic attack – proposal for a new definition. N Engl J Med 2002; 347: 1713-6.

Ali M, Bath PM, Curram J, Davis SM, Diener HC, Donnan GA, Fisher M, Gregson BA, Grotta J, Hacke W, Hennerici MG, Hommel M, Kaste M, Marler JR, Sacco RL, Teal P, Wahlgren NG, Warach S, Weir CJ, Lees KR. The virtual international stroke trials archive. Stroke 2007; 38(6): 1905-10.

Amarenco P, Cohen A, Tzourio C, Bertrand B, Hommel M, Besson G, Chauvel C, Touboul PJ, Bousser MG. Atherosclerotic disease of the aortic arch and the risk of ischemic stroke. N Engl J Med 1994; 331: 1474-9.

Aouizerat BE, Kulkarni M, Heilbron D, Drown D, Raskin S, Pullinger CR, Malloy MJ, Kane JP. Genetic analysis of a polymorphism in the human apoA-V gene: Effect on plasma lipids. J Lipid Res 2003; 44: 1167-73.

Austin MA, Talmud PJ, Farin FM, Nickerson DA, Edwards KL, Leonetti D, McNeely MJ, Viernes HM, Humphries SE, Fujimoto WY. Association of apolipoprotein A5 variants with LDL particle size and triglyceride in Japanese Americans. Biochimica et Biophysica Acta 2004; 1688: 1-9.

Bang OY, Saver JL, Liebeskind DS, Pineda S, Ovbiagele B. Association of serum lipid indices with large artery atherosclerotic stroke. Neurology 2008; 70(11): 841-7.

Baum L, Tomlinson B, Thomas GN. APOA5-1131T > C polymorphism is associated with triglyceride levels in Chinese men. Clin Genet 2003; 63: 337-9.

Benson RT, Sacco RL. Stroke prevention: Hypertension, diabetes, tobacco, and lipids. Neurologic Clinics 2000; 18(2): 309-19.

Bi N, Yan SK, Li GP, Yin ZN, Chen BS. A single nucleotide polymorphism -1131T > C in the apolipoprotein A5 gene is associated with an increased risk of coronary artery disease and alters triglyceride metabolism in Chinese. Molecular Genetics and Metabolism 2004; 83(3): 280-6.

Bots ML, Elwood PC, Nikitin Y, Salonen JT, Freire de Concalves A, Inzitari, D, Sivenius J, Benetou V, Tuomilehto J, Koudstaal PJ, Grobbee DE. Total and HDL cholesterol and risk of stroke. EUROSTROKE: a collaborative study among research centres in Europe. J Epidemiol Community Health 2002; 56: i19-i25.

Chandak GR, Ward KJ, Yajnik CS, Pandit AN, Bavdekar A, Joglekar CV, Fall CH, Mohankrishna P, Wilkin TJ, Metcalf BS, Weedon MN, Frayling TM, Hattersley AT. Triglyceride associated polymorphisms of the APOA5 gene have very different allele frequencies in Pune, India compared to Europeans. BMC Med Genet 2006; 7: 76.

Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, Jones DW, Materson BJ, Oparil S, Wright JT Jr, Roccella EJ; National Heart, Lung, and Blood Institute Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure; National High Blood Pressure Education Program Coordinating Committee. The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: the JNC 7 report. JAMA 2003; 289(19): 2560-72.

Collins R, MacMahon S. Blood pressure, antihypertensive drug treatment, and the risks of stroke and of coronary heart disease. Br Med Bull 1994; 50: 272-98.

Collins R, Peto R, MacMahon S, Hebert P, Fiebach NH, Eberlein KA, Godwin J, Qizilbash N, Taylor JO, Hennekens CH. Blood pressure, stroke and coronary heart disease, II: Short-term reductions in blood pressure: overview of randomised drug trials in their epidemiological context. Lancet 1990; 335: 827-38.

Cui R, Iso H, Toyoshima H, Date C, Yamamotof A, Kikuchi S, Kondo T, Watanabe Y, Koizumi A, Inaba Y, Tamakoshi A; JACC Study Group. Serum total cholesterol levels and risk of mortality from stroke and coronary heart disease in Japanese: The JACC study. Atherosclerosis 2007; 194: 415-20.

Elkind MS, Sacco RL. Stroke risk factors and stroke prevention. Semin Neurol 1998; 18(4): 429-40.

Endo K, Yanagi H, Araki J, Hirano C, Kobayashi KY, Tomura S. Association found between the promoter region polymorphism in the apolipoprotein A-V gene and the serum triglyceride level in Japanese schoolchildren. Hum Genet 2002; 111: 570-2.

Fisher CM. Occlusion of the internal carotid artery. Arch Neurol Psychiatry 1951; 65: 346-77.

Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB. The stroke data bank: Design, methods and baseline characteristics. Stroke 1988; 19: 547-54.

Futuyma DJ. Variation. In: evolution. Sunderland, Sinauer Associates, Inc. 2005. p. 189-224.

Gao J, Wei Y, Huang Y, Liu D, Liu G, Wu M, Wu L, Zhang Q, Zhang Z, Zhang R, Liang C. The expression of intact and mutant human apoAI/CIII/AIV/AV gene cluster in transgenic mice. J Biol Chem 2005; 280: 12559-66.

Gatchev O, Rastam L, Lindberg G, Gullberg B, Eklund GA, Isacsson SO. Subarachnoidal hemorrhage, cerebral hemorrhage and serum cholesterol concentration in men and women. Ann Epidemiol 1993; 3(4): 403-9.

Gubitz G, Sandercock P, Counsell C. Antiplatelet therapy for acute ischaemic stroke. Cochrane Database Syst Rev 2003; (2): CD000029.

Hankey GJ. Stroke: How large a public health problem, and how can the neurologist help? Arch Neurol 1999; 56: 748-54.

Harmesen P, Rosengren A, Tsipogianni A, Wilhelmsen L. Risk factors in middle-aged men in Gothenburg. Stroke 1990; 21(2): 223-9.

Havasi V, Szolnoki Z, Talian G, Bene J, Komlosi K, Maasz A, Somogyvari F, Kondacs A, Szabo M, Fodor L, Bodor A, Melegh B. Apolipoprotein A5 gene promoter region T-1131C polymorphism associates with elevated circulating triglyceride levels and confers susceptibility for development of ischemic stroke. J Mol Neurosci 2006; 29(2): 177-83.

Havel RJ, Goldstein JL, Brown MS. Lipoproteins and lipid transport. In: Bondy PK, Rosenberg LE, Eds. Metabolic Control and Disease. Philadelphia, PA: W. B. Saunders, 8th edition 1980. p. 393-494.

Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: A randomised placebo-controlled trial. Lancet 2002; 360: 7-22.

Hinkle JL, Guanci MM. Acute ischemic stroke review. J Neurosci Nurs 2007; 39(5): 285-93.

Hodoglugil U, Tanyolac S, Williamson DW, Huang Y, Mahley RW. Apolipoprotein A-V: a potential modulator of plasma triglyceride levels in Turks. J Lipid Res 2006; 47: 144-53.

Hsu LA, Ko YL, Chang CJ, Hu CF, Wu S, Teng MS, Wang CL, Ho WJ, Ko YS, Hsu TS, Lee YS. Genetic variations of apolipoprotein A5 gene is associated with the risk of coronary artery disease among Chinese in Taiwan. Atherosclerosis 2006; 185: 143-9.

Huang MC, Wang TN, Wang HS, Sung YC, Ko YC, Chiang HC. The -1131T>C polymorphism in the apolipoprotein A5 gene is related to hypertriglyceridemia in Taiwanese Aborigines. Kaohsiung J Med Sci. 2008; 24(4): 171-9.

Hubacek JA, Adamkova V, Ceska R, Poledne R, Horinek A, Vrablik M. New variants in the apolipoprotein AV gene in individuals with extreme triglyceride levels. Physiol Res 2004; 53: 225-8.

International SNP Map Working Group. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature 2001; 409(6822): 928-33.

Iso H, Jacobs DR Jr, Wentworth D, Neaton JD, Cohen JD. Serum cholesterol levels and six-year mortality from stroke in 350,977 men screened for the multiple risk factor intervention trial. N Engl J Med 1989; 320(14): 904-10.

Jones EF, Kalman JM, Calafiore P, Tonkin AM, Donnan GA. Proximal aortic atheroma: An independent risk factor for cerebral ischemia. Stroke 1995; 26: 218-24.

Kao JT, Wen HC, Chien KL, Hsu HC, Lin SW. A novel genetic variant in the apolipoprotein A5 gene is associated with hypertriglyceridemia. Human Molecular Genetics 2003; 12: 2533-9.

Karp G. The nature of the gene and the genome. In: cell and molecular biology. John Wiley & Sons, 3rd edition 2002. p. 397-438.

Komurcu-Bayrak E, Onat A, Poda M, Humphries SE, Palmen J, Guclu F, Can G, Erginel-Unaltuna N. Gender-modulated impact of apolipoprotein A5 gene (APOA5) -1131T>C and c.56C>G polymorphisms on lipids, dyslipidemia and metabolic syndrome in Turkish adults. Clin Chem Lab Med 2008; 46(6): 778-84.

Kronzon I, Tunick PA. Aortic atherosclerotic disease and stroke. Circulation 2006; 114: 63-75.

Kumral E, Ozkaya B, Sagduyu A, Sirin H, Vardarli E, Pehlivan M. The Ege stroke registry: A hospital-based study in the Aegean region, Izmir, Turkey. Analysis of 2,000 stroke patients. Cerebrovasc Dis 1998; 8(5): 278-88.

Lahiri DK, Schnabel B. DNA isolation by a rapid method from human blood samples: effects of $MgCl_2$, EDTA, storage time, and temperature on DNA yield and quality. Biochem Genet 1993; 31: 321-8.

Lai CQ, Demissie S, Cupples LA, Zhu Y, Adiconis X, Parnell LD, Corella D, Ordovas JM. Influence of the APOA5 locus on plasma triglyceride, lipoprotein subclasses, and CVD risk in the Framingham Heart Study. J.Lipid Res 2004; 45: 2096-105.

Lai CQ, Tai ES, Tan CE, Cutter J, Chew SK, Zhu YP, Adiconis X, Ordovas JM. The APOA5 locus is a strong determinant of plasma triglyceride

concentrations across ethnic groups in Singapore. Journal of Lipid Research 2003; 44: 2365-73.

Li GP, Wang JY, Yan SK, Chen BS, Xue H, Wu G. Genetic effect of two polymorphisms in the apolipoprotein A5 gene and apolipoprotein C3 gene on serum lipids and lipoproteins levels in a Chinese population. Clin Genet 2004; 65(6): 470-6.

Maasz A, Kisfali P, Horvatovich K, Mohas M, Marko L, Csongei V, Farago B, Jaromi L, Magyari L, Safrany E, Sipeky C, Wittmann I, Melegh B. Apolipoprotein A5 T-1131C variant confers risk for metabolic syndrome. Pathology Oncology Research 2007; 13: 243-7.

MacMahon S, Peto R, Cutler JA, *et al.* Blood pressure, stroke, and coronary heart disease, I: Effects of prolonged differences in blood pressure: Evidence from nine prospective observational studies corrected for the regression dilution bias. Lancet 1990; 335: 765-74.

Mahley RW, Innerarity TL, Rall SC, Weisgraber KH. Plasma lipoproteins: apolipoprotein structure and function. Journal of Lipid Research 1984; 25(12): 1277-94.

Mahley RW, Innerarity TL. Lipoprotein receptors and cholesterol homeostasis. Bkhim. Biophys. Acta 1983; 737: 197-222.

Martin S, Nicaud V, Humphries SE, Talmud PJ; EARS group. Contribution of APOA5 gene variants to plasma triglyceride determination and to the response to both fat and glucose tolerance challenges. Biochim Biophys Acta 2003; 1637: 217-25.

Mathews CK, Van Holde KE. Lipid metabolism I: fatty acids, triacylglycerols, and lipoproteins. In: biochemistry. The Benjamin/Cummings Publishing Company, 2nd edition 1995. p. 619-58.

Matsunaga A, Arishima H, Niimura H, Zhang B, Uehara Y, Ohwaki K, Morita M, Hayashida K, Saku K. Strong linkage disequilibrium and association of –1131T>C and c.553G>T polymorphisms of the apolipoprotein A5 gene with hypertriglyceridemia in a Japanese Population. Circ J 2007; 71: 746-52.

Merkel M, Heeren J. Give me A5 for lipoprotein hydrolysis! J Clin Invest 2005; 115: 2694-6.

Nabika T, Nasreen S, Kobayashi S, Masuda J. The genetic effect of the apoprotein AV gene on the serum triglyceride level in Japanese. Atherosclerosis 2002; 165: 201-4.

Najib JF, Bauge E, Niculescu LS, Pham T, Thomas B, Rommens C, Majd Z, Brewer B, Pennacchio LA, Fruchart JC. Mechanism of triglyceride lowering in mice expressing human apolipoprotein A5. Biochem Biophys Res Commun 2004; 319(2): 397-404.

National Institute of Neurological Disorders and Stroke (NINDS) Stroke: Hope Through Research. National Institutes of Health. (1999).

Nelson DL, Cox MM. Lipid biosynthesis. In: Lehninger principles of biochemistry. W. H. Freeman and Company, 4th edition 2005. p. 787-832.

Pennacchio LA, Olivier M, Hubacek JA, Cohen JC, Cox DR, Bruchart JC, Krauss RM, Rubin EM. An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science 2001; 294: 169-73.

Pennacchio LA, Olivier M, Hubacek JA, Krauss RM, Rubin EM, Cohen JC. Two independent apolipoprotein A5 haplotypes influence human plasma triglyceride levels. Hum Mol Gen 2002; 11: 3031-8.

Prospective Studies Collaboration. Cholesterol, diastolic blood pressure, and stroke: 13.000 strokes in 450.000 people in 45 prospective cohorts. Lancet 1995; 346: 1647-53.

Ribalta J, Figuera L, Ballart JF, Vilella E,Cabezas MC, Masana L, Joven J. Newly identified apolipoprotein AV gene predisposes to high plasma triglycerides in familial combined hyperlipidemia. Clin Chem 2002; 48: 1597-1600.

Rothwell PM, Eliasziw M, Gutnikov SA, Fox AJ, Taylor DW, Mayberg MR, Warlow CP, Barnett HJ; Carotid Endarterectomy Trialists' Collaboration. Analysis of pooled data from the randomised controlled trials of endarterectomy for symptomatic carotid stenosis. Lancet 2003; 361(9352): 107-16.

Rothwell PM, Warlow CP. Timing of TIAs preceding stroke: Time window for prevention is very short. Neurology 2005; 64(5): 817-20.

Sacco RL, Liao JK. Drug Insight: statins and stroke. Nat Clin Pract Cardiovasc Med 2005; 11: 576-84.

Sacco RL. American heart association prevention conference IV: Prevention and rehabilitation of stroke: Risk factors. Stroke 1997; 28: 1507-17.

Sacco RL. Identifying patient populations at high risk for stroke. Neurology 1998; 51: 27-30.

Sacco RL. Risk factors and outcomes for ischemic stroke. Neurology 1995; 45: 10-4.

Sanossian N, Tarlov NE. HDL-C and LDL-C: Their role in stroke pathogenesis and implications for treatment. Curr Treat Options Cardiovasc Med 2008; 10(3): 195-206.

Szalai C, Keszei M, Duba J, Prohaszka Z, Kozma GT, Csaszar A, Balogh S, Almassy Z, Fust G, Czinner A. Polymorphism in the promoter region of the apolipoprotein A5 gene is associated with an increased susceptibility for coronary artery disease. Atherosclerosis 2004; 173: 109-14.

Talmud PJ, Hawe E, Martin S, Olivier M, Miller GJ, Rubin EM, *et al.* Relative contribution of variation within the ApoC3/A4/A5 gene cluster in determining plasma triglycerides. Hum Mol Genet 2002; 11: 3039-46.

Talmud PJ, Hawe E, Martin S, Olivier M, Miller GJ, Rubin EM, Pennacchio LA, Humphries SE. Relative contribution of variation within the APOC3/A4/A5 gene cluster in determining plasma triglycerides. Hum Mol Genet 2002; 11(24): 3039-46.

Tang Y, Sun P, Guo D, Ferro A, Ji Y, Chen Q, Fan L. A genetic variant c.553G > T in the apolipoprotein A5 gene is associated with an increased risk of coronary artery disease and altered triglyceride levels in a Chinese population. Atherosclerosis 2006; 185: 433-7.

Tunick PA, Kronzon I. Protruding atherosclerotic plaque in the aortic arch of patients with systemic embolization: A new finding seen by transesophageal echocardiography. Am Heart J 1990; 120: 658-60.

Turek MC. Stroke risk factors: Recent evidence and new aspects. International Congress Series 2004; 1262: 466-9.

Ustrell X, Serena J. Stroke: Diagnosis and therapeutic management of cerebrovascular disease. Rev Esp Cardiol 2007; 60(7): 753-69.

van der Vliet HN, Sammels MG, Leegwater AC, Levels JH, Reitsma PH, Boers W, Chamuleau RA. Apolipoprotein A-V: A novel apolipoprotein associated with an early phase of liver regeneration. J Biol Chem 2001; 276: 44512-20.

van der Vliet HN, Schaap FG, Levels JH, Ottenhoff R, Looije N, Wesseling JG, Groen AK, Chamuleau RA. Adenoviral overexpression of apolipoprotein A-V reduces serum levels of triglycerides and cholesterol in mice. Biochem Biophys Res Commun 2002; 295(5): 1156-9.

Vance DE, Vance JE. Biochemistry of lipids and membranes. The Benjamin/Cummings Publishing Company, 1985.

Wannamethee SG, Shaper AG, Ebrahim S. HDL-Cholesterol, total cholesterol, and the risk of stroke in middle-aged British men. Stroke 2000; 31(8):1882-8.

Warlow CP, Sudlow C, Dennis M, Wardlaw J, Sandercock P. Stroke. Lancet 2003; 362: 1211-24.

Weaver RF, Philip WH. Genetics, 2nd ed. Dubuque, IA: WilliamC. Brown, 1992.

Weinberg RB Cook VR, Beckstead JA, Martin DD, Gallagher JW, Shelness GS, Ryan RO. Structure and interfacial properties of human apolipoprotein AV. J Biol Chem 2003; 278(36): 34438-44.

White H, Boden-Albala B, Wang C, Elkind MSV, Rundek T, Wright CB, Sacco RL. Ischemic stroke subtype incidence among Whites, Blacks, and Hispanics. The Nothern Manhattan Study. Circulation 2005; 111: 1327-31.

Zhai G, Wen P, Guo L, Chen L. Association of APOA5 c.553G>T polymorphism with type 2 diabetes mellitus in a Chinese population. Clin Chem Lab Med 2006; 44(11): 1313-6.

APPENDIX A

A.1 INFORMED CONSENT FOR PATIENTS

BİLGİLENDİRİLMİŞ ONAM (RIZA) FORMU

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 ya da bundan sonra kliniğimizde size karşı davranışlarımızda herhangi bir değişiklik olmayacaktır. Yine çalışmanın herhangi bir aşamasında onayınızı çekmek hakkına da sahipsiniz.

Hastanın Beyanı

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

Eğer bu araştırmaya katılırsam hekim ile aramda kalması gereken bana ait bilgilerin gizliliğine bu araştırma sırasında da büyük özen ve saygı ile

yaklaşılacağına inanıyorum. Araştırma sonuçlarının eğitim ve bilimsel amaçlarla kullanımı sırasında kişisel bilgilerimin ihtimamla korunacağı konusunda bana yeterli güven verildi.

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

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

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

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

Katılımcı

Adı, soyadı:

Adres:

Tel:

İmza:

Görüşme tanığı

Adı, soyadı:

Adres:

Tel:

İmza:

Katılımcı ile görüşen hekim

Adı soyadı, unvanı:

Adres:

Tel:

İmza:

A.2 INFORMED CONSENT FOR CONTROLS

GÖNÜLLÜ BİLGİLENDİRİLMESİ

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

Çalışmada yer aldığınız ve bilimsel gelişmelere katkılarınızdan dolayı teşekkür ediyoruz.

Açıklamaları Yapan Araştırmacının

Adı, Soyadı:

Görevi:

İmzası:

Açıklamayı başından sonuna kadar tanıklık eden kişinin

Adı, Soyadı:

Adresi:

İmzası:

Çalışmaya katılan gönüllünün

Adı, soyadı:

Adres:

İmzası:

APPENDIX B

ETHICAL COMMITTEE APPROVAL FORM

HİZMETE ÖZEL

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

Y. C. Şubat 2006

Y. ETİK KRL. : 1491 - [illegible] - 06
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

Al. Uğur ÜRAL
Prof. Tbp. Kd. Alb
GATA Etik Kurulu Başkanı

EK
1 Adet Etik Kurul Raporu

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. Şerel Demirkaya'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
Ali Uğur URAL
Prof.Tbp.Kd.Alb.

ÜYE
Ali İhsan UZAR
Prof.Hv.Tbp.Kd.Alb.

ÜYE
Ayhan KUBAR
Prof.Tbp.Alb.

ÜYE
Adnan ATAÇ
Doç.Dr.Ecz.Kd.Alb.

ÜYE
Mükerrem SAFALI
Doç.Tbp.Kd.Alb.

ÜYE
K. Melih AKAY
Doç.Tbp.Kd. Alb.

ÜYE
Mustafa ÖZER
Doç.Tbp.Alb.

ÜYE
Ergün TOZKOPARAN
Dpç.Tbp. Alb.

ÜYE
Nalan AKBAYRAK
Prof. Dr. Sağ. Yb

ÜYE
Muharrem UÇAR
Yrd.Doç.J.Tbp.Yb.

APPENDIX C

LIST OF STUDY POPULATION

Table A.1 List of study population composed of 198 stroke patients and 130 controls including demographic characteristics, lipid parameters and APO A5 genotypes. M: male; F: female; Y: yes; N: no; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; VLDL-C: very high density lipoprotein cholesterol.

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
1	Patient	75	M	Y	Y	Y	N	N	N	124	114	72	23	24	GG	TT
2	Patient	57	F	Y	Y	N	N	N	N	210	136	132	27	51	GG	TC
3	Patient	41	M	N	N	Y	N	N	100%	185	140	104	24	34	GG	TT
4	Patient	73	M	Y	N	N	N	N	N	143	64	74	12	57	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
5	Patient	53	M	Y	Y	N	N	N	N	401	231	296	46	59	GG	TC
6	Patient	66	M	Y	Y	Y	N	N	N	130	126	85	25	20	GG	TT
7	Patient	84	F	Y	N	N	N	N	N	145	59	70	12	63	GG	TT
8	Patient	56	F	Y	Y	N	N	N	N	138	212	68	30	38	GG	TT
9	Patient	54	M	Y	N	Y	N	Y	50%	200	217	115	43	42	GG	TT
10	Patient	67	F	Y	N	N	N	N	N	209	106	139	21	49	GG	TT
11	Patient	76	M	Y	N	Y	N	N	N	155	68	91	14	50	GG	TT
12	Patient	78	F	Y	Y	N	N	Y	50%	142	137	79	27	36	GG	TT
13	Patient	75	F	Y	N	N	N	N	N	182	104	127	21	34	GG	TT
14	Patient	74	F	Y	Y	N	N	N	50%	167	62	107	12	48	GG	TT
15	Patient	68	F	Y	N	Y	N	N	N	140	202	75	40	25	GG	TT
16	Patient	72	F	N	N	N	N	N	N	256	253	165	51	40	GG	TC
17	Patient	84	M	Y	N	N	N	N	N	159	173	93	35	31	GG	TT
18	Patient	81	F	Y	N	N	N	N	70%	231	156	115	31	42	GG	TT
19	Patient	73	F	Y	Y	N	N	N	N	207	202	125	40	42	GG	TT
20	Patient	73	F	Y	N	N	Y	N	N	180	88	107	18	55	GG	TT
21	Patient	67	F	Y	N	N	N	N	N	208	124	143	25	40	GG	TT
22	Control	69	F	N	N	N	N	N	N	244	145	91	29	74	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
23	Control	71	M	Y	N	N	N	N	N	209	119	82	24	42	GG	TC
24	Patient	61	M	Y	Y	N	N	N	100%	219	122	108	54	37	GG	TT
25	Patient	40	M	N	N	Y	N	N	N	187	104	78	20	47	GG	TT
26	Control	61	F	Y	N	N	N	N	N	242	83	46	16	68	GG	TT
27	Patient	60	F	Y	Y	N	Y	N	0,9	192	150	119	30	43	GG	TT
29	Patient	75	M	N	N	N	N	N	50%	175	96	71	19	85	GG	TT
30	Patient	76	F	Y	N	N	N	N	N	158	135	50	58	46	GG	TT
31	Control	76	M	Y	Y	N	N	N	50%	132	93	74	19	39	GG	TT
32	Control	51	M	N	N	Y	N	N	N	142	115	80	54	35	GG	TT
33	Control	50	F	N	N	N	N	N	N	164	180	73	87	30	GG	TT
34	Control	42	F	N	N	N	N	N	N	124	113	57	72	24	GG	TT
35	Control	45	F	N	N	Y	N	N	N	191	53	73	59	57	GG	TC
36	Patient	70	M	Y	N	N	N	N	0,4	126	73	59	25	42	GG	TT
37	Patient	76	M	N	N	Y	N	Y	N	128	124	73	25	30	GG	TT
38	Control	63	M	Y	Y	Y	N	N	0,4	150	100	60	43	37	GG	TT
39	Patient	83	F	N	N	N	N	N	70%	279	210	182	42	55	GG	TT
40	Control	63	F	N	N	N	N	N	N	200	221	123	87	34	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
42	Control	75	M	Y	Y	N	N	N	N	251	153	100	43	51	GG	TT
43	Control	58	F	Y	N	N	N	N	N	186	97	43	22	42	GG	TC
44	Control	78	F	Y	N	N	N	N	0,3	139	95	71	19	49	GG	TT
46	Control	74	M	N	N	N	N	N	50%	182	82	100	16	51	GG	TC
47	Patient	71	M	Y	N	N	N	N	50%	207	232	126	46	35	GG	TC
50	Control	61	M	N	N	N	N	N	N	228	166	155	33	40	GG	TT
51	Control	85	M	Y	N	N	N	N	50%	254	160	24	32	63	GG	TC
52	Control	65	F	N	Y	N	N	N	50%	235	126	126	25	84	GG	TT
53	Control	65	M	N	N	N	N	N	N	191	57	128	11	52	GG	TC
54	Control	58	M	N	Y	Y	N	N	N	229	107	169	21	39	GG	TC
55	Control	61	F	N	N	N	Y	N	N	262	163	170	33	59	GG	TT
56	Control	80	F	Y	N	N	N	Y	50%	233	202	83	40	24	GG	TT
57	Control	80	F	N	N	N	N	N	N	244	63	46	12	71	GG	TT
58	Control	67	F	Y	Y	N	N	Y	N	151	127	85	25	41	GG	TT
59	Control	76	M	N	N	N	N	N	50%	231	206	143	41	47	GG	TT
61	Patient	61	M	N	Y	N	N	Y	50%	148	144	76	29	43	GG	TC
62	Control	69	F	Y	N	N	N	N	N	241	157	74	31	46	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
63	Control	66	M	N	Y	Y	N	N	N	268	349	160	70	38	GG	TT
64	Control	60	F	Y	N	N	N	N	N	191	123	121	25	45	GG	TT
66	Patient	64	F	N	N	Y	Y	N	50%	166	94	99	19	48	GG	TT
67	Patient	58	F	Y	Y	N	N	N	N	350	360	228	72	50	GG	TT
68	Patient	74	F	Y	N	N	Y	N	50%	241	154	162	31	48	GG	TC
69	Control	71	M	N	N	N	N	N	50%	148	140	80	30	55	GG	TT
71	Patient	80	F	Y	N	N	Y	N	100%	145	86	90	17	38	GG	TT
72	Patient	62	M	Y	Y	N	N	N	70%	188	107	128	21	39	GG	TT
73	Control	68	M	N	N	N	N	N	N	210	115	123	23	64	GG	TT
74	Control	65	F	N	N	N	N	N	N	294	157	123	31	46	GG	TC
76	Control	72	F	Y	N	N	N	N	50%	234	149	158	30	46	GG	TT
77	Control	65	M	N	N	N	N	N	N	152	80	64	16	37	GG	TT
78	Control	63	F	Y	Y	N	Y	Y	50%	193	328	66	84	43	GG	TT
79	Control	70	F	N	N	N	N	N	50%	187	132	118	26	43	GG	TT
80	Control	70	F	Y	N	N	N	N	50%	202	105	130	21	51	GG	TT
81	Control	65	F	Y	N	N	N	N	N	229	157	147	31	51	GG	TC
82	Patient	63	M	Y	N	N	N	N	N	131	89	72	18	41	GG	TT
83	Control	65	F	Y	N	N	N	N	N	174	113	85	23	66	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
84	Control	78	F	Y	N	N	N	N	50%	175	111	93	22	54	GG	TT
85	Patient	68	M	Y	N	N	N	N	N	178	80	95	25	60	GG	TT
86	Control	47	M	N	N	N	N	N	N	241	272	130	54	57	GG	TT
87	Patient	77	F	Y	N	N	N	N	0,9	195	95	137	19	39	GG	TT
88	Control	77	F	Y	Y	N	N	N	N	156	87	103	17	36	GG	TT
89	Patient	80	F	Y	N	N	N	N	N	201	94	120	19	62	GG	TT
90	Control	71	M	N	N	N	N	N	N	168	131	90	26	52	GG	TC
91	Patient	55	M	Y	N	N	N	Y	50%	157	124	95	25	37	GG	TC
92	Control	73	M	Y	N	N	N	N	50%	140	220	76	44	20	GG	TT
93	Control	61	M	N	N	Y	N	N	N	180	35	114	7	59	GG	TT
94	Control	37	M	N	Y	N	N	N	N	170	120	110	30	50	GG	TT
95	Patient	62	M	N	N	N	N	N	50%	142	90	54	18	43	GG	TT
96	Patient	77	M	N	N	N	N	N	50%	113	54	68	11	68	GG	TT
97	Patient	24	M	N	N	N	N	N	N	204	255	143	51	41	GT	TC
98	Patient	53	F	N	N	Y	N	N	N	273	192	23	110	35	GG	TT
99	Patient	61	M	N	N	N	N	N	N	186	142	121	28	37	GG	TT
101	Control	52	M	N	N	N	N	N	N	232	103	183	21	63	GG	TT
102	Patient	78	M	Y	N	Y	N	N	50%	201	183	120	37	44	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
103	Control	65	M	N	N	N	Y	N	N	205	76	113	15	32	GG	TT
105	Patient	81	M	Y	N	N	N	N	70%	150	270	37	54	42	GG	TT
106	Patient	80	F	Y	N	N	Y	N	70%	221	113	57	23	25	GG	TC
107	Control	50	F	Y	N	N	N	N	N	167	76	84	15	24	GT	TC
108	Control	87	F	Y	N	N	Y	N	50%	244	91	54	18	34	GG	TT
109	Control	67	M	N	N	N	N	N	N	168	102	82	20	6	GG	TT
110	Patient	84	M	Y	N	N	N	N	70%	143	98	74	20	49	GG	TT
111	Control	38	M	N	N	Y	N	N	N	184	50	111	10	63	GG	TT
112	Control	50	M	N	N	N	N	N	N	147	55	79	11	57	GG	TC
113	Patient	75	F	Y	N	N	N	Y	70%	110	58	52	12	46	GG	TT
114	Patient	26	M	N	N	N	N	N	N	162	95	80	19	63	GG	TT
115	Patient	55	M	Y	N	N	N	N	N	170	83	123	20	54	GG	TT
116	Patient	26	M	N	N	N	N	N	N	156	64	114	13	29	GG	TT
117	Patient	73	F	Y	N	N	N	N	70%	154	101	99	20	35	GG	TT
119	Control	80	F	N	Y	N	N	N	N	153	123	77	25	51	GG	TT
120	Patient	36	M	N	N	N	N	N	N	187	140	116	28	43	GG	TC
121	Patient	56	M	N	N	N	N	N	N	167	109	100	22	45	GG	TT
122	Patient	47	F	N	N	N	N	N	N	184	123	123	25	36	GG	TC

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
123	Patient	21	M	N	N	Y	N	N	N	101	38	46	8	47	GG	TT
124	Patient	81	F	Y	Y	N	N	Y	N	139	193	69	39	31	GG	TC
125	Patient	84	M	Y	Y	N	N	N	N	183	87	118	17	48	GG	TT
126	Patient	73	M	Y	Y	N	N	N	N	198	224	118	45	35	GG	TT
127	Patient	73	F	Y	N	N	N	N	70%	154	101	99	20	35	GG	TT
128	Patient	66	F	N	N	N	N	N	70%	129	248	58	50	21	GG	TT
129	Patient	74	F	Y	N	N	N	N	70%	230	127	154	25	51	GG	TT
130	Control	44	M	N	N	Y	N	N	N	200	212	96	42	62	GG	TT
131	Control	51	F	N	N	N	N	N	N	168	51	110	10	48	GG	TT
132	Control	67	M	Y	N	N	N	N	N	172	148	101	30	41	GG	TT
133	Control	73	F	Y	Y	N	N	N	N	227	206	143	41	43	GG	TT
134	Control	88	F	Y	N	N	N	N	50%	154	71	100	14	40	GG	TT
136	Patient	66	M	Y	Y	N	N	N	N	129	254	49	51	29	GG	TC
138	Patient	61	F	Y	Y	N	N	N	50%	200	178	63	13	25	GG	TT
139	Patient	78	M	N	N	Y	N	N	N	130	110	70	20	40	GG	TT
140	Control	59	M	N	N	N	N	N	N	157	110	90	20	45	GG	TC
141	Control	69	F	N	Y	N	N	N	N	241	119	157	24	60	GG	TC
142	Patient	80	M	Y	N	N	N	N	N	304	74	239	15	50	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
143	Patient	76	F	Y	Y	N	N	N	0,9	158	110	98	22	38	GG	TT
144	Patient	79	F	Y	N	N	N	N	N	202	136	129	27	46	GG	TT
146	Control	51	F	N	N	N	N	N	N	110	90	54	16	38	GG	TC
147	Patient	21	M	N	N	N	N	N	N	157	83	100	12	32	GG	TT
148	Patient	76	M	N	N	N	Y	N	N	193	106	132	21	40	GG	TC
149	Patient	68	F	Y	N	N	N	N	N	180	140	110	30	45	GG	TT
150	Control	35	M	N	N	Y	N	N	N	148	114	79	23	46	GG	TT
151	Patient	28	M	N	N	Y	N	N	N	151	146	78	29	44	GG	TT
152	Patient	20	M	N	N	Y	N	N	N	180	80	100	16	50	GG	TT
153	Patient	80	F	Y	N	N	N	N	N	193	251	86	50	57	GG	TC
154	Patient	64	F	Y	N	N	N	N	N	145	144	77	29	39	GG	TT
155	Patient	71	M	N	N	Y	N	N	50%	190	81	132	16	42	GG	TT
156	Patient	67	F	Y	Y	N	Y	N	N	391	226	297	45	49	GG	TC
157	Patient	58	F	Y	Y	N	N	N	N	274	256	169	51	54	GG	TT
158	Control	77	M	N	N	N	N	N	N	179	167	80	13	46	GG	TC
159	Patient	49	M	N	N	Y	N	N	100%	226	204	139	41	46	GG	TT
161	Patient	78	F	N	N	N	N	N	50%	178	88	103	18	57	GG	TT
162	Patient	65	M	N	N	N	N	N	100%	154	164	79	33	42	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
163	Patient	75	M	Y	N	N	N	N	0,9	195	142	130	28	37	GG	TT
164	Patient	79	M	N	N	Y	N	N	50%	105	80	54	16	35	GG	TC
165	Patient	73	F	Y	Y	N	N	N	0,9	320	315	205	70	23	GG	TC
166	Patient	84	F	Y	N	N	N	N	0,9	258	78	170	16	72	GG	TT
167	Patient	25	M	N	N	N	N	N	N	180	140	107	27	43	GG	TT
168	Patient	73	M	Y	N	N	N	N	N	195	79	119	16	60	GG	TT
169	Patient	74	M	Y	N	N	N	N	0,9	128	118	70	24	34	GG	TT
170	Patient	78	F	Y	Y	N	Y	Y	0,9	181	138	109	28	44	GG	TT
172	Patient	56	M	Y	Y	N	N	N	N	177	157	105	31	41	GG	TT
173	Patient	67	M	Y	N	N	N	N	N	150	154	89	33	43	GG	TT
174	Patient	74	M	Y	N	N	N	N	N	183	201	79	30	50	GG	TT
175	Patient	64	M	Y	Y	N	N	N	N	168	170	98	17	41	GG	TT
176	Patient	73	M	Y	Y	N	N	N	50%	192	173	100	40	43	GG	TC
177	Control	52	F	N	N	N	N	N	50%	120	100	73	25	54	GG	TT
179	Patient	57	M	N	N	Y	N	N	N	187	149	116	30	41	GG	TT
180	Patient	76	M	N	N	N	N	N	50%	161	171	94	34	33	GG	TT
181	Patient	61	M	N	N	Y	N	N	100%	187	124	116	25	46	GG	TT
184	Control	77	F	Y	N	N	N	N	50%	162	178	79	36	47	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
185	Patient	62	F	N	Y	N	N	N	N	157	145	91	29	37	GG	TT
186	Patient	73	M	N	N	Y	N	N	N	304	265	204	53	47	GG	TT
187	Patient	63	M	N	N	Y	N	Y	50%	133	103	66	21	46	GG	TT
188	Patient	52	M	N	N	Y	N	N	100%	158	98	108	19	31	GG	TT
189	Control	79	M	N	N	N	N	N	N	86	77	150	70	37	GG	TT
190	Patient	61	F	Y	N	N	N	N	70%	121	124	62	25	34	GG	TT
191	Control	46	M	Y	N	Y	N	N	N	304	252	207	50	47	GG	TT
193	Patient	45	M	N	N	Y	N	N	N	151	333	51	67	33	GG	TT
194	Patient	64	M	N	N	Y	N	Y	0,9	166	97	103	19	36	GG	TT
195	Patient	56	F	N	Y	N	N	N	N	270	284	169	57	44	GG	TT
196	Patient	67	M	Y	Y	N	N	N	70%	266	253	171	51	44	GG	TT
197	Patient	53	M	Y	N	N	N	N	N	284	302	176	60	48	GG	TT
198	Control	35	M	N	N	N	N	N	N	157	175	88	35	34	GG	TC
200	Control	36	M	N	N	N	N	N	N	121	69	75	14	32	GG	TT
201	Control	38	M	N	N	Y	N	N	N	169	129	100	60	52	GG	TT
202	Patient	80	M	N	N	N	N	N	70%	220	119	146	24	50	GG	TC
203	Patient	62	M	Y	Y	N	N	N	50%	200	110	143	25	46	GG	TT
204	Patient	83	F	Y	N	N	N	N	100%	140	98	77	20	43	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
206	Control	34	F	N	N	Y	N	N	N	154	87	105	17	32	GG	TT
207	Control	41	F	N	N	N	N	N	N	150	130	80	30	40	GG	TT
209	Patient	80	M	N	N	N	N	N	N	280	150	123	30	50	GG	TT
210	Patient	61	M	N	N	Y	N	N	50%	279	120	75	22	34	GG	TC
211	Patient	67	F	N	Y	N	N	N	N	190	135	125	27	38	GG	TT
212	Patient	64	M	N	N	Y	N	N	0,9	166	97	103	19	44	GG	TC
213	Control	48	F	N	N	N	Y	N	N	100	86	55	17	28	GG	TT
214	Patient	65	F	N	N	Y	N	N	50%	222	105	149	21	52	GG	TC
215	Control	35	F	N	N	N	N	N	N	135	148	75	30	30	GG	TT
216	Patient	79	F	Y	Y	N	N	N	50%	164	150	103	30	31	GG	TC
217	Patient	61	M	Y	N	Y	N	N	50%	181	107	119	21	41	GG	TT
218	Patient	36	F	N	N	Y	N	N	70%	246	173	148	35	63	GG	TT
219	Patient	80	M	Y	Y	N	N	N	70%	289	377	173	75	41	GG	TT
221	Patient	61	M	N	Y	Y	N	N	N	143	102	84	20	39	GG	TT
222	Patient	69	M	Y	N	N	N	N	100%	156	77	105	15	36	GG	TT
223	Patient	76	F	Y	Y	N	N	N	70%	158	68	104	14	40	GG	TT
224	Patient	69	F	N	N	N	N	N	50%	123	99	70	20	33	GG	TC
225	Control	43	F	N	N	Y	N	N	N	222	164	137	33	52	GG	TC

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
226	Patient	77	M	Y	Y	Y	N	N	100%	148	73	102	15	31	GG	TT
227	Patient	82	M	N	N	N	N	N	50%	251	148	80	20	35	GG	TT
229	Control	45	F	N	N	N	N	N	N	201	245	101	49	51	GG	TT
231	Control	38	F	N	N	N	N	N	N	220	115	139	23	58	GG	TT
232	Patient	78	F	Y	N	N	N	N	N	188	62	124	12	52	GG	TT
233	Control	64	F	Y	N	N	Y	N	N	269	133	186	27	56	GG	TT
234	Patient	58	F	N	N	N	N	N	N	166	98	94	20	52	GG	TT
235	Control	66	M	N	N	N	N	N	50%	204	62	135	12	57	GG	TT
236	Patient	59	F	Y	Y	N	N	N	70%	200	167	133	34	33	GG	TT
237	Control	69	F	Y	N	N	N	N	N	159	82	91	16	52	GG	TC
239	Patient	53	M	N	N	Y	N	N	50%	139	33	86	7	46	GG	TT
240	Control	42	M	N	N	Y	N	N	N	178	244	96	49	33	GG	TT
241	Control	65	M	Y	N	N	N	N	N	148	115	76	23	49	GG	TT
242	Control	87	M	Y	Y	N	N	N	50%	104	110	38	22	44	GG	TT
244	Control	75	F	N	N	Y	Y	N	50%	184	81	113	16	55	GG	TT
245	Patient	54	M	N	N	N	N	N	N	170	59	121	12	37	GG	TC
246	Control	77	M	Y	N	N	N	N	N	139	60	95	12	32	GG	TT
247	Control	69	F	N	N	N	N	N	N	178	115	106	23	49	GG	TC

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
248	Patient	75	F	N	Y	N	N	N	N	189	87	118	17	54	GG	TT
250	Patient	84	F	N	N	N	N	N	100%	175	71	102	14	59	GG	TT
251	Patient	78	M	Y	Y	N	N	Y	70%	163	135	100	27	36	GG	TT
252	Patient	40	F	N	N	Y	Y	N	N	192	94	127	19	46	GG	TT
253	Patient	48	M	Y	N	N	N	N	N	211	72	147	14	50	GG	TT
254	Patient	80	F	Y	Y	N	N	N	N	156	77	112	18	54	GG	TT
255	Patient	41	M	N	N	N	N	N	N	145	120	88	24	33	GG	TT
256	Patient	77	F	Y	Y	N	N	N	70%	192	129	123	26	43	GG	TT
257	Patient	63	F	Y	Y	N	Y	N	50%	151	69	100	14	37	GG	TT
258	Patient	55	M	Y	N	Y	N	N	50%	277	163	207	33	37	GG	TC
259	Patient	79	M	Y	Y	N	N	N	70%	120	68	61	14	45	GG	TT
260	Patient	67	F	Y	Y	N	N	Y	50%	197	127	114	25	58	GG	TT
261	Patient	63	M	Y	Y	N	N	N	70%	145	157	83	31	31	GG	TT
262	Patient	75	F	Y	N	N	N	N	50%	228	169	152	34	42	GG	TC
263	Control	65	M	N	N	N	N	N	50%	159	62	103	12	44	GG	TT
264	Control	78	M	N	N	N	N	N	N	158	82	87	16	55	GG	TT
265	Control	81	M	Y	N	N	N	N	N	108	54	48	11	49	GG	TT
266	Control	56	M	N	Y	N	N	N	N	177	122	110	24	43	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
267	Control	64	F	Y	Y	N	N	N	N	166	246	86	49	31	GG	TT
268	Control	79	F	Y	N	N	N	N	N	181	110	110	22	49	GG	TT
269	Control	67	F	N	N	N	N	N	N	298	102	228	20	50	GG	TT
270	Control	76	F	Y	N	N	N	N	50%	166	110	85	22	59	GG	TT
271	Control	64	M	N	N	Y	N	N	N	130	91	86	18	26	GG	TT
272	Control	77	M	N	Y	N	N	N	N	338	158	243	32	63	GG	TT
273	Control	75	M	N	N	N	N	N	N	191	149	109	30	52	GG	TT
274	Patient	54	F	N	N	N	N	N	100%	201	301	114	60	27	GG	TT
275	Patient	61	F	Y	N	N	N	N	50%	180	110	126	22	35	GG	TC
276	Patient	71	M	Y	N	N	N	N	0,9	125	71	73	14	38	GG	TT
277	Control	68	F	Y	N	N	Y	N	N	186	170	97	34	55	GG	TT
278	Patient	74	F	Y	N	N	N	N	70%	211	165	123	33	55	GG	TT
279	Patient	59	M	N	N	N	N	Y	50%	128	127	60	45	23	GG	TT
280	Patient	62	M	N	N	Y	N	N	N	154	70	103	14	37	GG	TC
281	Patient	82	F	Y	Y	N	N	N	50%	179	105	120	21	38	GG	TT
282	Control	71	F	Y	N	N	N	N	50%	149	97	90	19	40	GG	TT
283	Control	52	F	N	N	N	N	N	50%	154	71	100	14	40	GG	TT
284	Control	78	M	Y	N	N	N	N	N	159	124	97	25	37	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
285	Patient	70	M	Y	N	N	N	N	50%	198	76	134	15	49	GG	TT
286	Patient	69	M	Y	N	N	N	N	50%	170	83	111	17	42	GG	TT
287	Control	80	M	Y	N	N	N	N	50%	197	86	126	17	54	GG	TC
288	Patient	57	F	N	Y	N	N	N	100%	210	154	135	31	44	GG	TC
289	Patient	58	F	Y	N	N	N	N	N	123	112	60	22	41	GG	TT
292	Patient	80	M	Y	N	N	N	N	70%	136	62	78	12	46	GG	TT
293	Patient	84	M	Y	N	N	N	N	50%	132	164	71	33	28	GG	TT
294	Control	78	F	N	N	N	N	N	50%	186	84	98	18	41	GG	TT
295	Patient	62	M	Y	Y	Y	N	Y	100%	131	192	57	38	36	GG	TC
296	Control	57	F	N	N	N	N	N	N	231	93	19	14	56	GG	TT
297	Control	60	F	Y	N	N	N	N	50%	250	198	112	48	54	GG	TT
298	Control	63	F	Y	N	N	N	Y	50%	257	246	160	49	48	GG	TT
299	Patient	74	M	N	Y	N	N	N	50%	130	65	65	13	52	GG	TT
300	Patient	50	M	Y	N	N	N	N	50%	217	75	24	20	38	GG	TT
301	Control	58	M	Y	N	N	N	N	50%	190	200	80	50	30	GG	TT
302	Control	54	M	Y	N	N	N	N	N	166	103	94	21	51	GG	TC
304	Control	78	M	N	N	N	N	N	50%	130	60	80	18	50	GG	TT
305	Patient	57	M	N	Y	N	N	N	100%	193	127	139	25	29	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
306	Control	75	M	Y	Y	N	N	N	50%	185	71	115	14	56	GG	TT
307	Control	77	M	Y	N	N	N	N	50%	118	88	64	18	36	GG	TT
309	Patient	62	M	Y	N	N	N	N	50%	195	233	119	47	29	GG	TC
310	Patient	81	F	Y	Y	N	N	N	70%	161	85	96	17	48	GG	TC
311	Patient	77	F	Y	Y	N	N	N	50%	116	187	61	37	18	GG	TT
312	Patient	54	M	Y	Y	N	N	N	50%	159	119	111	24	24	GG	TT
315	Patient	82	F	Y	Y	N	N	N	70%	167	90	102	18	47	GG	TT
316	Patient	71	F	Y	Y	N	N	N	50%	163	172	91	34	38	GG	TT
317	Patient	84	M	Y	Y	N	N	N	100%	111	134	58	27	26	GG	TT
318	Patient	80	F	Y	N	N	N	N	50%	165	139	90	28	47	GG	TT
319	Control	59	F	N	N	N	N	N	N	213	126	126	25	62	GG	CC
320	Control	57	F	N	N	N	N	N	N	168	166	100	33	35	GG	TC
321	Control	79	F	Y	Y	N	N	N	50%	214	147	86	29	48	GG	TT
322	Control	52	F	N	N	N	N	N	N	233	114	163	23	47	GG	TT
323	Control	79	F	Y	N	N	N	N	N	59	99	23	20	16	GG	TT
324	Patient	69	M	Y	Y	N	N	N	50%	207	223	109	45	53	GG	TT
325	Patient	84	M	N	N	N	N	N	N	181	146	78	43	53	GG	TT
326	Control	54	M	N	Y	N	N	N	N	282	220	191	44	47	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
327	Patient	54	F	Y	Y	N	N	N	50%	198	107	98	43	54	GG	TT
328	Patient	70	F	Y	N	N	N	N	50%	181	145	95	29	57	GG	TT
329	Control	67	F	N	N	N	N	N	N	160	70	82	16	52	GG	TT
330	Control	50	F	N	N	N	N	N	N	58	246	68	49	14	GG	TC
331	Patient	31	F	N	N	N	N	N	N	246	268	141	54	51	GG	TT
332	Patient	55	F	N	N	N	N	N	N	273	62	190	12	71	GG	TT
333	Control	90	F	N	Y	N	N	N	50%	214	146	133	29	52	GG	TT
334	Patient	71	M	Y	Y	N	N	N	70%	200	178	146	28	34	GG	TT
336	Control	77	F	Y	N	N	N	N	50%	314	173	202	35	77	GG	TT
337	Patient	83	F	Y	N	N	N	N	50%	161	69	80	14	67	GG	TC
338	Control	37	M	N	N	Y	N	N	N	202	71	14	51	71	GG	TT
339	Patient	77	M	Y	Y	N	N	Y	50%	176	193	92	39	45	GG	TT
340	Control	57	M	Y	Y	N	N	N	N	174	49	103	10	61	GG	TT
341	Control	68	M	N	N	N	N	N	N	136	52	72	10	54	GG	TT
342	Patient	74	F	Y	Y	N	N	N	50%	285	277	173	55	57	GG	TT
343	Patient	47	M	Y	Y	N	N	N	N	175	129	95	26	54	GG	TC
344	Patient	43	M	N	N	Y	N	N	N	301	310	185	62	54	GG	TT
346	Patient	67	M	Y	Y	N	N	N	70%	212	87	151	17	44	GG	TT

		Demographic Characteristics								Lipid Parameters					APO A5 genotypes	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C	c.553 G>T	-1131 T/C
347	Patient	69	F	N	N	N	N	N	70%	248	147	170	29	49	GG	TT
348	Control	77	M	Y	Y	Y	N	N	N	201	81	127	16	58	GG	TT
349	Control	60	M	Y	N	N	N	N	N	154	89	92	18	44	GG	TT
351	Patient	74	M	N	N	Y	N	N	70%	154	96	106	19	29	GG	TT
352	Patient	84	M	Y	Y	N	N	N	70%	212	90	154	15	42	GG	TC
353	Patient	71	M	Y	Y	Y	N	N	70%	229	234	141	47	41	GG	TT
354	Patient	74	M	Y	N	N	N	N	50%	140	122	80	24	36	GG	TC
355	Patient	76	F	Y	Y	N	N	Y	70%	170	174	86	35	49	GG	TT
356	Control	44	M	N	N	N	Y	N	N	171	109	110	21	39	GG	TT
357	Patient	68	F	Y	Y	Y	N	N	N	117	109	55	22	40	GG	TT
359	Control	52	F	Y	N	N	N	N	50%	213	173	111	43	54	GG	TT
360	Patient	68	F	Y	N	N	N	N	70%	205	92	140	18	46	GG	TT
361	Patient	74	F	Y	N	N	Y	Y	N	150	70	97	14	39	GG	TC
363	Control	58	F	N	N	N	N	N	N	157	149	90	30	37	GG	TT
364	Patient	57	M	Y	Y	Y	N	N	70%	237	253	152	51	34	GG	TT
365	Control	79	M	Y	Y	N	N	N	N	199	173	126	38	35	GG	TT
366	Control	73	F	N	N	N	N	N	N	203	111	141	22	40	GG	TT
367	Patient	34	F	N	N	N	N	N	N	223	174	103	47	24	GG	TT

		Demographic Characteristics										Lipid Parameters						APO A5 genotypes	
	No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Statin use	Stenosis		Total Cholesterol	Triglycerides	LDL-C	VLDL-C	HDL-C		c.553 G>T	-1131 T/C
368		Patient	61	M	Y	N	N	N	N	100%		176	132	114	26	36		GG	TT
369		Patient	62	M	N	N	N	N	N	100%		181	188	105	38	38		GG	TT