HUMAN SERUM ARYLESTERASE AND GLUTATHIONE S-TRANSFERASE ACTIVITIES IN PATIENTS WITH ISCHEMIC STROKE COMPARED TO HEALTHY CONTROLS

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ABSTRACT

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Stroke is an important public health problem and the third leading cause of death after coronary heart diesase and all cancers in all over the world. Free radicals and oxidative stress play important role in the pathogenesis of several diseases including atherosclerosis, stroke, cancer, neurodegenerative diseases such as Alzheimer's dementia.

The activity of paraoxonase (PON1) aganist phenylacetate is known as arylesterase (ARE). Paraoxonase is an esterase associated with high-density lipoprotein (HDL) and contributes to the protective role of this lipoprotein on low-density lipoprotein (LDL) oxidation. Oxidized LDL is known to play a central role in early events in the progression of atherosclerosis which is a risk factor for stroke. Glutathione S-transferases (GSTs) catalyze the conjugation of nonpolar compounds to reduced glutathione (GSH) and detoxify toxic metabolites produced within the cell by oxidative stress to protect cells from oxidant injury.

The maximum ARE enzyme activity was detected at 10 mM Tris-HCl buffer, pH 8.0 and at 45 °C. ARE enzyme was saturated with its substrate phenylacetate around 20 mM concentration. The apparent K_m and V_{max} values of human blood serum ARE for phenylacetate were found as 1.66 mM and 3300 nmol/min/mg, respectively. The maximum GST enzyme activity was detected at 2 mM potassium phosphate buffer, pH 5.5 and at 65 °C. GST enzyme was saturated with its substrate, CDNB around 4.5 mM concentration and with its cofactor, GSH around 8 mM concentration. The apparent K_m and V_{max} values of human blood serum GST for CDNB substrate were found as 2.8 mM and 0.43 nmol/min/mg and for GSH were found as 4.11 mM and 0.23 nmol/min/mg, respectively. In addition, effects of three different heavy metal ions, Cd^{+2}, Hg^{+2} and Ni^{+2}, on human blood serum ARE and GST activity were studied and half maximal inhibitory concentrations (IC_{50}) were determined.

The main objective of this study was to investigate the human blood serum ARE and GST activities in patient and control groups using the optimized conditions. For this purpose, blood samples were collected from 172 ischemic stroke patients and 105 controls. Then serum obtained from blood samples were used to determine ARE and GST activities. The mean of ARE activity in patient group (n=172, 109.9 ± 32.5 U/mL) was insignificantly lower than the mean of ARE activity in control group (n=105, 113.5 ± 33.1 U/mL, *P*=0.284). GST activity of the patients (10.8 ± 4.4 U/L) was insignificantly higher than that of controls (10.5 ± 4.2 U/L, *P*=0.483). In addition, statistical analysis showed hypertension, diabetes and HDL as significant risk factors of stroke.

Key words: Ischemic stroke, Arylesterase, Glutathione S-transferase, Paraoxonase, Oxidative stress

ÖZ

İNSAN SERUM ARİLESTERAZ VE GLUTATYON S-TRANSFERAZ AKTİVİTELERİNİN İSKEMİK İNMELİ HASTALARDA SAĞLIKLI KONTROLLERLE KARŞILAŞTIRILMASI

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İnme tüm dünyada bütün kanserlerden ve koroner kalp hastalıklarından sonra üçüncü ölüm sebebi ve önemli bir halk sağlığı problemidir. Serbest radikaller ve oksidatif stres aterosklerozis, inme, kanser, Alzheimer gibi nöron hasarı hastalıklarının patogenezinde önemli rol oynarlar.

Paraoksonazın (PON1) fenilasetata karşı aktivitesi arilesteraz olarak bilinmektedir. Paraoksonaz yüksek yoğunluklu lipoproteine (HDL) bağlı bir esterazdır ve HDL'nin düşük yoğunluklu lipoproteinin (LDL) oksidasyonuna karşı koruyucu etkisine katkıda bulunur. Okside olmuş LDL'nin inme için risk faktörü olan aterosklerozun erken safhalarında önemli bir rol oynadığı bilinmektedir. Glutatyon S-transferazlar (GSTs) polar olmayan moleküllerin indirgenmiş glutatyon (GSH) ile konjugasyonlarını katalizlemekte ve hücreleri oksidan hasarından korumak için oksidatif stres sonucu oluşan toksik metabolitlerin detoksifikasyonunu gerçekleştirmektedirler.

En yüksek ARE enzim aktivitesi 10 mM Tris-HCl tampon çözeltisi, pH 8.0 ve 45 °C'de tespit edildi. ARE enzimi kendi substratı fenilasetat ile 20 mM konsantrasyon civarında doyuruldu. İnsan serum ARE enziminin substratı için görünen K_m and V_{max} değerleri sırasıyla 1.66 mM and 3300 nmol/min/mg olarak bulundu. En yüksek GST enzim aktivitesi 2 mM potasyum fosfat tampon çözeltisi, pH 5.5 ve 65 °C'de tespit edildi. GST enzimi kendi substratı, CDNB ile 4.5 mM konsantrasyon civarında ve kofaktörü, GSH ile 8 mM konsantrasyon civarında doyuruldu. İnsan serum GST enziminin CDNB substratı için görünen K_m and V_{max} değerleri sırasıyla 2.8 mM ve 0.43 nmol/min/mg, ve GSH kofaktörü için 4.11 mM ve 0.23 nmol/min/mg olarak bulundu.

Ayrıca, üç farklı ağır metal iyonunun, Cd^{+2}, Hg^{+2} ve Ni^{+2}, insan serum ARE ve GST enzimleri üzerine etkileri çalışıldı ve enzim aktivitesinin %50'sini inhibe eden konsantrasyon (IC_{50}) değerleri belirlendi.

Çalışmanın esas amacı ise insan serum ARE ve GST aktivitelerini optimize edilmiş koşulları kullanarak hasta ve kontrol gruplarında araştırmaktır. Bu amaçla, 172 iskemik inme hastası ve 105 kontrolden kan örnekleri toplanmıştır. ARE ve GST aktivitelerini belirlemek için toplanan kan örneklerinden serum elde edilmiştir. Hasta grubunun ortalama ARE aktivitesi (n=172, 109.9 ± 32.5 U/mL) kontrol grubunun ortalama ARE aktivitesinden (n=105, 113.5 ± 33.1 U/mL, *P*=0.284) istatistiksel olarak önemsiz bir derecede düşük bulunmuştur. Hastaların GST aktivitesi (10.8 ± 4.4 U/L) kontrollerden daha yüksek bulundu (10.5 ± 4.2 U/L, *P*=0.483). Ayrıca, istatistiksel analizler hipertansiyon, diabet ve HDL'nin inme için önemli risk faktörleri olduğunu göstermektedir.

Anahtar kelimeler: İskemik inme, Arilesteraz, Glutatyon S-transferaz, Oksidatif stres, Paraoksonaz

Dedicated to my father,

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ARE	Arylesterase
BSA	Bovine Serum Albumin
CDNB	1-Chloro-2,4-dinitrobenzene
CT	Computed Tomography
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-Transferase
HDL	High Density Lipoprotein
H_2O_2	Hydrogen Peroxide
IC_{50}	Half maximal inhibitory concentration
LDL	Low Density Lipoprotein
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PAHs	Polycyclic Aromatic Hydrocarbons
PON1	Paraoxonase1
ROS	Reactive Oxygen Species
TRIS	Tris (hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

1.1 Stroke

Stroke is a sudden interruption in the blood supply of the brain. Most strokes are caused by an abrupt blockage of arteries leading to the brain known as ischemic stroke. Other strokes are caused by bleeding in the brain tissue when a blood vessel bursts (hemorrhagic stroke). Stroke remains the third most common cause of death, particularly in elderly. The mortality rate of stroke in acute phase is as high as 20% (Sarti *et al*., 2000) and it remains higher for several years after the acute event in stroke patients than in the general population (Ayala *et al*., 2001).

1.1.1 Ischemic Stroke

Ischemic stroke accounts for about 75% of all cases while hemorrhagic stroke is responsible for almost 15% of all strokes (Lyden and Zivin, 1993). Although ischemic and hemorrhagic stroke have different risk factors and pathophysiological mechanisms, there is evidence of an increased generation of free radicals and other reactive species in both conditions, leading to oxidative stress (Kochanek and Hallenbeck, 1992, Alexandrova *et al*., 2004, Cherubini *et al*., 2005).

Ischemic stroke is the consequence of the interruption or severe reduction of blood flow in cerebral arteries. According to degree of hyperfusion it is possible to identify an area with complete absence of flow,

namely the core, where neuronal death occurs within a few minutes, and a surrounding area, called penumbra, which suffers from a moderate reduction of blood flow and contains functionally imparied but still viable brain tissue (Yu *et al*., 1998, Cuzzocrea *et al*., 2001).

Many different symptoms can occur, depending on which part of the brain is deprived of blood and oxygen. When the arteries that branch from the internal carotid artery are affected, blindness in one eye or abnormal sensations and weakness in one arm or leg or on one side of the body are most common. When the arteries that branch from the vertebral arteries in the back of the brain are affected, dizziness and vertigo, double vision, and generalized weakness of both sides of the body are more common. Many other symptoms, such as difficulty in speaking (for example, slurred speech) and loss of coordination, can occur.

1.1.2 Risk Factors of Ischemic Stroke

The risk factors of stroke can be classified with respect to subtype of stroke, modifiability of risk factor and scientific certainty of relation between risk factor and stroke. The risk factors of stroke are given in Table 1.1.

Hypertension has been defined 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).

Table 1.1 Risk factors of stroke.

A- Certain Risk Factors of Stroke

1- Nonmodifiable Risk Factors

Age

Heredity and Race

Sex (gender)

Prior stroke

Transient ischemic attack or heart attack

2- Modifiable Risk Factors

High blood pressure

Cigarette smoking

Diabetes mellitus

Carotid or other artery disease

Atrial fibrillation

Other heart disease

Sickle cell disease

High blood cholesterol

Poor diet

Physical activity

Obesity

B- Uncertain Risk Factors of Stroke

Geographic location

Socioecenomic factors

Alcohol abuse

Drug abuse

1.2 Free radicals and oxidative stress

A free radical can be defined as any species that has at least one unpaired electron in the outermost shell (Karlsson, 1997). Free radicals are highly reactive because of the presence of unpaired electron(s). Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO.) (Chiou and Tzeng, 2000). The production of ROS from partially reduced O_2 is an unavoidable consequence of aerobic respiration. Free radicals arise through oxidative phosphorylation, although 5-lipoxygenase, cyclooxygenase, cytochrome P450 and xanthene oxidase catalyzed reactions are also a source (Hayes and McLellan, 1999). In addition, radicals can be produced in the cells and tissues of our bodies by various processes and reactions. For example; the impact of radiation, ionizing radiation, visible light with photosensitizes and thermal degredation of organic materials can produce hemolytic fission, by the loss of a single electron from a non-radical, or by the gain of a single electron, where the electron pair of a covalent bond is shared between two atoms resulting in each having an odd electron (Halliwell and Gutteridge, 1989).

There are enzymatic such as superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and nonenzymatic such as α-tocopherol, ascorbic acid, glutathione, bilirubin, antioxidant defense systems against free radicals. Despite these antioxidant defenses, ROS inflict damage on membrane lipids, DNA, proteins and carbohydrates. Oxidation of these macromolecules gives rise to cytotoxic and mutagenic degradation products (Marnett *et al*., 2003).

Free radicals interact with cellular compartment and cause cellular and genetic damage. The involvement of ROS has been implicated in several

diseases such as atherosclerosis, HIV/AIDS, cancer, chronic fatique syndrome, asthma, arthritis, cystic fibrosis, multiple sclerosis, fibromyalgia, and other immune disorders (McCord, 1985, Schmidley, 1990, Loft and Poulsen, 1997).

The condition occuring when the physiological balance between oxidants and antioxidants is disrupted in favor of the former with potential damage for the organism is called as oxidative stress (Sies, 1985). In principle, oxidative stress can be caused by increased production of free radicals or diminished antioxidants. The tissue level of antioxidants critically influences susceptibility of various tissues to oxidative stress. Enhanced oxidative stress and oxidative damage to tissues are general features of some chronic diseases such as Alzheimer disease, Parkinson's disease, cancer, atherosclerosis and diabetes mellitus (Yoritaka *et al*., 1996, Berlett and Stadtman, 1997, Beal, 2002). If mild oxidative stress occurs, tissues often respond by making extra antioxidant defenses. However, severe oxidative stress can cause cell injury and death (Sarafian and Bredesen, 1994). Some oxidative stress related conditions in different body parts are given in Figure 1.1.

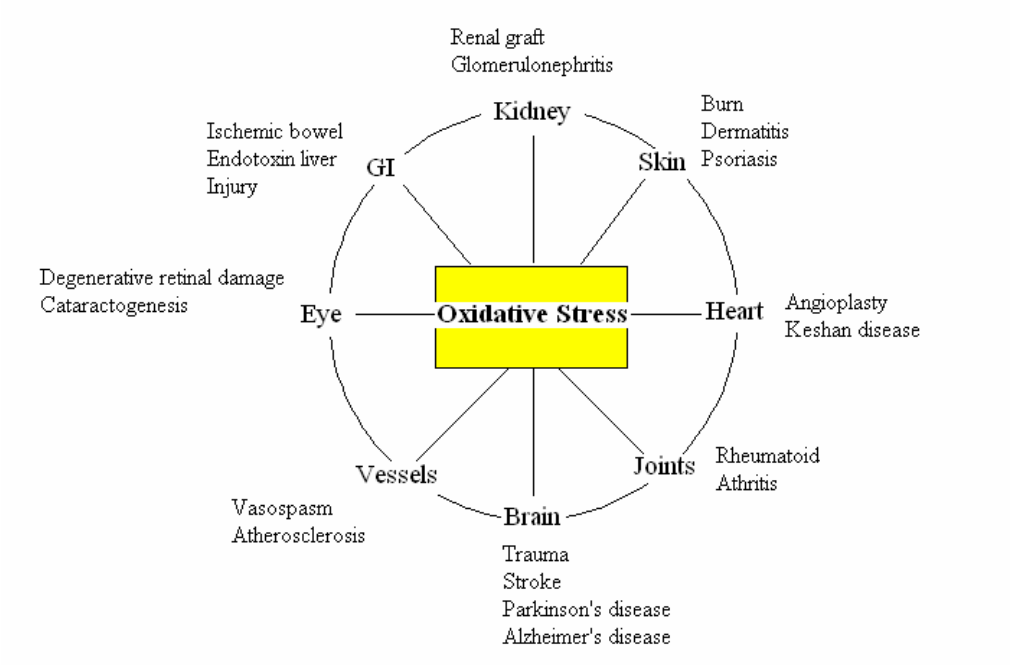

Figure 1.1 Oxidative stress related conditions (Taken from lecture notes of Miral Dizdaroğlu).

1.2.1 Atherosclerosis

Atherosclerosis is a major health problem in many industrialized countries and contributes significantly to morbidity and mortality. Atherosclerosis is the build up of fatty deposits called plague on the inside walls of arteries. Plaques can grow large enough to significantly reduce the blood's flow through an artery. As an artery becomes more and more narrowed, less blood can flow through as shown in Figure 1.2. The artery may also become less elastic called ''hardening of the arteries''. Atherosclerosis is the main cause of a group of cardiovascular diseases such as heart failure, heart attack (Hirai *et al*., 1989). Epidomiological studies have shown that

hypercholesterolemia, hypertension and smoking are major risk factors for atherosclerosis (Marenberg *et al*.,1994, Aslan *et al*., 2007).

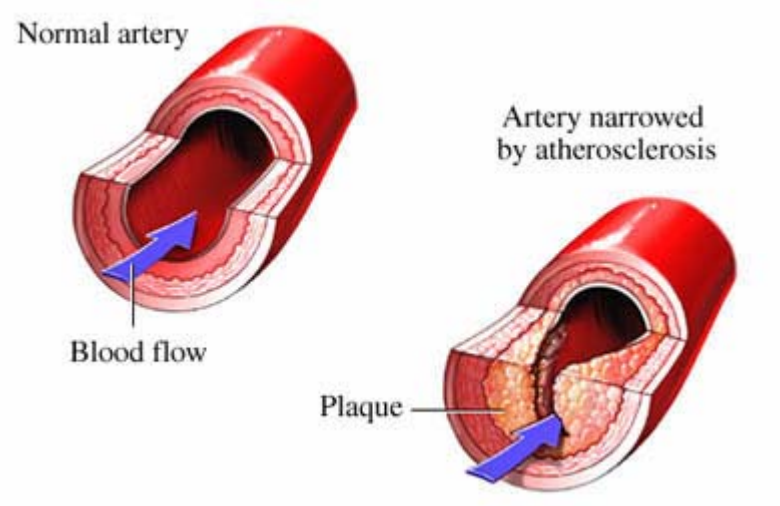

Figure 1.2 A normal artery with normal blood flow and an artery containing plaque build up (atherosclerosis).

Atherosclerosis plaque itself consists of fatty substances, cholesterol, cellular waste products and calcium. If part of this plaque breaks away, it can travel in the bloodstream to a point where the artery is narrow enough for the plaque to completely block it. If the affected artery feeds the heart, a heart attack may result, and if it feeds the brain, a stroke may result.

Atherosclerosis also causes high blood pressure (hypertension). As the blood is forced through the narrowed arteries, it exerts more pressure on the walls. This also increases the risk of stroke and heart attack. Angina (chest pain) is another condition caused by atherosclerosis.

The oxidative modification of low-density lipoprotein (LDL) in the artery wall is currently believed to be central to the pathogenesis of atherosclerosis (Steinberg *et al*., 1989). One such mechanism is prevention of LDL oxidation by high-density lipoprotein (HDL) (Mackness and Durrington, 1995). HDL acts at a specific point in the lipid peroxidation cascade and by a mechanism that was enzymatic in nature (Mackness *et al*., 1993). The HDL associated enzyme PON1 (arylesterase) is responsible for HDL's ability to prevent the accumulation of lipid-peroxides in LDL and plays an important role in preventing atherosclerosis (Mackness *et al*., 1991, Mackness *et al*., 1993).

1.3 PON1 (Arylesterase)

Arylesterase (ARE) (EC 3.1.1.2) is an enzyme present in the blood of mammals where it is associated with high density lipoproteins (HDL) (Mackness *et al*., 1998). It is also classified as paraoxonase (PON) (EC 3.1.8.1) (Adkins *et al*., 1993). PON has at least three isozymes called as PON1, PON2, PON3.

PON1 is a high density lipoprotein associated enzyme with three activities: paraoxonase, arylesterase and diazoxonase (McElveen *et al*., 1986, Abbott *et al*., 1995). It is a Ca^{+2} dependent serum esterase that is synthesized in the liver and is a protein of 354 aminoacids (43 kDa) (Blatter *et al*., 1993). The activity of PON1 aganist phenylacetate is called arylesterase and activity of arylesterase the level of PON 1 in serum.

PON1 enzyme is known mainly for its ability to hydrolyse toxic organophosphorus esters including insecticides such as paraoxon and diazoxon and nerve gases such as sarin, soman, and tabun (Debord *et al*., 2003, Aslan *et al*., 2006) . Other synthetic esters like phenylacetate are also hydrolysed with a high catalytic efficiency (Mackness *et al*., 1995). Some substrates of PON1 are given in Figure 1.3.

In addition PON1 (arylesterase) also plays a role in lipid metabolism, since as a HDL associated antioxidant enzyme it can prevent lipid peroxide accumulation in low-density lipoproteins (LDL) that are involved in the initiation of atherosclerosis (Mackness *et al.*, 1993). Oxidized LDL plays an important role in the initial stage of atherosclerotic lesions (Witztum, 1994). So, PON 1 is one such enzyme that is known to contribute to the protective effects of HDL. In fact , the enzyme PON1 has a significant role in prevention of atherosclerosis (Mackness *et al*., 1991, Mackness *et al*., 1993, Watson *et al*., 1995, Ng *et al*., 2005, Aslan *et al*., 2007). In addition, a number of studies have evaluated the oxidative status by determining the level and activity of antioxidant enzyme PON1 in patients with ulcerative colitis, myocardial infarction, diabetes mellitus, high grade gliomas and meningiomas, chronic hepatitis, Parkinson's disease and stroke. According to these studies, the serum PON1 activities in disease condition such as, ulcerative colitis, high grade gliomas and meningiomas, chronic hepatitis, were lower than serum PON1 activities in control group (Baskol *et al*., 2004, Kafadar *et al*., 2005, Kilic *et al*., 2005).

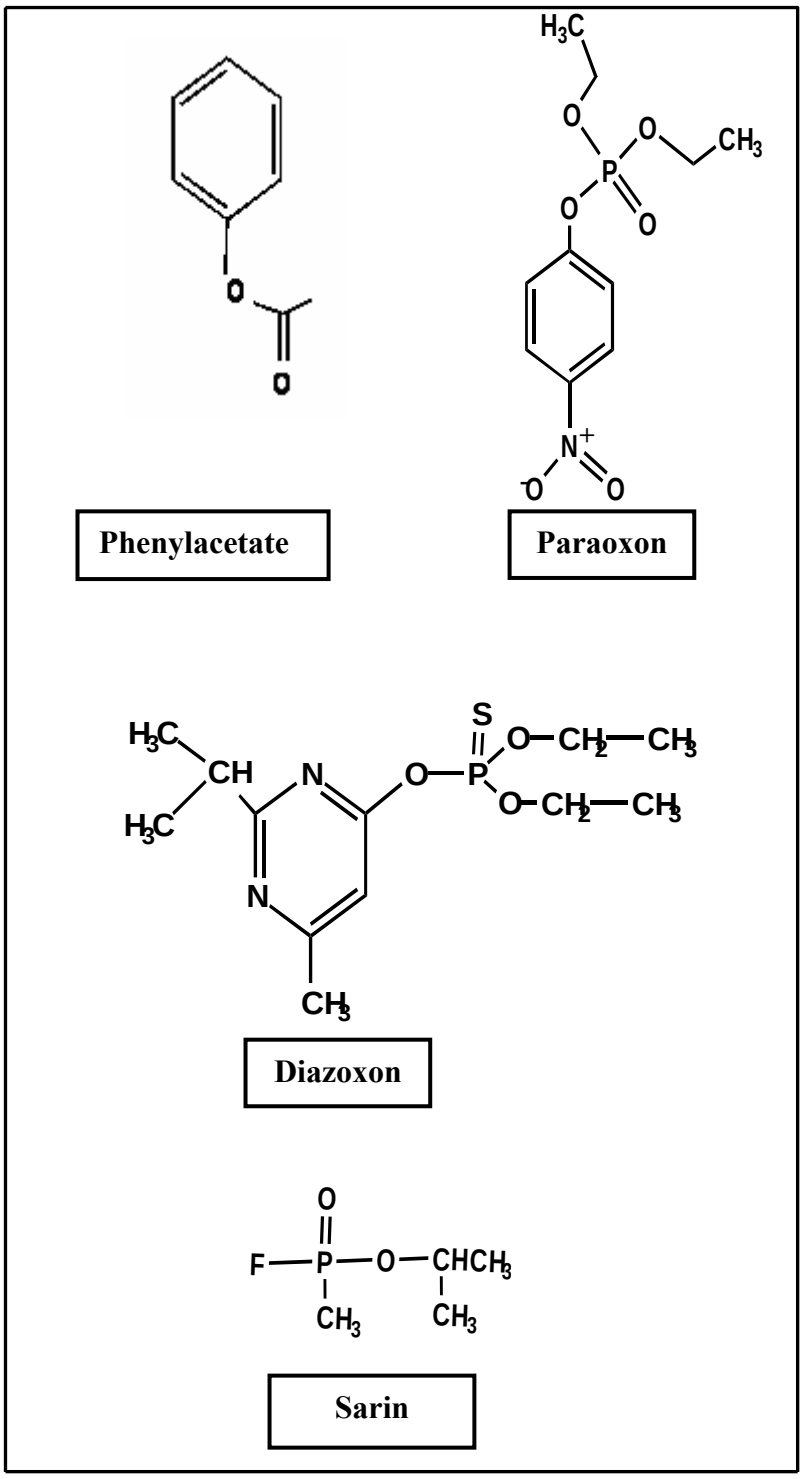

Figure 1.3 Some Substrates of Paraoxonase.

1.3.1 Structure of Paraoxonase 1

The three dimensional structure of human PON1 has not been solved yet. However three dimensional structure of a hybrid mammalian recombinant PON1 (a synthetic construct created by mixing bits of rabbit, mouse, rat and human PON1 genes expressed in E.coli) was recently determined (Harel *et al*., 2004). This structure corresponds to an enzyme differing by ~ 16% of aminoacids from the human enzyme, some of the mutations being in the vicinity of the region involved in substrate binding, and in binding to HDL and partner lipoproteins. The crystal structure shows that the protein is a six-bladed β propeller. A disulfide bridge between C42 and C353 stabilizes the structure. Overall structure of recombinant PON1 is given in Figure 1.4.

Each blade consists of four β-sheets. Two calcium atoms are in the central tunnel of the enzyme. One calcium at the top of the tunnel is assigned as the catalytic calcium; one calcium in the central section is likely a structural calcium involved in stabilization of the structure. In addition, there is an unexpected phosphate ion bound to the catalytic calcium in the active site. Three α-helices are located at the top of the propeller. Thus, in human PON1, the three α-helices are likely involved in the anchoring to the HDL particle. Schematic illustration of interaction of HDL particle with associated proteins is given in Figure 1.5.

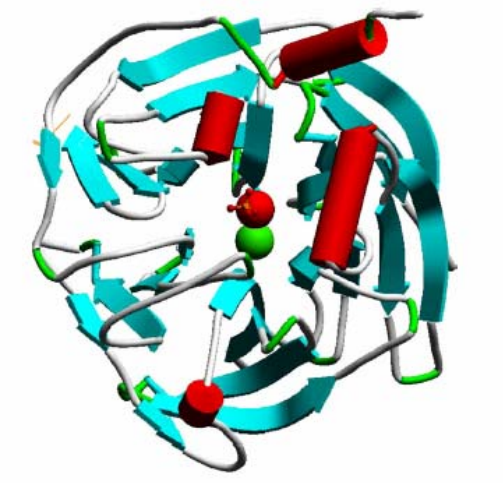

Figure 1.4 Overall structure of rPON1 showing the six-bladed β-propeller conformation, the three α-helices involved in anchoring to HDL (red influence cylinders), the structural calcium cation (green), and catalytic calcium cation (red) to which a phosphate anion (sticks) is bound (Taken from Rochu *et al*., 2007).

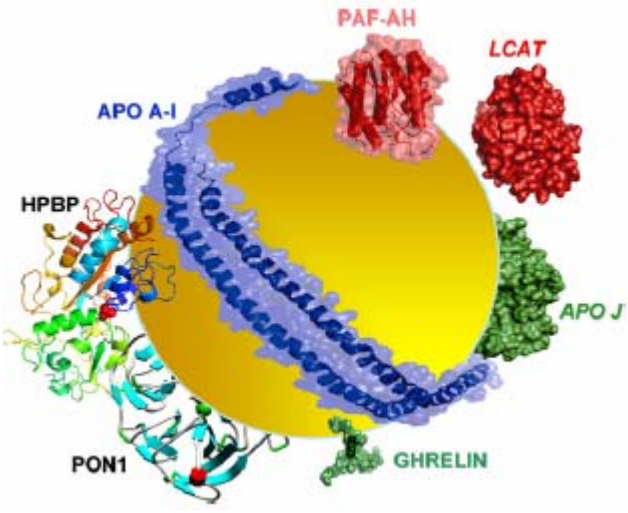

Figure 1.5 Schematic illustration of HDL particle with durably and transiently associated proteins (Taken from Rochu *et al*., 2007).

1.4 Glutathione S- Transferases

Glutathione S-transferases (GSTs; EC 2.5.1.18) are soluble proteins with typical molecular masses of around 50 kDa (Wilce and Parker, 1994). They consist of a superfamily of dimeric enzymes (Mannervik, 1985) and play a key role in phase II of enzymic detoxification (Figure 1.6). Phase II enzymes catalyse the conjugation of activated xenobiotics to an endogenous water soluble substrate, such as reduced glutathione (GSH), UDP-glucuronic acid or glycine. Quantitatively, conjugation to GSH, which is catalysed by the GSTs, is the major phase II reaction in many species (Sheehan *et al*., 2001). GSTs catalyze nucleophilic attack by reduced glutathione on nonpolar compounds that contain an electrophilic carbon, nitrogen or sulphur atom (Keen *et al*., 1978, Armstrong, 1997, Hayes *et al*., 2005), resulting in the formation of oxidized glutathione (GSSG) .

Glutathione transferases are of interest to pharmacologists and toxicologists because they provide targets for antiasthmatic and antitumor drug therapies (Evans *et al*., 1991, Matsushita *et al*., 1998, Jakobsson *et al*., 1999), and they metabolize cancer chemotherapeutic agents, insecticides, herbicides, carcinogens, and by-products of oxidative stress. Overexpression of GST in mammalian tumor cells has been implicated with resistance to various anticancer agents and chemical carcinogens (Hayes and Pulford, 1995). Furthermore, elevated levels of GST have been associated with tolerance of insecticides and with herbicide selectivity (Ranson *et al*., 2001, Edwards and Dixon, 2004).

The cancer chemotherapeutic agents adriamycin, 1,3-*bis*(2-chloroethyl)-1 nitrosourea (BCNU), busulfan, carmustine, chlorambucil, *cis*-platin, crotonyloxymethyl-2 cyclohexenone (COMC-6), cyclophosphamide, ethacrynic acid, melphalan, mitozantrone, and thiotepa are detoxified by GST

(Hayes and Pulford, 1995, Lien *et al*., 2002, Hamilton *et al*., 2003). Environmental chemicals and their metabolites detoxified by GST include acrolein, atrazine, DDT, inorganic arsenic, lindane, malathion, methyl parathion, muconaldehyde, and tridiphane (Hayes and Pulford, 1995, Abel *et al*., 2004).

A large number of epoxides, such as the antibiotic fosfomycin and those derived from environmental carcinogens, are detoxified by GST. The latter group includes epoxides formed from aflatoxin B1, 1-nitropyrene, 4-nitroquinoline, polycyclic aromatic hydrocarbons (PAHs), and styrene by the actions of cytochromes P450 in the liver, lung, gastrointestinal tract, and other organs. Conjugation of aflatoxin B1-8,9-epoxide with GSH is a major mechanism of protection against the mycotoxin, at least in rodents (Kelly *et al*., 2000). The PAHs are ubiquitous, found in cigarette smoke and automobile exhaust fumes, and represent an ever-present threat to health. Those that are metabolized by GST include ultimate carcinogenic bay- and fjord region diol epoxides produced from, benzo[*a*]pyrene benzo[*c*]chrysene, benzo[*g*]chrysene, benzo[*c*]phenanthrene, dibnez[*ah*]anthracene, dibenzo[*a*,*l*]pyrene, chrysene and methylchrysene (Sundberg *et al*., 1997, Hu *et al*., 1998, Dreij *et al*., 2002).

Heterocyclic amines, produced by cooking protein-rich food, represent another important group of carcinogens. One of the major heterocyclic amines found in cooked food is 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and cytosolic GST isoenzymes have been shown to detoxify the activated metabolite, *N*-acetoxy-PhIP (Coles *et al*., 2001).

Glutathione S-transferases are involved in endogenous metabolism such as detoxification of products of oxidative stress, degredation of aromatic amino acids, synthesis of steroid hormones and synthesis and inactivation of eicosanoids.

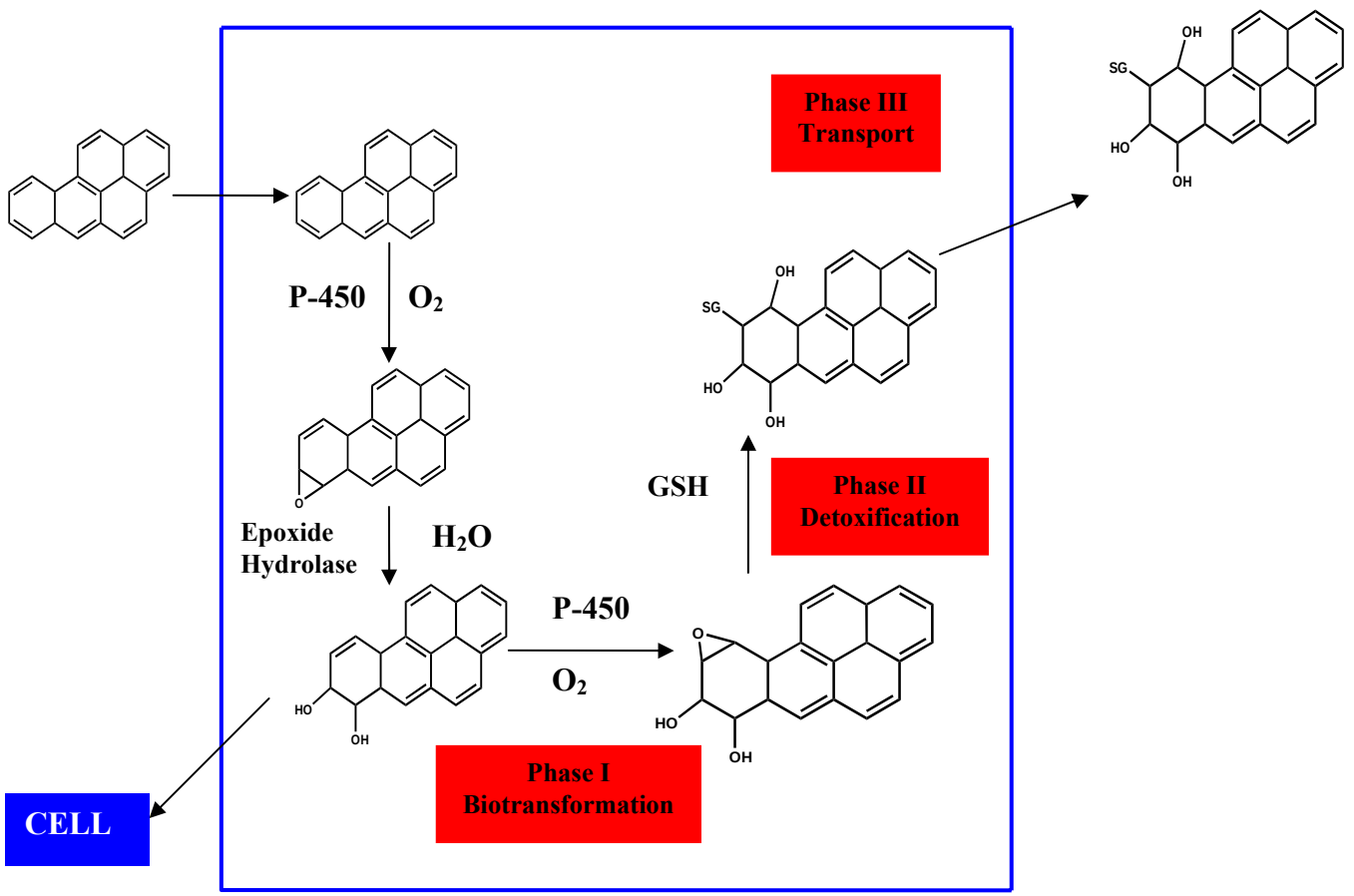

Figure 1.6 Overview of enzymic biotransformation by phase I (cytochrome P450 and epoxide hydrolase), phase II (glutathione S-transferase) and phase III mechanisms.

The glutathione redox cycle given in Figure 1.7 is important to protect normal cells from oxidant injury. In the glutathione redox cycle, glutathione is oxidized by hydrogen peroxide to glutathione disulfide by the selenium-containing enzyme, glutathione peroxidase (GPx), and also by other enzymes that may use lipid peroxides rather than hydrogen peroxide as the oxidant. Thus, glutathione can detoxify both soluble and lipid peroxides. Glutathione disulfide is subsequently reduced by glutathione reductase (GR), using NADPH as the reductant. Cellular NADPH, produced by the pentose-P pathway and other cytoplasmic sources, provides the major source of reducing power for detoxifying many peroxides. The concentration of cellular glutathione has a major effect on its antioxidant function and it varies considerably as a result of nutrient limitation, exercise, and oxidative stress. Under oxidative conditions, the concentration of glutathione can be considerably diminished through conjugation to xenobiotics, and by secretion of both the glutathione conjugates and glutathione disulfide from the affected cells. A considerable amount of glutathione may also become protein-bound during severe oxidative stress. During oxidative stress, large families of proteins that contain reactive sulfhydryls are modified by oxidation to mixed-disulfides with attached glutathione (S-thiolation). Three antioxidant enzymes are important and working in glutathione redox cycle, these are; Glutathione Peroxidase (GPx), Glutathione S-Transferase (GST) and Gluatathione Reductase (GR).

Three major families of widely distributed proteins that exhibit GST activity have been described as cytosolic, mitochondrial (Ladner *et al*., 2004) and microsomal GST. Microsomal GST is designated as membrane associated protein in eicosanoid and glutathione metabolism (Jakobsson *et al*., 1999). Initially, GST subunits were classified into five independent classes alpha, mu, pi, sigma and theta (Ketterer *et al*., 1993, Neuefiend, 1997), but later studies

have shown that there also exist beta, delta, tau, zeta form of these family (Dixon *et al*., 2002). In drug metabolism GSTs alpha, mu and pi forms are active and sigma form plays a role in the prostoglandin synthesis (Rowsey *et al*., 2001). Zeta and theta GSTs are found in both animals and plants but tau and phi are plant specific (Dixon *et al*., 2002).

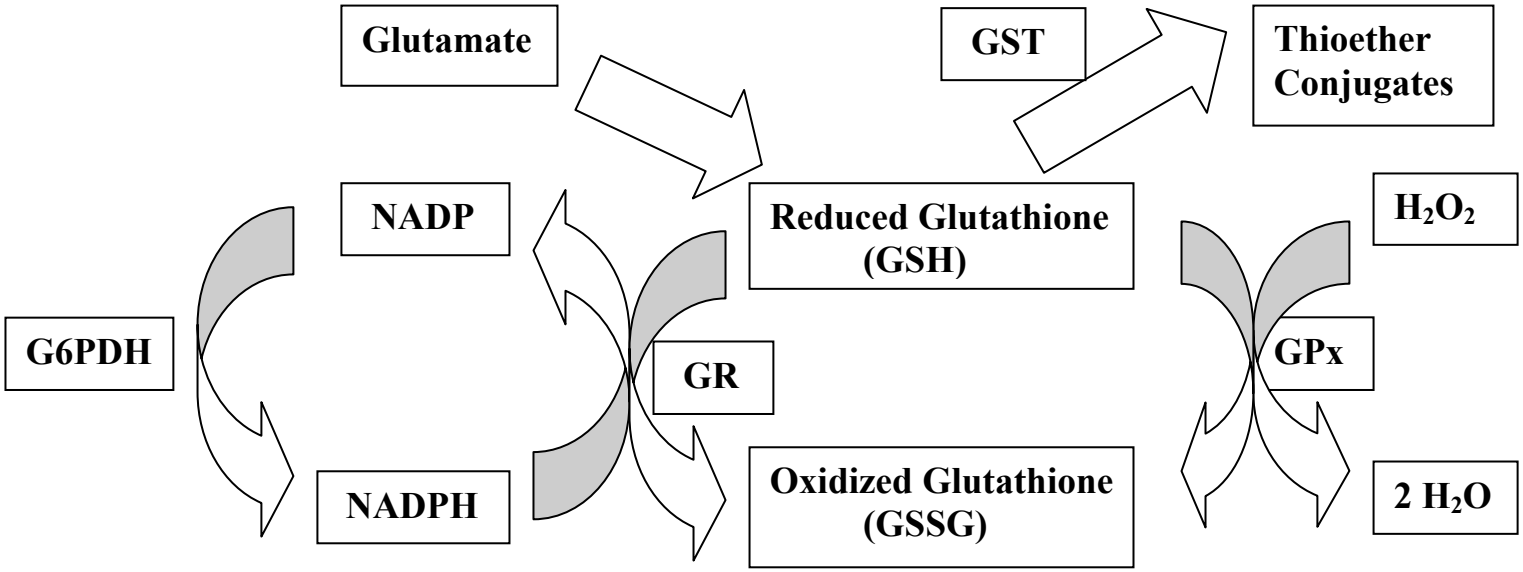

Figure 1.7 Glutathione Redox Cycle.

1.4.1 Structure of Glutathione S-Transferases

Each GST subunit of the protein dimer contains an independent catalytic site composed of two components (Figure 1.8). The first is a binding site specific for GSH or closely related homolog (G site) formed from a conserved group of amino acid residues in the amino terminal domain of the polypeptide. The second component is a site that binds hydrophobic substrate (H site), which is much more structurally variable and is formed from residues in the carboxy terminal domain. There is a linker region of 5-10 residues between the two domains. The G and H sites of the enzyme are quite mobile when the crystal structure is determined, suggesting that the GST subunits undergo significant conformational changes on binding the substrates (Dixon *et al*., 2002).

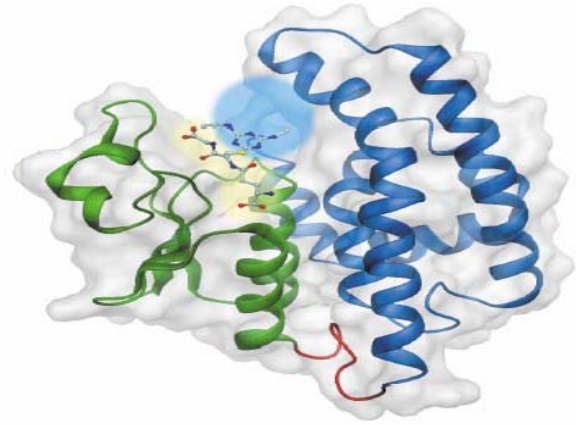

Figure 1.8 Overview of GST dimer structure and substrate binding. The amino terminal domain in green, the linker region in red, the carboxy terminal domain in blue and the protein surface in gray. A glutathione conjugate of the herbicide in ball-and-stick representation is shown binding at the active site; the GSH binding site (G site) is highlighted in yellow and the hydrophobic site (H site) is highlighted in blue (Taken from Dixon *et al*., 2002).

1.5 The Aim of This Study

Oxidative stress plays an important role in the pathogenesis of atherosclerosis and carotid atherosclerosis is a risk factor for stroke. Human serum paraoxonase (PON1) which is known as HDL associated enzyme protects LDL from oxidation by the hydrolysis of biologically active lipidperoxides. Oxidized LDL plays an important role in the early stage of atherosclerosis, so PON1 has a protective role against atherosclerosis. Phenylacetate hydrolysis activity of PON1 which is known as arylesterase (ARE) is directly correlated with concentration of PON1 in blood. Compared to atherosclerosis, a limited number of studies in literature have shown the relation of arylesterase (PON1) and ischemic stroke.

In addition, glutathione S-transferases (GST) are involved in phase II detoxification reactions of xenobiotics and detoxify metabolites produced within the cell by oxidative stress and protect cells from oxidant injury. Recent studies have shown that, glutathione S-transferases play an important role against atherosclerosis. However, no data is available in the literature showing the relation of the glutathione S-transferases and stroke.

The aim of this study is first to characterize and optimize the conditions for human blood serum arylesterase and glutathione S-transferase enzymes and then to investigate whether there is an association between human serum ARE and GST enzyme activities and ischemic stroke risk. To accomplish this purpose, first, the effects of temperature, pH, substrate concentration on human serum ARE and GST enzyme activities were determined. Then ARE and GST enzyme activities were measured at optimum conditions in blood serum samples obtained from both ischemic stroke patients and healthy volunteers.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Subjects and Blood Sample Collection

A total of 172 serum samples were obtained from consecutive unrelated adult Caucasian patients with acute hemispheric ischemic stroke and 105 serum samples were obtained from symptom-free Caucasian controls from the same geographic region (central Anatolia, Turkey) with the collaboration of Gülhane Military Medical Academy, Department of Neurology, Ankara. Informed consent was obtained from all participants before study entry. Copy of the informed consent forms are given in Appendix 1 and Appendix 2. The study was approved by the ethical committee of the medical faculty and a copy of approval form is given in Appendix 3. Cases were selected among patients suffering atherothrombotic ischemic stroke admitted to the neurology services of Gülhane Military Medical Academy within 24 h after onset. The cerebral infarction was initially diagnosed on the basis of neurological examination and brain computed tomography (CT) and then transthoracic echocardiographic examination, Holter study and Transcranial Doppler emboli detection procedure to rule out emboli source. The control group was selected randomly from the neurology outpatient clinics.

A detailed history of conventional vascular risk factors and conditions such as hypertension, diabetes, obesity, smoking status was taken from each participant. Routine laboratory tests, including electrocardiogram, chest X-ray,

complete blood count, leukocyte differential, erythrocyte sedimentation rate, routine biochemistry tests including fasting glucose, lipid profile (triglyceride, total cholesterol, LDL-C, HDL-C), creatinine, sodium, potassium, bilirubin, and liver function tests, routine urine tests and rheumatologic screening tests were performed for all participants. Clinical tests given above were carried out in Gülhane Military Medical Academy, Department of Neurology, Ankara.

10 ml of blood samples from subjects was taken to test tubes containing no anticoagulant. Then serum was isolated from whole blood by centrifugation at 2000 g for 10 minutes and stored at -20 C° until use for measurement of human serum ARE and GST activities in our laboratory. Activity measurements were completed in 7 days after blood collection. During storage of serum samples at -20 C° , no significant changes in activities of both enzymes were observed (Data no given).

2.1.2 Chemicals

Dipotassium hydrogen phosphate (K_2HPO_4; 05101), potassium dihydrogen phoshate (KH_2PO_4; 04871), calcium chloride ($CaCl_2$; 728487), cupper sulphate penta hydrate ($CuSO_4 . 5H_2O$; 2787), sodium hydroxide (NaOH; 06462), sodium acetate (6268) were the products of E. Merck, Darmstadt, Germany.

1-Chloro-2,4-dinitrobenzene (CDNB; C-6396), reduced glutathione (GSH; G-4251), sodium-potassium tartarate (S-2377), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T-1503), phenylacetate (108723), bovine serum albumine (BSA; A7511) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Absolute ethanol (32221) was the products of Riedel de Haën, Seelze.

All chemicals were analytical grade and were obtained from commercial sources at the highest purity avaliable.

2.2 Methods

2.2.1 Protein Determination

Principle:

The protein concentrations of human serum determined by the method of Gornall *et al*., (1949). This method depends on the formation of violet colored complexes between cupric ions and protein in alkaline medium. The cupric ions that are introduced into the solution as cupric sulfate react with the peptide bonds of serum proteins in alkaline solution to form a colored complex which gives maximum absorbance at 540 nm. The intensity of the color is directly proportional with the total protein concentration.

Reagents:

1. Biuret reagent: 2.4 g $CuSO_4 . 5H_2O$
   9.6 g Na-K tartarate
   300 ml of 5 N NaOH final volume completed to 1 Liter
2. Standard : 10 mg/ml of Bovine serum albumine

Assay:

A typical reaction medium is given in Table 2.1. Crystalline bovine serum albumine was used as a standard and standarts and samples were run in

separate duplicate tubes. 0.1 ml standard was added into the standard tube, 0.1 ml distilled water was added to the blank tube and 0.1 ml serum was added to the sample tube. Then, 3 ml of Biuret reagent was added to each tube. All tubes were mixed and let stand for 30 minutes at room temperature. The intensity of resulting color was measured at 540 nm using Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Analytical Instruments Divison, Kyoto, Japan) against blank cuvette. The protein amount of the sample was calculated by using the following formula:

$$\text{Protein Concentration (mg/ml)} = [\text{Standard (mg/ml)}] \times \frac{OD_{540}\ \text{Serum}}{OD_{540}\ \text{Standard}}$$

Table 2.1 The Constituents of Reaction Mixture for Protein Determination in Human Serum.

Tube	**dH_2O (ml)**	**Standard (ml)**	**Serum (ml)**	**Biuret reagent (ml)**
Blank	0.1	------	------	3
Standard (BSA)	------	0.1	------	3
Sample (Serum)	------	------	0.1	3

2.2.2 Determination of Human Serum Arylesterase Activities

Principle:

Arylesterase activity of serum samples from human was determined using phenylacetate as substrate and the conversion of phenylacetate to phenol followed by monitoring the increase in absorbance at 270 nm at 37 C° according to the method described by Furlong, *et al.,* (1988). Reaction catalyzed by arylesterase enzyme (PON1) is given in Figure 2.1.

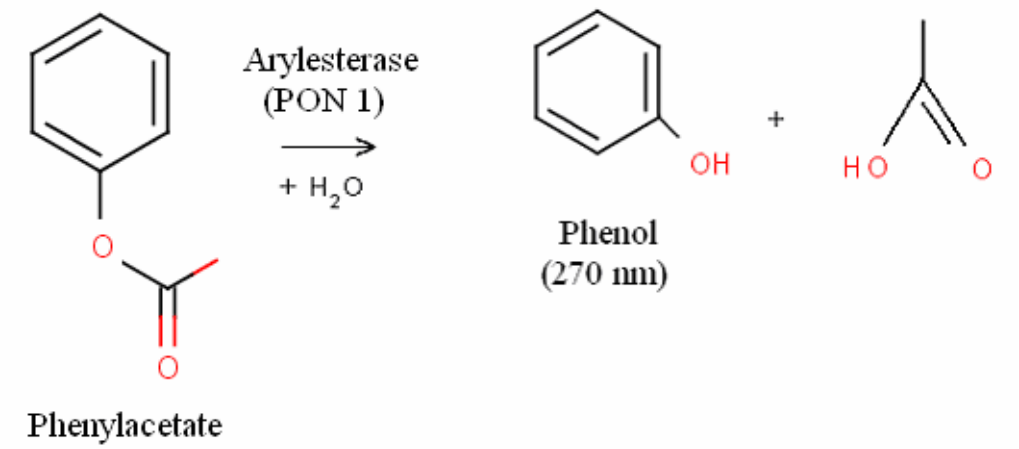

Figure 2.1 Reaction catalyzed by arylesterase enzyme.

Reagents:

1. Tris -HCl Buffer: 10 mM, pH 8.0
2. $CaCl_2$: 50 mM
3. Phenylacetate: 108 mM
   (Note: Stock phenylacetate substrate solution should be prepared fresh each hour, kept in a tightly closed bottle.)

Assay:

A typical reaction mixture is given in Table 2.2. Into the 3 ml quartz cuvette , 2700 µl Tris-HCl buffer, 54 µl $CaCl_2$, 20 µl enzyme (1/50 diluted human serum) and 126 µl dH_2O were added and the reaction was started with the addition of 100 µl phenylacetate solution . Then phenol formation was followed at 270 nm at 37 C° for 5 minutes using Shimadzu UV-160A UV-visible spectrophotometer (Shimadzu Corporation, Analytical Instruments Divison, Kyoto, Japan). Arylesterase enzyme activity was calculated using 1310 M^{-1} x cm^{-1} as an extinction coefficient of phenol formed as a reaction product. ARE activity was expressed in U/mL and 1 unit was defined as 1µmol phenol formed per minute under the given conditions.

Table 2.2 The Constituents of the Incubation Mixture for Determination of Arylesterase Activity in Human Serum.

Constituents	**Stock Solution**	**Volume to be added (µl)**	**Final Concentration in 3 ml incubation mixture**
Tris-HCl Buffer pH 8.0	10 mM	2700	9 mM
$CaCl_2$	50 mM	54	0.9 mM
Serum (1/50 diluted)	-------	20	--------
Phenylacetate	108 mM	100	3.6 mM
Distilled water	-------	To 3 ml	--------

2.2.2.1 Effect of pH on Human Serum Arylesterase Activity

- Enzyme activities at acidic region were measured using 10 mM acetate buffer, pH 5, 6.
- Enzyme activities at neutral region were measured using 10 mM tris-HCl buffer , pH 7, 8, 9.
- Enzyme activities at basic region were measured using 10 mM glycine buffer, pH 10.

Reactions were followed as described in section 2.2.2.

2.2.2.2 Effect of Temperature on Human Serum Arylesterase Activity

After mixing all constituents of the assay medium, except phenylacetate, 2 minutes of preincubation was carried out in a circulatory water bath at temperatures of 20 C°, 25 C°, 30 C°, 37 C°, 45 C°, 50 C°, 60 C°. Then reaction was started by adding phenylacetate and followed continuously at 270 nm at indicated temperatures given above for 5 minutes.

2.2.2.3 Effect of Substrate Concentration on Human Serum Arylesterase Activity

Kinetic constants, Km and Vmax were determined by changing the phenylacetate concentrations from 1 mM to 25 mM. The other conditions were same as described under "Methods". The reaction rates obtained by increasing substrate (phenylacetate) concentration were used to draw Michaelis-Menten and Lineweaver-Burk plots for K_m and V_{max} determination.

2.2.2.4 Effects of Heavy Metal Ions on Human Serum Arylesterase Enzyme Activity

In this study, the effects of heavy metal ions on human serum arylesterase enzyme activity were determined by using varying concentrations of 3 different metal ions. In the same experiment, substrate phenylacetate concentration was fixed at 3.6 mM during the conversion of phenylacetate to phenol.

$NiCl_2$ was used at 10 µM, 50 µM, 100 µM, 1 mM and 10 mM concentrations. $CdCl_2$ was added to the reaction medium at final concentrations of 1 µM, 2.5 µM, 5 µM, 10 µM, 50 µM and 100 µM. $HgCl_2$ was used at 2.5 µM, 5 µM, 10 µM, 50 µM and 100 µM concentrations.

The reactions were carried out using a fixed phenylacetate concentration and changing metal ion concentration in the range given above. Reactions were initiated by the addition of phenylacetate and rates were recorded at 270 nm for 5 minutes. Then, half maximal inhibitory concentrations (IC_{50}) of Ni, Cd and Hg for ARE activity were determined .

2.2.3 Determination of Human Serum Glutathione S-Transferase Activities

Principle:

Total Glutathione S-Transferase activity was determined according to the method of Habdous *et al*., (2002) which was modified from Habig *et al*., (1974).

1-Chloro-2,4 dinitrobenzene (CDNB) is a common substrate for all isozymes of glutathione S-transferases. CDNB can be used as a substrate for total glutathione S-transferase catalyzed glutathione (GSH) oxidation which is monitored by the increase in the absorbance at 340 nm due to the 1-(S-glutathionyl)-2,4-dinitrobenzene (DNB-SG) formation. Mechanism and reaction catalyzed by glutathione S-transferase enzyme is given in Figure 2.2.

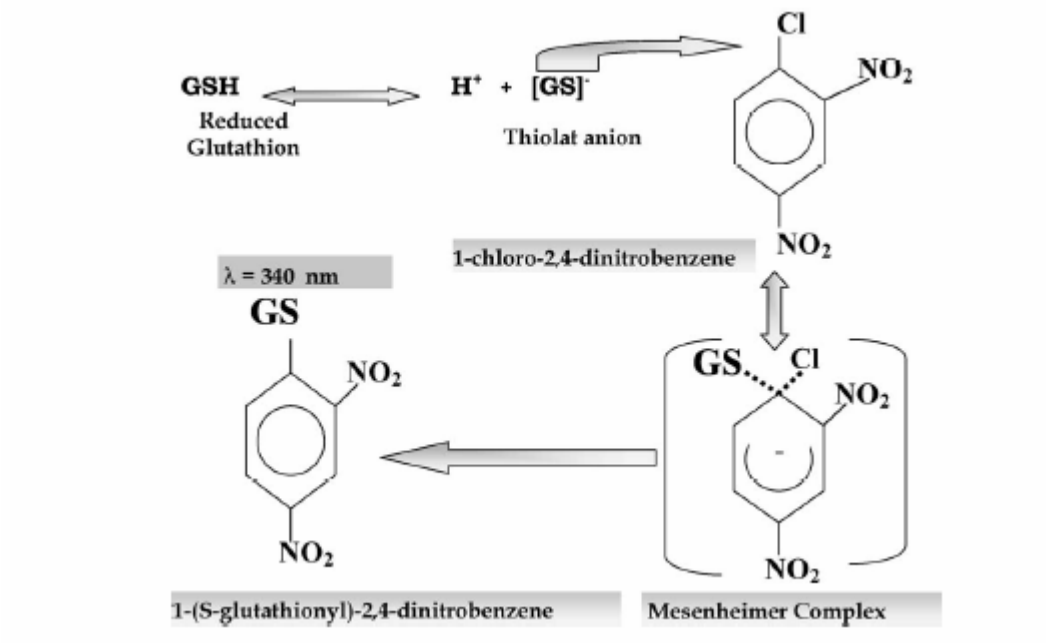

Fig.2.2 Mechanism of the glutathione conjugation to the CDNB. Step 1: ionization of the reduced glutathione in proton (H^+) and thiolate anion $[GS]^-$. Step 2: nucleophilic attack of thiolate anion on C-1 of the aromatic nucleus; formation of Mesenheimer complex and of 1-(S-glutathionyl)- 2,4-dinitrobenzene (GS-DNB) which was followed at 340 nm (Taken from Habdous *et al*., 2002).

Reagents:

* 1-CDNB: 25 mM

  Note: It should be first dissolved in ethanol and then appropriate amount of dH_2O (2/3 of ethanol) is added before storing in dark.

2-Potassium Phosphate Buffer: 0.2 M, pH 5.5

* 3-GSH: 50 mM

*Note: It should be prepared fresh everyday.

Assay:

A typical reaction mixture is given in Table 2.3. Into the 1 ml quartz cuvette, followings were added in the order of 500 µl potassium phosphate buffer pH 5.5, 200 µl distilled water, 100 µl GSH, 100 µl CDNB and the reaction was started by adding 100 µl of serum sample. Then, thioeter formation was followed continuously at 340 nm for 5 minutes at 37 C° using Shimadzu UV-160A UV-visible spectrophotometer (Shimadzu Corporation, Analytical Instruments Divison, Kyoto, Japan). Each time, a blank (reaction with no enzyme) was used and the absorbance obtained was subtracted from absorbance of enzymatic reaction to eliminate the non-enzymatic product formation.

The enzyme activity was calculated by using 0.0096 μM^{-1} x cm^{-1} as an extinction coefficient of thioether formed by GST. GST activity was expressed in U/L and 1 unit was defined as 1 µmol thioether formed per minute under the given conditions.

Table 2.3 The Constituents of the Incubation Mixture for Determination of Glutathione S-Transferase Activity in Human Serum.

Constituents	Stock Solution	Volume to be added (µl)	Final Concentration in 1ml incubation mixture
Potassium Phosphate Buffer , pH 5.5	0.2 M	500	0.1 M
CDNB	25 mM	100	2.5 mM
GSH	50 mM	100	5 mM
Serum	-------	100	-------
Distilled water	-------	To 1 ml	-------

2.2.3.1 Effect of pH on Human Serum Glutathione S-Transferase Activity

- Enzyme activities at acidic region were measured using 0.2 M acetate buffer, pH 3, 4 and 0.2 M potassium phosphate buffer , pH 5, 5.5, 6.
- Enzyme activities at neutral region were measured using 0.2 M potassium phosphate buffer, pH 7, 7.5, 8, 9.

Reactions were followed as described in section 2.2.3.

2.2.3.2 Effect of Temperature on Human Serum Glutathione S-Transferase

Activity

The effect of reaction temperature on human serum glutathione S-transferase activity was determined by changing the temperature of the reaction medium from 5 °C to 75 °C. The temperatures used were 5 C°, 10 C°,

15 C°, 20 C°, 30 C°, 37 C°, 45 C°, 55 C°, 65 C° and 75 C°. Assays were conducted at pH 5.5 using 100 µl human serum and the other conditions remained same as described in section 2.2.3.

2.2.3.3 Effect of Substrate Concentration on Human Serum Glutathione S-Transferase Activity

The effect of both substrates, GSH and CDNB, were studied and individual Km and Vmax values were calculated independently by fixing one substrate concentration at saturating level and changing the concentration of second substrate . Km of GST against GSH was determined by changing the GSH concentrations from 0.5 mM to 10 mM. Km of GST against CDNB was determined by changing the CDNB concentrations from 0.1 mM to 5 mM. Assays were conducted at pH 5.5 and the other conditions remained same as described in section 2.2.3.

2.2.3.4 Effects of Heavy Metal Ions on Human Serum Glutathione S-Transferase Enzyme Activity

The effects of nickel concentration on human serum GST activity were determined by using, 10 µM, 20 µM, 50 µM, 100 µM, 1 mM, 2 mM, 5 mM, and 10 mM final concentration of $NiCl_2$ in the reaction medium. The effects of cadmium concentration on human serum GST activity were determined by using, 10 µM, 30 µM, 50 µM, 100 µM, 300 µM, 500 µM and 1 mM final concentration of $CdCl_2$ in the reaction medium. The effects mercury concentration on human serum GST activity were determined by using, 10 µM, 50 µM, 100 µM, 200 µM, 500 µM and 1 mM final concentration of $HgCl_2$ in the reaction medium. The reactions were carried out using a fixed CDNB concentration and changing metal ion concentration in the range given above.

Reactions were initiated by the addition of human serum and rates were recorded at 340 nm for 5 minutes. Then, half maximal inhibitory concentrations (IC_{50}) of Ni, Cd and Hg for GST activity were determined .

2.2.4 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. Depending on the shape of the distribution curves, differences of continuous variables were evaluated by the Student's t-test or Mann-Whitney U test. Categorical variables were expressed as proportions and compared using χ^2 test.

CHAPTER 3

RESULTS & DISCUSSION

Stroke is the clinical designation for a rapidly developing loss of brain function due to an interruption in the blood supply to all or part of the brain. It is an important public health problem and the third leading cause of death after coronary heart diesase and all cancers in all over the world. Risk factors for stroke include hypertension, diabetes mellitus, high cholesterol, cigarette smoking and atherosclerosis. Free radicals and oxidative stress play important role in aging as well as in the pathogenesis of several diseases including atherosclerosis, stroke, cancer, neurodegenerative diseases such as Alzheimer's dementia.

Human blood serum paraoxonase (PON1, arylesterase) is a calcium dependent esterase and binds to high density lipoprotein and contributes to the detoxification of organophosphorus compounds, such as paraoxon, and carcinogenic lipid soluble radicals from lipid peroxidation. PON1 is recognized as an antioxidant enzyme, because it hydrolyses lipid peroxides (La Du *et al*., 1993, La Du, 1996). PON1 plays an important role in prevention of atherosclerosis.

Glutathione S-transferase (GST) is a multigene family of isoenzymes that catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophilic compounds as the first step in a detoxification pathway leading to mercapturic acid formation (Ngo and Nutter, 1994). It is also detoxify toxic metabolites produced within the cell by oxidative stress and protect cells from oxidant injury. For this reason, GSTs have an important role against atherosclerosis.

This study was aimed to investigate the association between human serum ARE and GST enzyme activities and ischemic stroke risk.

3.1 Clinical and Biochemical Characteristics of Controls and Stroke Patients

The study population consisted of 172 ischemic stroke patients and 105 controls. Clinical tests such as blood serum HDL, LDL, total cholesterol and triglyceride level and biochemical characteristics such as ARE and GST activities were measured in stroke patients and controls . In addition, conventional risk factors including hypertension, diabetes and cigarette smoking in acute ischemic stroke subjects and controls were evaluated. Raw data including these parameters obtained from study participants are listed in Appendix 4.

3.1.1 Clinical Laboratory Data and Conventional Risk Factors of Acute Ischemic Stroke Patients and Controls

The results of clinical laboratory tests and some risk factors of acute ischemic stroke patients and control subjects are summarized in Table 3.1. There was no difference in mean age of the patient (66.6 ± 14.8) and control groups (64.5 ± 12.8, P=0.093). Patient group contained more males (56.4 %) than control group (49.5 %, P=0.266). As expected the risk factors of acute ischemic stroke were found to be higher in patient group. Hypertension was significantly higher in patient group (61.0 %) when compared to control group (42.9 %, P=0.003). Diabetic people have high risk of ischemic stroke (29.1 %, P=0.025). Similarly smoking were found to be higher in patient group (22.7 %) than control group (13.3 %, P=0.055). According to clinical tests, triglyceride level of patients (1.5 ± 0.7 mmol/L) was insignificantly higher than

control group (1.4 ± 0.7 mmol/L, *P*=0.376). In the same way, LDL level was insignificantly higher in patient group (2.8 ± 1.1 mmol/L) when compared to controls (2.6 ± 1.0 mmol/L, *P*=0.296). As given in Table 3.1, total cholesterol level was found to be lower in patients (4.8 ± 1.3 mmol/L) than controls (4.9 ± 1.2 mmol/L, *P*=0.449). In addition, HDL level was significantly lower in ischemic stroke patients (1.1 ± 0.3 mmol/L) when compared to control group (1.2 ± 0.3 mmol/L, *P*=0.007).

Table 3.1 Clinical Laboratory Data and Conventional Risk Factors of Acute Ischemic Stroke Patients and Controls.

Parameter	**Patients (n=172)**	**Controls (n=105)**	***P***	**OR (95% CI)**
Age (years)[a]	66.6 ± 14.8	64.5 ± 12.8	0.093	
Male, n (%)[b]	97 (56.4)	52 (49.5)	0.266	1.318 (0.810-2.145)
Hypertension, n (%)[b]	105 (61.0)	45 (42.9)	0.003	2.090 (1.276-3.422)
Diabetes mellitus, n (%)[b]	50 (29.1)	18 (17.1)	0.025	1.981 (1.082-3.627)
Smokers, n (%)[b]	39 (22.7)	14 (13.3)	0.055	1.906 (0.979-3.711)
Total cholesterol (mmol/L)*	4.8 ± 1.3	4.9 ± 1.2	0.449	
Triglycerides (mmol/L)*	1.5 ± 0.7	1.4 ± 0.7	0.376	
HDL-cholesterol (mmol/L)*	1.1 ± 0.3	1.2 ± 0.3	0.007	
LDL-cholesterol (mmol/L)*	2.8 ± 1.1	2.6 ± 1.0	0.296	

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

[a] Mann Whitney U test is applied

[b] Chi-square test is applied

* Independent Samples T-test is applied

3.1.2 Human Serum Arylesterase (ARE) and Glutathione S-Transferase (GST) Activities of Ischemic Stroke Patients and Controls

Human serum ARE and GST activities were measured in 172 ischemic stroke patients and 105 controls, and results are given in Table 3.2. The comparison of arylesterase and glutathione S-transferase activities in patients and controls were evaluated by using Student's t-test. According to statistical analysis, arylesterase activity of the patients (109 ± 32.5 U/mL) was lower than that of controls (113.5 ± 33.1 U/mL, *P*=0.284). On the other hand, GST activity was insignificantly higher in the patient group (10.8 ± 4.4 U/L) when compared to control group (10.5 ± 4.2 U/L, *P*=0.483).

Table 3.2 Human Serum Arylesterase (ARE) and Glutathione S-Transferase (GST) Activities of Ischemic Stroke Patients and Controls.

Enzyme Activity	**Patients (n=172)**	**Controls (n=105)**	***P***
ARE (U/mL)	109 ± 32.5	113.5 ± 33.1	0.284
GST (U/L)	10.8 ± 4.4	10.5 ± 4.2	0.483

Data are mean ± SD Independent Samples T-test is applied for calculation of *P* values.

The variations of human serum ARE and GST activities against their substrates, phenylacetate and CDNB, respectively, are given in Table 3.3. Arylesterase activities showed a 4x (range: 49-200 U/mL) and 5x (range: 43-218 U/mL) variation among stroke patients and controls, respectively. GST

activities showed a 5x (range: 4.2-22.3 U/L) and 4x (range: 4.2-17.7 U/L) variation among patients and controls, respectively.

Table 3.3 Range of Human Serum ARE and GST activities.

Enzyme activity	Patients (n=172)			Controls (n=105)		
	Lowest	Highest	Variation fold	Lowest	Highest	Variation fold
ARE (U/mL)	49	200.0	4	43.0	218.0	5
GST (U/L)	4.2	22.3	5	4.2	17.7	4

3.1.2.1 ARE and GST Enzyme Activities in Different Subgroups of Ischemic Stroke Patients and Controls

Effects of vascular risk factors; age, gender, hypertension, diabetes and smoking on ARE and GST enzyme activities were analyzed in total population (patient + control) and in patients and controls separately.

3.1.2.1.1 Effect of Age

Study population was analyzed considering the age of subjects and differences in enzyme activities between patients and controls were determined. As can be seen in Table 3.4, arylesterase activity of older subjects (>59 years) (108.3 U/mL) was lower than those of the younger group (<60 years) (117.6 U/mL; *P*=0.035). On the other hand, glutathione S-transferase activity of the elderly group was 11.1 U/L, which was insignificantly higher

than glutathione S-transferase activity of the younger group (9.5 U/L; *P*=0.170).

Table 3.4 Human Serum ARE and GST Activities in Elderly and Younger Subjects.

Enzyme Activity	**Elderly group (>59) (n=203)**	**Younger group (<60) (n=74)**	***P***
ARE (U/mL)	108.3 ± 33.9	117.6 ± 28.4	0.035
GST (U/L)	11.1 ± 4.1	9.5 ± 4.6	0.170

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

Comparison of ARE and GST enzyme activities of patients and controls conducted separately in the elderly and younger age groups is given in Table 3.5. In both age groups, arylesterase activity was lower in the stroke patients than the respective control group. The difference in arylesterase activities between patients and controls was almost lost in the elderly group (107.3 ± 33.2 U/mL vs. 110.0 ± 35.1 U/mL, *P*=0.579). But, this difference was more pronounced in the younger group (114.7 ± 29.8 U/mL vs. 121.7 ± 26.3 U/mL), although still not significant (*P*=0.296). GST activity in elderly group was higher in the stroke patients than the respective control group. On the other hand, GST activity was lower in younger patient group than that of control group. The difference in glutathione S-transferase activities between patients and controls was very close both in elderly group (11.4 ± 4.3 U/L vs. 10.7 ± 3.8 U/L, *P*=0.295) and in younger group (9.3 ± 4.4 U/L vs. 9.8 ± 4.9 U/L).

Table 3.5 Human Serum ARE and GST Activities in Stroke Patients and Controls Compared by Age Groups.

Enzyme Activity	Elderly group (>59)			Younger group (<60)		
	Stroke patient (n=129)	Control (n=74)	*P*	Stroke patient (n=43)	Control (n=31)	*P*
ARE (U/mL)	107.3 ± 33.2	110.0 ± 35.1	0.579	114.7 ± 29.8	121.7 ± 26.3	0.296
GST (U/L)	11.4 ± 4.3	10.7 ± 3.8	0.295	9.3 ± 4.4	9.8 ± 4.9	0.623

Data are mean ± SD Independent Samples T-test is applied for calculation of *P* values.

3.1.2.1.2 Effect of Gender

ARE and GST enzyme activities of stroke patients and controls were analyzed considering the gender of subjects. As can be seen in Table 3.6, arylesterase and glutathione S-transferase activities of male subjects were lower than female subjects. The difference in ARE activities of males (106.6± 29.2 U/mL) and females (115.6 ± 35.9 U/mL) was statistically significant (*P*=0.026).

Table 3.6 Human Serum ARE and GST Activities in Male and Female Subjects.

Enzyme Activity	**Male (n=149)**	**Female (n=128)**	***P***
ARE (U/mL)	106.6 ± 29.2	115.6 ± 35.9	0.026
GST (U/L)	10.6 ± 4.5	10.8 ± 4.0	0.751

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

ARE and GST enzyme activity comparisons between stroke patients and controls were carried out in males and females separately. As given in Table 3.7, in both gender group, GST activity of stroke patients was higher than those of controls. ARE activity of male patients was higher than the respective control group. In contrast to male group, in females, ARE activity of patients was lower than those of control. However, all of the differences were statistically insignificant.

Table 3.7 Human Serum ARE and GST Activities in Stroke Patients and Controls Compared by Gender.

Enzyme Activity	**Male (n=149)**			**Female (n=128)**		
	Stroke patients (n=97)	Controls (n=52)	*P*	Stroke patients (n=75)	Controls (n=53)	*P*
ARE (U/mL)	107.1 ± 29.5	105.8 ± 28.9	0.808	111.8 ± 36.1	120.9 ± 35.4	0.155
GST (U/L)	10.6 ± 4.5	10.5 ± 4.7	0.907	11.1 ± 4.2	10.4 ± 3.6	0.321

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

3.1.2.1.3 Effect of Hypertension

ARE and GST activities of hypertensive and normotensive subjects are compared in Table 3.8.

Table 3.8 Human Serum ARE and GST in Hypertensive and Normotensive Subjects.

Enzyme Activity	Hypertensive (n=150)	Normotensive (n=127)	*P*
ARE (U/mL)	110 ± 32	111.6 ± 33.6	0.694
GST (U/L)	11 ± 3.8	10.4 ± 4.7	0.239

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

Hypertensives had insignificantly lower ARE activities (110 ± 32 U/mL) than those of the normotensive subjects (111.6 ± 33.6 U/mL). GST activities of hypertensives (11 ± 3.8 U/L) were higher than those of the normotensive subjects (10.4 ± 4.7 U/L).

Comparison of ARE and GST enzyme activities of patients and controls conducted separately in the hypertensive and normotensive groups is given in Table 3.9. In hypertensives, ARE activity of stroke patients and controls were found to be very close. In normotensives, however, patients had slightly lower arylesterase activities than controls. In addition, GST activity of stroke patients and controls were very close in both hypertensive and normotensive groups. However, all results were found to be statistically insignificant.

Table 3.9 Human Serum ARE and GST Activities in Stroke Patients and Controls Compared by Being Hypertensive or Normotensive.

Enzyme Activity	Hypertensive (n=150)			Normotensive (n=127)		
	Stroke patients (n=105)	Controls (n=45)	*P*	Stroke patients (n=67)	Controls (n=60)	*P*
ARE (U/mL)	110.3 ± 32.7	109.5 ± 30.8	0.881	107.2 ± 32.4	116.5 ± 34.6	0.123
GST(U/L)	11.2 ± 4.0	10.6 ± 3.7	0.386	10.3 ± 5.0	10.4 ± 4.5	0.947

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

3.1.2.1.4 Effect of Diabetes

Study population was analyzed considering the disease diabetes. ARE and GST enzyme activities of diabetic and non-diabetic subjects among the patients and controls were also compared. As can be seen in Table 3.10, arylesterase and glutathione S-transferase activities of diabetic subjects were insignificantly lower than those of the non-diabetic subjects.

Table 3.10 Human Serum ARE and GST Activities in Diabetic and Non-diabetic Subjects.

Enzyme Activity	Diabetic (n=68)	Non-diabetic (n=209)	*P*
ARE (U/mL)	107.5 ± 35.8	111.8 ± 31.7	0.343
GST (U/L)	10.1 ± 3.7	11 ± 4.4	0.154

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

ARE and GST enzyme activity comparisons between stroke patients and controls were conducted in diabetic and non-diabetic subjects separately. As given in Table 3.11, in diabetics, ARE activity was insignificantly lower in patients (105.2 ± 33.4 U/mL) than controls (114 ± 41.9 U/mL; *P*=0.374). GST activity of diabetic patients (10.1 ± 3.6 U/L) was higher than GST activity of diabetic controls (9.9 ± 4.3 U/L, *P*=0.871).

Table 3.11 Human Serum ARE and GST Activities in Stroke Patients and Controls Compared by Being Diabetic or Nondiabetic.

Enzyme Activity	**Diabetic (n=68)**			**Non-diabetic (n=209)**		
	Stroke patients (n=50)	Controls (n=18)	*P*	Stroke patients (n=122)	Controls (n=87)	*P*
ARE (U/mL)	105.2 ± 33.4	114 ± 41.9	0.374	110.7 ± 32.1	113.4 ± 31.2	0.556
GST(U/L)	10.1 ± 3.6	9.9 ± 4.3	0.871	11.1 ± 4.6	10.6 ± 4.2	0.364

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

Diabetic stroke patients had lower arylesterase (105.2 ± 33.4 U/mL) and glutathione S-transferase (10.1 ± 3.6 U/L) activities than arylesterase (110.7 ± 32.1 U/mL) and glutathione S-transferase activities (11.1 ± 4.6 U/L) of non-diabetic stroke patients. In diabetic controls, ARE activity was slightly higher than non-diabetic controls. On the other hand, diabetic controls had lower GST activity (9.9 ± 4.3 U/L) than GST activity (10.6 ± 4.2 U/L) of non-diabetic controls. But, all results were found to be statistically insignificant.

3.1.2.1.5 Effect of Smoking

Comparison of ARE and GST enzyme activities conducted separately in the smoker and non-smoker groups is given in Table 3.12. ARE and GST activities were lower in smokers than in non-smokers.

Table 3.12 Human Serum ARE and GST Activities in Smokers and Non-smokers.

Enzyme Activity	Smoker (n=53)	Non-smoker (n=224)	*P*
ARE (U/mL)	105.2 ± 27.5	112.1 ± 33.8	0.123
GST (U/L)	10.2 ± 5.2	10.8 ± 4.1	0.440

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

ARE activities of stroke patients were lower when compared to those of controls, both in smokers and non-smokers (Table 3.13). Smoker stroke patients had higher GST activity (11 ± 4.6 U/L) than smoker control group (8.3 ± 6.3 U/L, P=0.167). On the other hand, the GST activity of non-smoker patients (10.8 ± 4.3 U/L) was same as the GST activity of non-smoker controls (10.8 ± 3.7 U/L, P=0.980) .

Table 3.13 Human Serum ARE and GST Activities in Stroke Patients and Controls Compared by Being Smoker or Non-smoker.

Enzyme Activity	Smoker (n=53)			Non-smoker (n=224)		
	Stroke patients (n=39)	Controls (n=14)	*P*	Stroke patients (n=133)	Controls (n=91)	*P*
ARE (U/mL)	101.7 ± 27.7	115.1 ± 25.1	0.118	111.3 ± 33.6	113.2 ± 34.2	0.676
GST (U/L)	11 ± 4.6	8.3 ± 6.3	0.167	10.8 ± 4.3	10.8 ± 3.7	0.980

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

As given in Table 3.13, ARE activity of smoker stroke patients was lower than non-smoker stroke patients. However ARE activity of smoker control group was higher than ARE activity of the non-smoker control group. In addition, GST activity of the smoker patients was found to be higher as compared to non-smoker patients. On the contrary, GST activity of the smoker controls was lower than GST activity of the non-smoker controls. All results were found to be statistically insignificant.

In the present study, the relation between human serum ARE and GST activity levels and ischemic stroke risk was examined. We observed that, ARE activity of the patients was lower than that of controls. In addition, HDL level was significantly lower in ischemic stroke patients compared to control subjects. Serum PON1 (arylesterase) is carried in circulation as bound to HDL particles. So, there is a significant correlation between human serum ARE and HDL particles. In recent years, it was shown that serum PON1 (ARE) activity was found to be reduced in patients with diseases such as myocardial infarction, high grade gliomas and meningiomas, diabetes mellitus and

hypercholesterolaemia in comparison to healthy subjects (McElveen *et al*., 1986, Mackness *et al*., 1991, Kafadar *et al*., 2005). Sudha *et al*., 2004, have reported that erythrocyte membrane lipid peroxidation was significantly elevated in thrombotic and hemorrhagic stroke patients and the levels of antioxidant enzymes, glutathione reductase and superoxide dismutase, were also significantly reduced in these patients compared to controls. GST is also known as an important antioxidant enzyme and involved in the the process of detoxification of toxic products generated as a result of oxidative damage (Esterbauer, 1982, Beckett and Hayes, 1993, Kaur and Bansal, 2003). However, the results obtained in this study showed that GST activity was insignificantly higher in the patient group when compared to control group. So we can say that there are no changes in the levels of GST activity of stroke patients and controls due to the role of other antioxidants although not investigated in this study. Superoxide dismutase, catalase, glutathione, vitamins E and C are known as important antioxidants and may prevent any changes in the levels of GST by taking charge. In addition, this study was designed to determine only the total GST activity in subjects and no difference was found between control group and stroke patient group. However it was known that GST has a number of isozymes with different functions, therefore further studies are needed to clarify the contribution of each isozyme to total GST and moreover the role of each isozyme in stroke risk. Besides, other antioxidant such as glutathione peroxidase, superoxide dismutase and catalase activity in patients and controls may be studied.

The risk factors of ischemic stroke such as hypertension, diabetes and smoking were also examined in this study. Arylesterase activities in hypertensives were found to be lower than normotensives. In addition, arylesterase activities in diabetics were found to be decreased when compared to nondiabetic individuals. These results were in accordance with studies that have reported lower paraoxonase activities in subjects with diabetes (Abbott *et*

al.,1995, Mackness *et al*., 2002). Similarly, reduced arylesterase activities were found in hypertensives and smokers as compared to normotensive and nonsmoker individuals, respectively. Nishio and Watanabe, 1997, have shown that paraoxonase activity was inhibited by cigarette smoke extract.

3.2 Characterization of Human Serum Arylesterase Enzyme Activity

3.2.1 Effect of pH on Human Serum ARE Activity

The effect of changing pH on ARE activity is shown in Figure 3.1. The pH of the reaction mixture was varied between 5 and 10 in the presence of acetate, tris-HCl and glycine buffers. The maximal activity of ARE was obtained at pH 8.0 and after that all the ARE activity measurements in blood serum samples obtained from ischemic stroke patients and control group were carried out by using 10 mM Tris-HCl buffer pH 8.0.

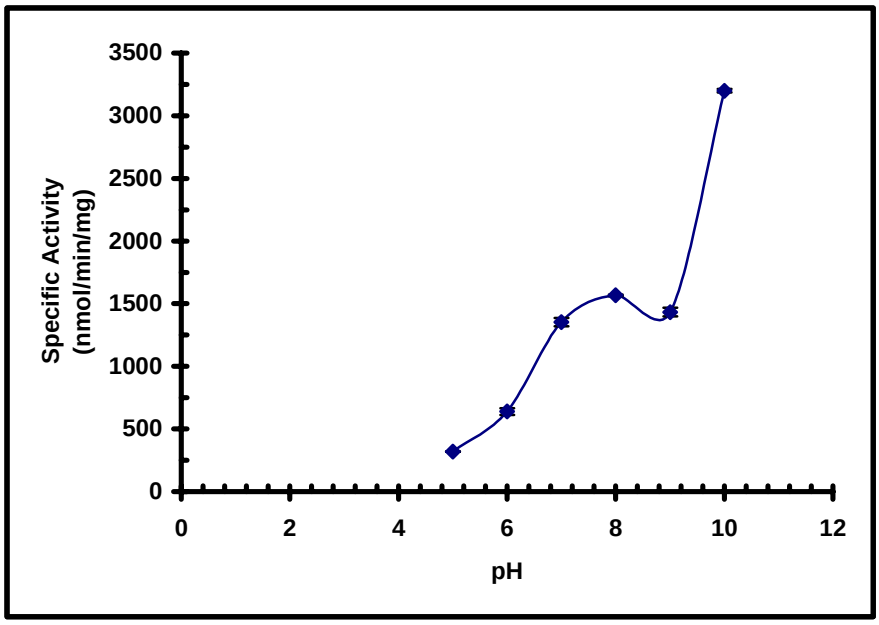

Figure 3.1 The effect of pH on human serum ARE activity. The reactions were carried out at the indicated pH values, using 10 mM acetic acid-Na acetate buffer for pH 5.0 and pH 6.0; 10 mM Tris-HCl buffer for pH 7.0, pH 8.0 and pH 9.0; 10 mM glycine buffer for pH 10.0. The other conditions were the same as described under "Methods". The points are the means of duplicate measurements.

3.2.2 Effect of Temperature on Human Serum ARE Activity

The effect of reaction temperature on ARE activity was determined by incubating the reaction mixture at seven different temperatures namely 20 C°, 25 C°, 30 C°, 37 C°, 45 C°, 50 C°, 60 C°. Maximum ARE activity was observed at 45 C°. However, throughout this study human serum ARE activity measurements of patients and control subjects were carried out at 37 C°. Effect of the temperature on ARE activity is shown in Figure 3.2.

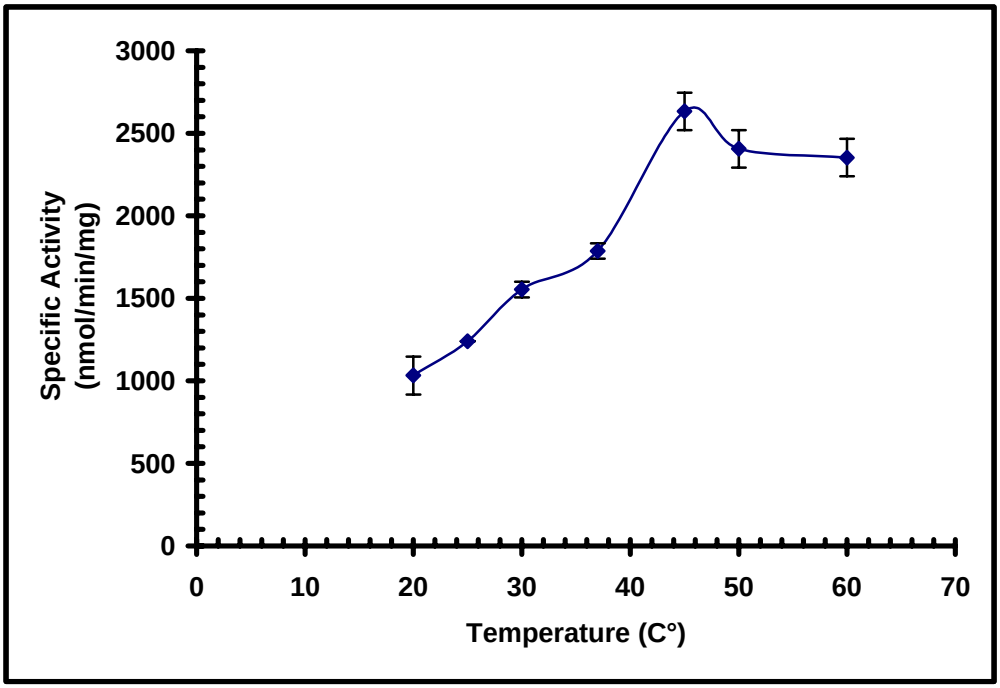

Figure 3.2 The effect of reaction temperature on human serum ARE activity. The reaction mixture contained 9 mM Tris-HCl buffer (pH 8.0), 0.9 mM $CaCl_2$, 20 µl 1/50 diluted human serum, 3.6 mM phenylacetate and 126 µl distilled water in a final volume of 3.0 ml. The assay mixture was warmed to the indicated temperature in a spectrophotometric cuvette and the endogenous rate was recorded. Reactions were initiated by the addition of phenylacetate to the cuvette and the increase in absorbance at 270 nm was recorded for 5 minutes. The points are the means of duplicate measurements.

3.2.3 Effect of Substrate Concentration on Human Serum ARE Activity

The effect of substrate concentration on ARE activity was measured by changing phenylacetate concentrations between 1 mM and 25 mM in the 3 ml reaction mixture. The effect of substrate concentration is shown in Figure 3.3 and as it seen from the hyperbolic curve, at concentration of approximately

20 mM phenylacetate, ARE was fully saturated by substrate. Therefore, 3.6 mM phenylacetate concentration was used throughout this study for measurement of ARE activity of blood serum samples obtained from human subjects. In addition, Km and Vmax kinetic constants of human serum ARE were calculated for phenylacetate reaction by constructing Lineweaver-Burk plot (Figure 3.4). According to Lineweaver-Burk plot, Km and Vmax values of ARE were calculated as 1.66 mM and 3300 nmol/min/mg, respectively.

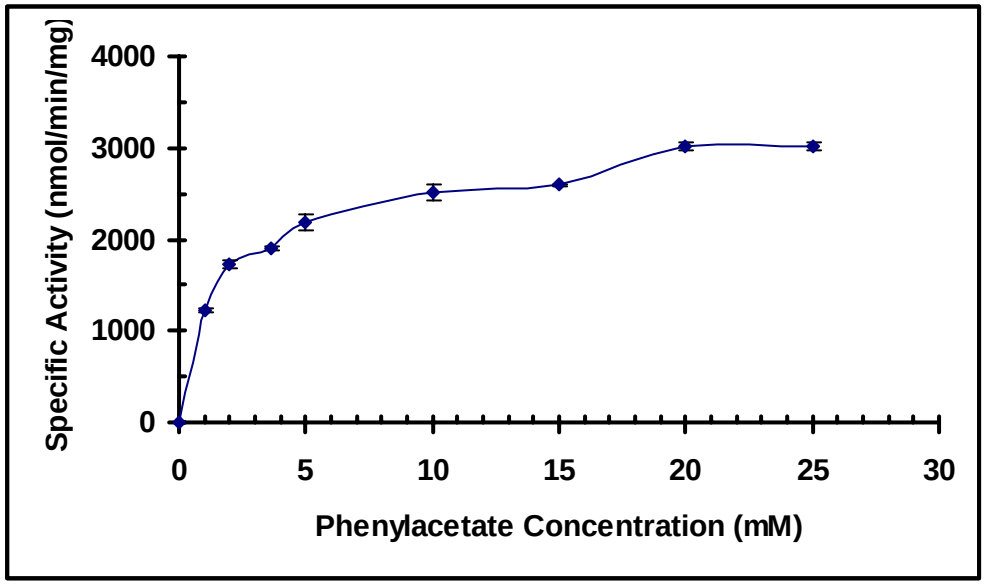

Figure 3.3 The effect of phenylacetate concentration on human serum ARE activity. Eight different concentrations of phenylacetate, changing from 1.0 to 25 mM were used in the reaction medium. The reactions were followed at 270 nm for 5 minutes at 37 °C.

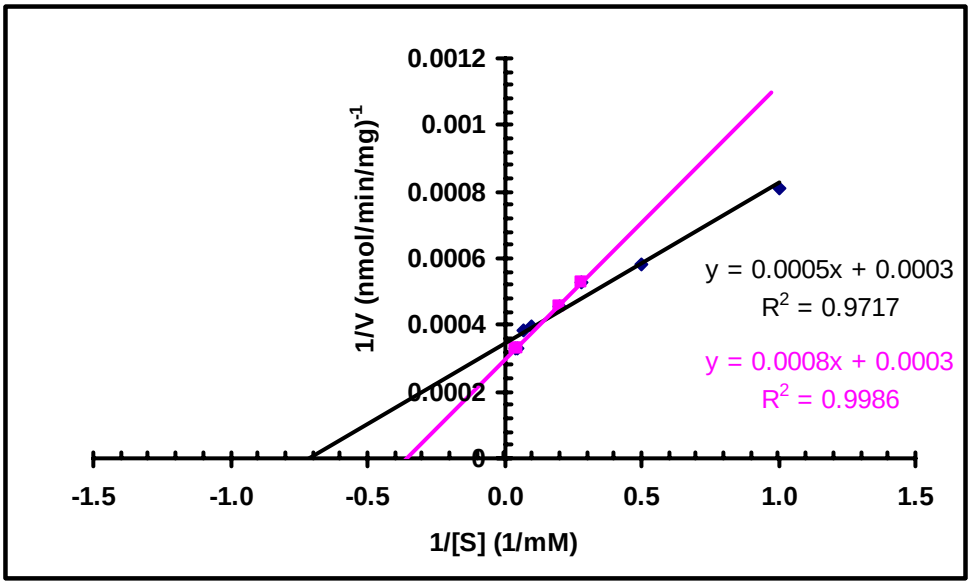

Figure 3.4 Lineweaver-Burk plot for human serum ARE. The conditions were identical to those given in the legend of Figure 3.2.

A summary of the properties of human serum arylesterase (ARE) is given in Table 3.14.

Table 3.14 A Summary of the Properties of Human Blood Serum ARE.

Optimum Temperature	45 °C
Optimum pH	8.0
K_m 1	1.66 mM
V_{max} 1	3300 nmol/min/mg
K_m 2	2.66 mM
V_{max} 2	3300 nmol/min/mg

3.3 Characterization of Human Serum Glutathione S-Transferase Enzyme Activity

3.3.1 Effect of pH on Human Serum GST Activity

The effect of changing pH on GST activity is shown in Figure 3.5. The pH of the reaction mixture was varied between 3 and 9 in the presence of acetate and potassium phosphate buffers. The maximal activity of GST was obtained at pH 5.5 and after that all the GST activity measurements in blood serum samples obtained from ischemic stroke patients and control group were carried out by using 2 mM potassium phosphate buffer, pH 5.5. Habdous *et al*., 2002, optimum pH of human serum GST activity measurement were found to be pH 5.5. At pH 4.0, pH 7.0 and pH 9.0 the human serum GST activity was not followed.

3.3.2 Effect of Temperature on Human Serum GST Activity

The effect of reaction temperature on GST activity was determined by incubating the reaction mixture at 10 different temperatures namely 5 C°, 10 C°, 15 C°, 20 C°, 30 C°, 37 C°, 45 C°, 55 C°, 65 C°, 75 C°. Maximum GST activity was observed at 65 C°. Human serum GST enzyme activity was completely lost at 75 °C due to enzyme denaturation. Habdous *et al.,* 2002, have reported the optimum temperature for human serum GST activity measurement as 37 °C.

Therefore, throughout this study human serum GST activity measurements of patients and control subjects were carried out at 37 C°. Effect of the temperature on GST activity is shown in Figure 3.6.

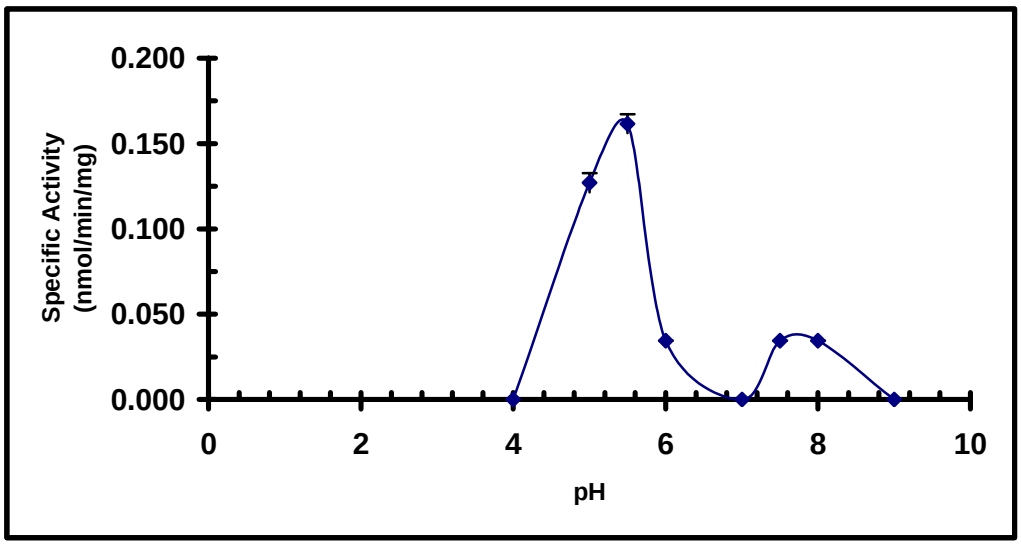

Figure 3.5 The effect of pH on human serum GST activity. The reactions were carried out at the indicated pH values, using 0.2 M acetic acid-Na acetate buffer for pH 4.0; 0.2 M potassium phosphate buffer for pH 5.0, pH 5.5, pH 6.0, pH 7.0, pH 7.5, pH 8.0 and pH 9.0. The other conditions were the same as described under "Materials and Methods". The points are the means of duplicate measurements.

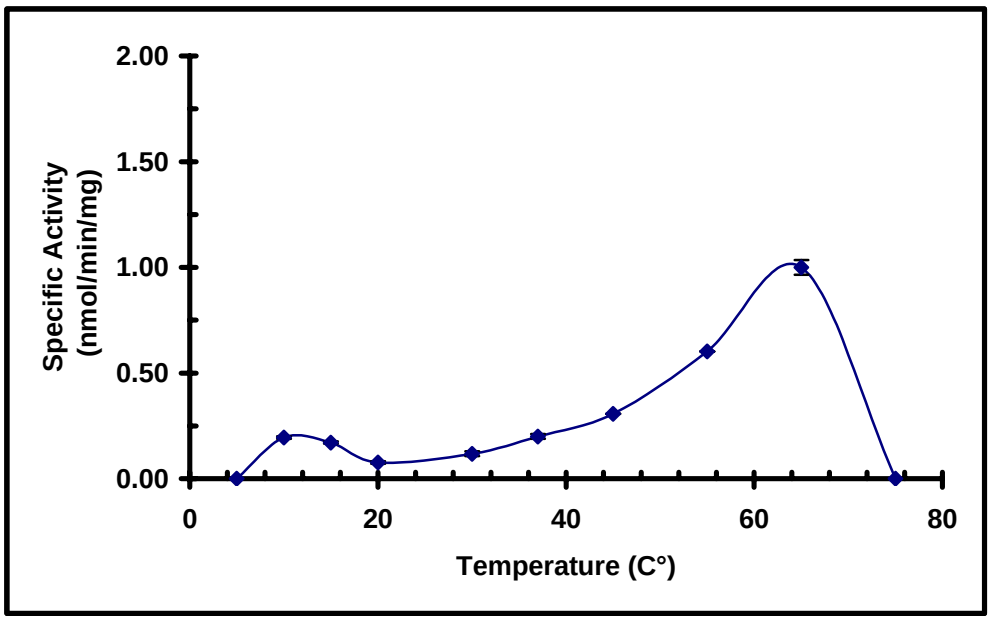

Figure 3.6 The effect of reaction temperature on human serum GST activity. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 5.5), 2.5 mM CDNB, 5.0 mM GSH, 100 µl human serum, and 200 µl distilled water in a final volume of 1.0 ml. The assay mixture was warmed to the indicated temperature in a spectrophotometric cuvette and the endogenous rate was recorded. Reactions were initiated by the addition of human serum to the cuvette and the increase in absorbance at 340 nm was recorded for 5 minutes. The points are the means of duplicate measurements.

3.3.3 Effect of Substrate Concentration on Human Serum GST Activity

3.3.3.1 Reduced Glutathione (GSH) as a Substrate

The effect of substrate concentration on GST activity was measured by changing reduced glutathione concentrations between 0.5 mM and 10 mM in the 1 ml reaction mixture. The effect of GSH concentration is shown in Figure 3.7 and as it is seen from the hyperbolic curve, at concentration of

approximately 8 mM reduced glutathione, GST was fully saturated by substrate. Therefore, 5 mM GSH concentration was used throughout this study for the measurement of GST activity of blood serum samples obtained from human subjects. In addition, Km and Vmax kinetic constants of human serum GST were calculated for GSH reaction by constructing Lineweaver-Burk plot (Figure 3.8). According to Lineweaver–Burk plot, Km and Vmax values of GST were calculated as 4.11 mM and 0.23 nmol/min/mg, respectively.

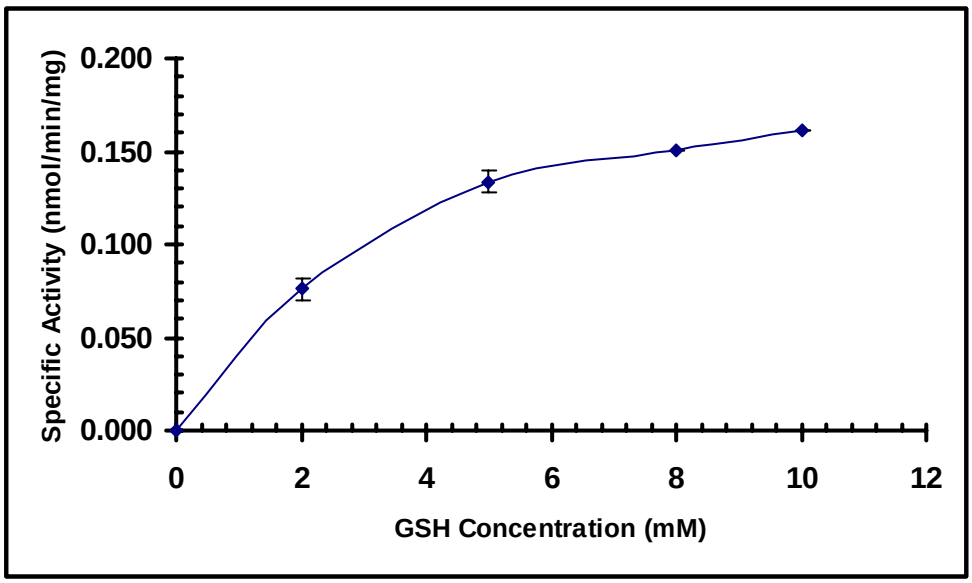

Figure 3.7 The effect of GSH concentration on human serum GST activity. Six different concentrations of GSH, changing from 0.5 to 10 mM were used in the reaction medium. The reactions were followed at 340 nm for 5 minutes at 37 °C. The points are the means of duplicate measurements.

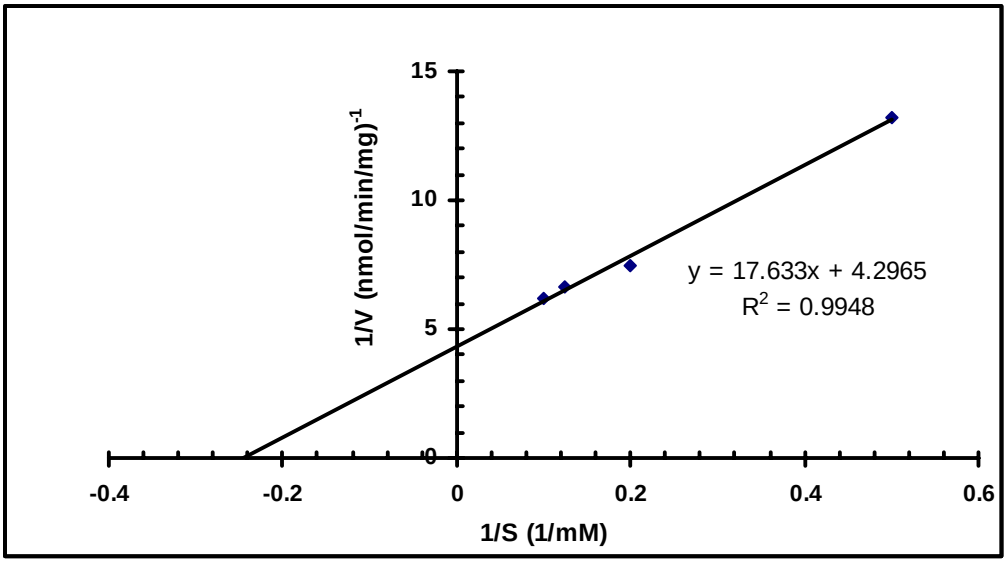

Figure 3.8 Lineweaver-Burk plot of human serum GST. The conditions were identical to those given in the legend of Figure 3.6.

3.3.3.2 1-Chloro-2,4- dinitrobenzene (CDNB) as a Substrate

The effect of substrate concentration on GST activity was measured by changing CDNB concentrations between 0.1 mM and 5 mM in the 1 ml reaction mixture. The effect of CDNB concentration is shown in Figure 3.9 and it is seen from the curve, at concentration of approximately 4.5 mM CDNB, GST was fully saturated by substrate. Therefore, 2.5 mM CDNB concentration was used throughout this study for measurement of GST activity of blood serum samples obtained from human subjects. In addition, Km and Vmax kinetic constants of human serum GST were calculated for CDNB reaction by constructing Lineweaver-Burk plot (Figure 3.10). According to Lineweaver–Burk plot, Km and Vmax values of GST were calculated as 2.8 mM and 0.43 nmol/min/mg, respectively.

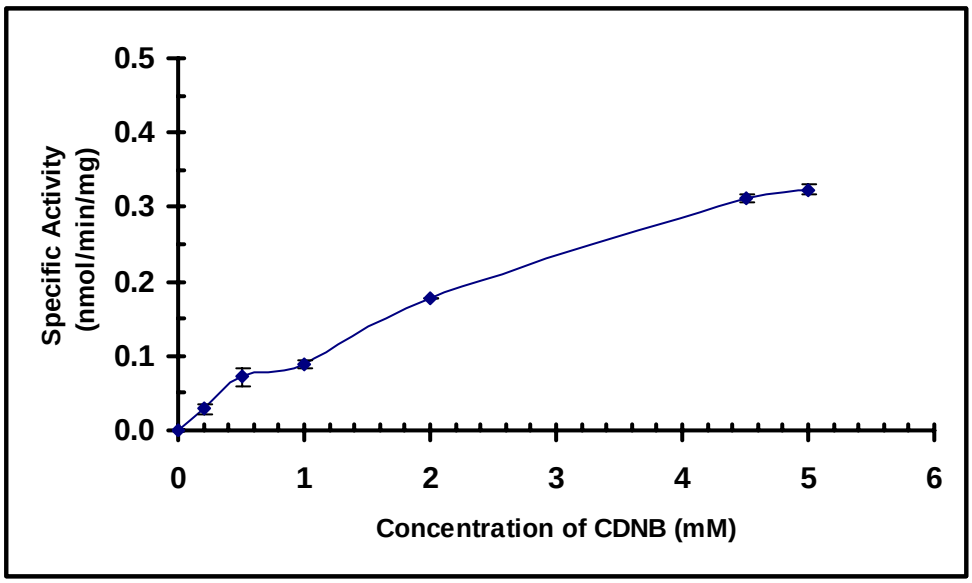

Figure 3.9 The effect of CDNB concentration on human serum GST activity. Seven different concentrations of CDNB, changing from 0.1 to 5 mM were used in the reaction medium. The reactions were followed at 340 nm for 5 minutes at 37 °C. The points are the means of duplicate measurements.

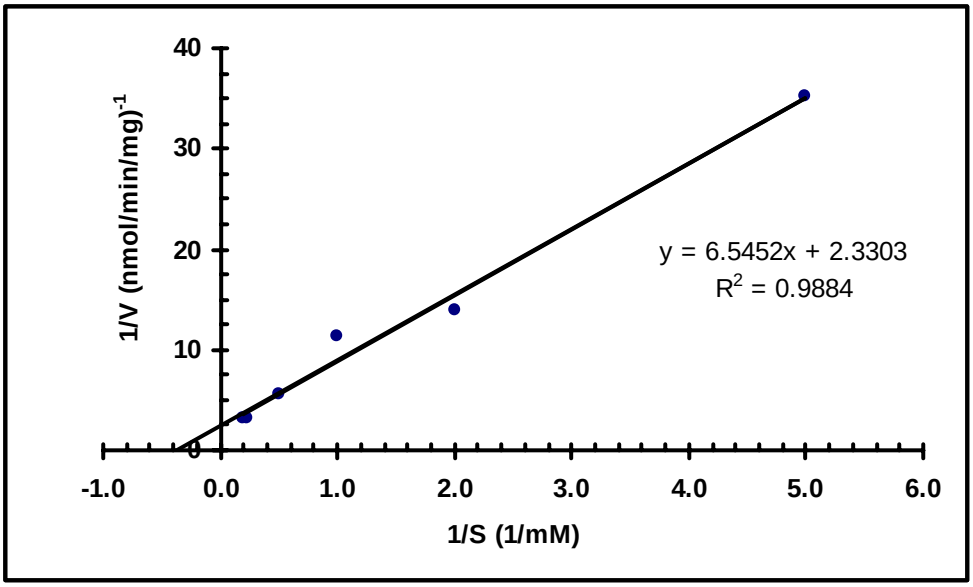

Figure 3.10 Lineweaver-Burk plot of human serum GST. The conditions were identical to those given in the legend of Figure 3.6.

A summary of the properties of human serum GST is given in Table 3.15.

Table 3.15 A Summary of the Properties of Human Blood Serum GST.

Optimum Temperature	65 °C
Optimum pH	5.5
K_m for CDNB	2.8 mM
V_{max} for CDNB	0.43 nmol/min/mg
K_m for GSH	4.11 mM
V_{max} for GSH	0.23 nmol/min/mg

3.4 Effect of Metals on Human Serum Arylesterase Activity

Metals are the oldest toxins known to human. Cellular targets of heavy metals are mostly specific biochemical processes and membrane of cells and organelles. Mercury is one of the most toxic heavy metal in the environment. It is mostly used in the industry for chlor-alkali production and in the manufacture of electrical apparatus. Mercurials are attracted to sulfhydryl groups in the body and are bound to proteins, onto membranes, and into enzymes, altering their normal functions (Altindag et al., 2003). Another heavy metal ion, cadmium, is also toxic when inhaled or digested. Cadmium poisoning associated with industrial exposure, ingestion of contaminated shellfish, or drinking acidic beverages from contaminated vessels. Nickel is a constituent of the atmosphere as a result of fosil fuel combustion. It is very toxic when inhaled. All the organisms are susceptible to heavy metal exposure since heavy metals can easily enter water supplies through industrial and consumer waste, or even from acidic rain. Therefore, it is important to understand the effects of heavy metal ions on enzyme activities.

3.4.1 Effect of $CdCl_2$ on human serumARE activity

The effect of $CdCl_2$ on ARE activity was measured by changing $CdCl_2$ concentrations between 1 μM and 100 μM in the 3 ml reaction mixture. The effect of $CdCl_2$ concentration is shown in Figure 3.11 and as it is seen from the figure, half maximal inhibitory concentration of $CdCl_2$ was found to be 4 μM.

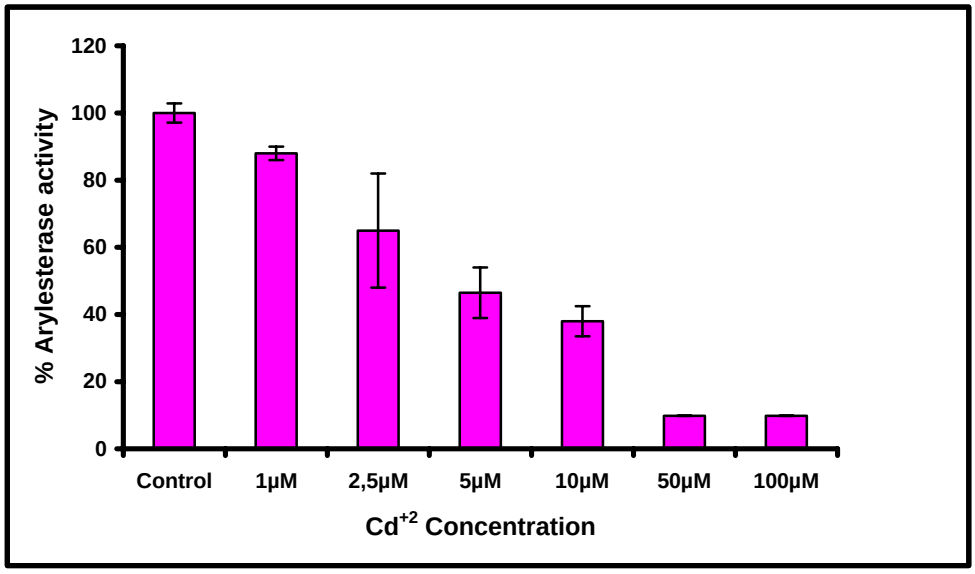

Figure 3.11 The effect of $CdCl_2$ concentration on human serum ARE activity. Six different concentrations of $CdCl_2$, changing from 1.0 to 100 μM were used in the reaction medium. The reactions were followed at 270 nm for 5 minutes at 37 °C.

3.4.2 Effect of $HgCl_2$ on human serum ARE activity

The effect of $HgCl_2$ on ARE activity was measured by changing $HgCl_2$ concentrations between 2.5 µM and 100 µM in the 3 ml reaction mixture. The effect of $HgCl_2$ concentration is shown in Figure 3.12 and as it is seen from the figure, half maximal inhibitory concentration of $HgCl_2$ was calculated as 4.7 µM.

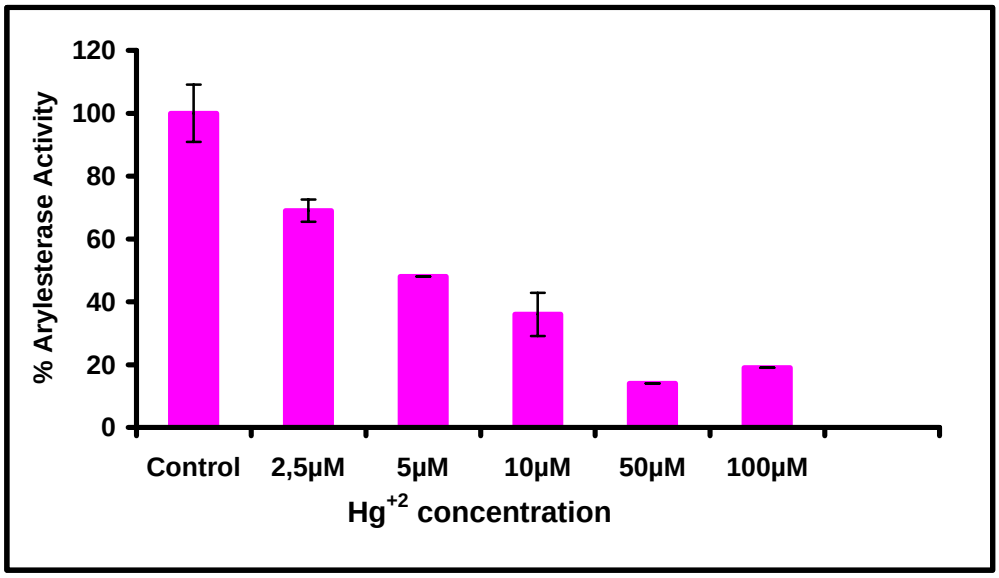

Figure 3.12 The effect of $HgCl_2$ concentration on human serum ARE activity. Five different concentrations of $HgCl_2$, changing from 2.5 µM to 100 µM were used in the reaction medium. The reactions were followed at 270 nm for 5 minutes at 37 °C.

3.4.3 Effect of $NiCl_2$ on human serum ARE activity

The effect of $NiCl_2$ on ARE activity was measured by changing $NiCl_2$ concentrations between 10 μM and 10 mM in the 3 ml reaction mixture. The effect of $NiCl_2$ concentration is shown in Figure 3.13 and as it is seen from the figure, half maximal inhibitory concentration of $NiCl_2$ was found to be 57 μM.

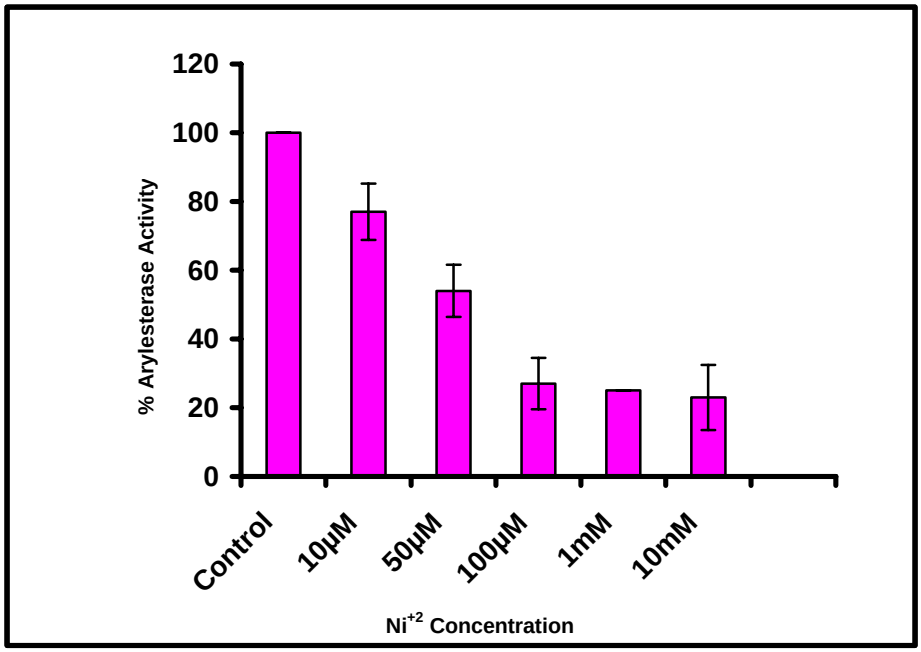

Figure 3.13 The effect of $NiCl_2$ concentration on human serum ARE activity. Five different concentrations of $NiCl_2$, changing from 10μM to 10 mM were used in the reaction medium. The reactions were followed at 270 nm for 5 minutes at 37 °C.

A summary of the half maximal inhibitory concentrations of Cd^{+2}, Hg^{+2} and Ni^{+2} for human serum ARE is given in Table 3.16.

Table 3.16 A Summary of the Half Maximal Inhibitory Concentrations of Cd^{+2}, Hg^{+2} and Ni^{+2} for Human Serum ARE and GST.

Heavy metal ions	IC_{50} values for ARE	IC_{50} values for GST
Cd^{+2}	4 μM	30 μM
Hg^{+2}	4.7 μM	73 μM
Ni^{+2}	57 μM	42 μM

3.5 Effect of Metals on Human Serum Glutathione S- Transferase Activity

3.5.1 Effect of $CdCl_2$ on human serum GST activity

The effect of $CdCl_2$ on GST activity was measured by changing $CdCl_2$ concentrations between 10 μM and 1 mM in the 1 ml reaction mixture. The effect of $CdCl_2$ concentration is shown in Figure 3.14 and as it is seen from the figure, half maximal inhibitory concentration of $CdCl_2$ was found to be 30 μM.

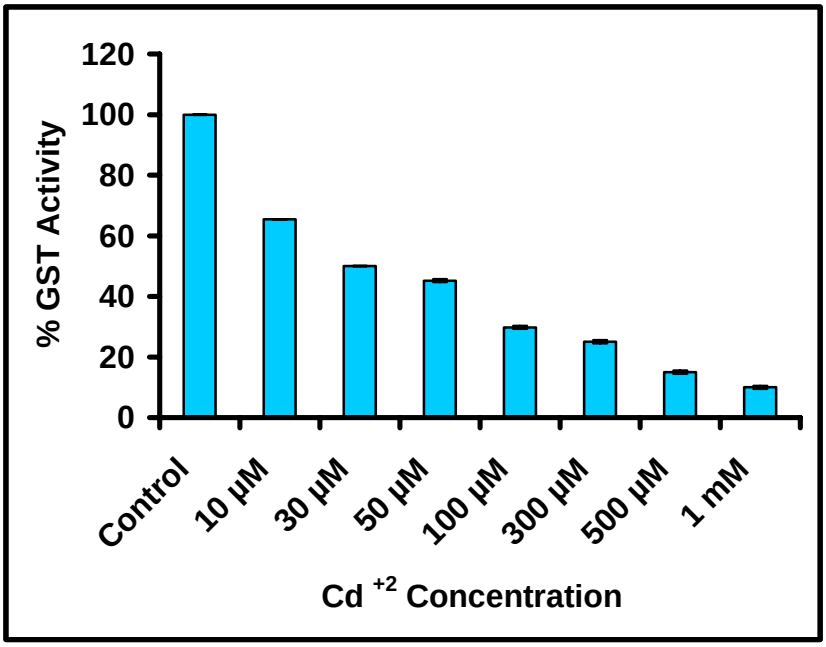

Figure 3.14 The effect of $CdCl_2$ concentration human serum GST activity. Seven different concentrations of $CdCl_2$, changing from 10 µM to 1.0 mM were used in the reaction medium. The reactions were followed at 340 nm for 5 minutes at 37 °C.

3.5.2 Effect of $HgCl_2$ on human serum GST activity

The effect of $HgCl_2$ on GST activity was measured by changing $HgCl_2$ concentrations between 10 µM and 1 mM in the 1 ml reaction mixture. The effect of $HgCl_2$ concentration is shown in Figure 3.15 and as it is seen from the figure, half maximal inhibitory concentration of $HgCl_2$ was calculated as 73 µM.

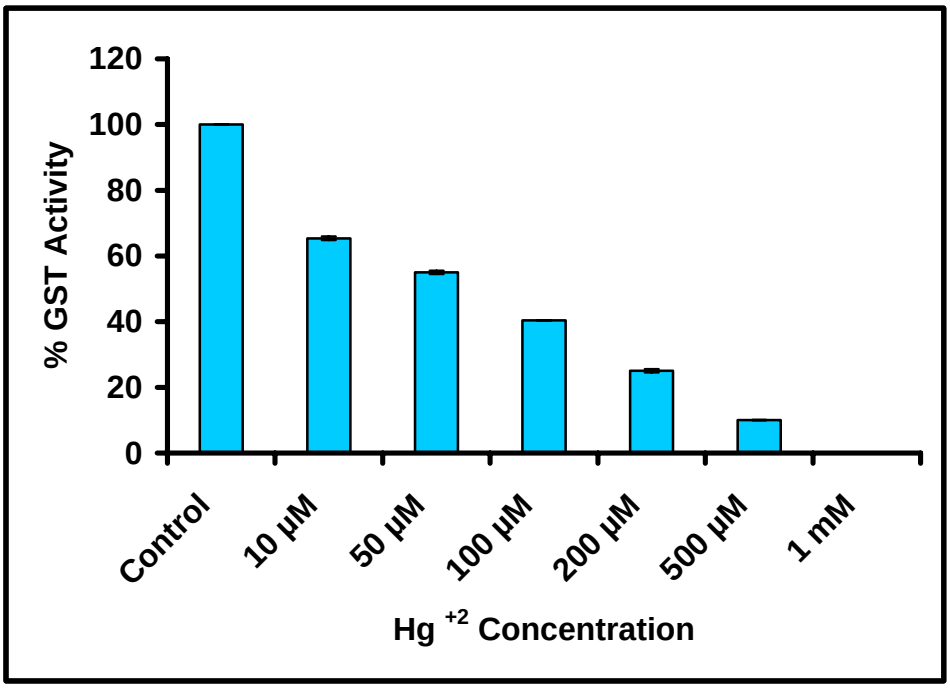

Figure 3.15 The effect of $HgCl_2$ concentration human serum GST activity. Six different concentrations of $HgCl_2$, changing from 10 µM to 1.0 mM were used in the reaction medium. The reactions were followed at 340 nm for 5 minutes at 37 °C.

3.5.3 Effect of $NiCl_2$ on human serum GST activity

The effect of $NiCl_2$ on GST activity was measured by changing $NiCl_2$ concentrations between 10 µM and 10 mM in the 1 ml reaction mixture. The effect of $NiCl_2$ concentration is shown in Figure 3.16 and as it is seen from the figure, half maximal inhibitory concentration of $NiCl_2$ was found to be 42 µM.

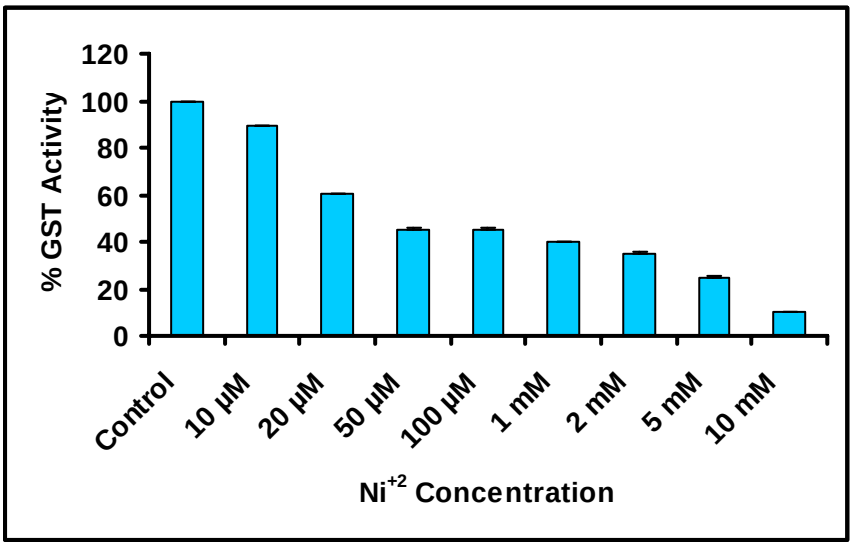

Figure 3.16 The effect of $NiCl_2$ concentration human serum GST activity. Eight different concentrations of $NiCl_2$, changing from 10 µM to 10 mM were used in the reaction medium. The reactions were followed at 340 nm for 5 minutes at 37 °C.

A summary of the half maximal inhibitory concentrations of Cd^{+2}, Hg^{+2} and Ni^{+2} for human serum GST is given previously in Table 3.16.

According to the results of metal effect on ARE and GST enzyme activities obtained in this study, the order of inhibiting potency of metals for ARE was $Cd^{+2} > Hg^{+2} > Ni^{+2}$ and for GST was $Cd^{+2} > Ni^{+2} > Hg^{+2}$. A number of studies regarding the potential effect of many metal ions on enzyme activities have been found (Güner and Colak, 1996, Can Demirdöğen and Adalı, 2005, Bozcaarmutlu and Arinç, 2007). This study revealed the inhibitory potency of Cd^{+2} , Hg^{+2} and Ni^{+2} for ARE similar to findings of Debord *et al*., 2002. In the present study, cadmium exhibited much higher inhibitory effect on both human serum ARE and GST activities. The inhibitor concentration providing 50% inhibition of initial ARE activity by Cd^{+2} occured at 4 µM concentration. However, GST has a half maximal inhibitory

concentration of 30 μM for Cd^{+2} which was 7.5 fold higher than half maximal inhibitory concentration of Cd^{+2} for ARE. The present study showed that Hg^{+2} was much more stronger inhibitor of ARE than Ni^{+2} . On the other hand, GST activity was inhibited much more strongly by Ni^{+2} when compared to Hg^{+2}.

CHAPTER 4

CONCLUSION

In this study, first, the biochemical properties of human blood serum arylesterase and glutathione S-transferase enzymes were characterized. Then ARE and GST activities were measured in serum samples obtained from ischemic stroke patients and control subjects. The maximum ARE enzyme activity was detected at 10 mM Tris-HCl buffer, pH 8.0 and at 45 °C. ARE was saturated with its substrate phenylacetate around 20 mM concentration. The apparent K_m and V_{max} values of human blood serum ARE for phenylacetate substrate were found as 1.66 mM and 3300 nmol/min/mg, respectively. The maximum GST enzyme activity was detected at 2 mM potassium phosphate buffer, pH 5.5 and at 65 °C. GSTs were saturated with its substrate (CDNB) around 4.5 mM concentration and with its cofactor (GSH) around 8 mM concentration. The apparent K_m and V_{max} values of human blood serum GST for CDNB substrate were found as 2.8 mM and 0.43 nmol/min/mg and for GSH were found as 4.11 mM and 0.23 nmol/min/mg, respectively.

The effect of cadmium (Cd^{+2}) on ARE and GST enzyme activities were determined and IC_{50} values were calculated as 4 μM for ARE and 30 μM for GST. The IC_{50} values for mercury (Hg^{+2}) for ARE and GST were found to be 4.7 μM and 73 μM, respectively. IC_{50} values for nickel (Ni^{+2}) came out to be 57 μM for ARE and 42 μM for GST.

In present work, human serum ARE and GST activities of both stroke patients and controls were measured. ARE activities were insignificantly lower in patient group, while GST activities were insignificantly higher in patients

when compared to controls. Statistical analysis showed hypertension, diabetes and HDL as significant predictors of stroke. In addition, ARE and GST enzyme activities in different subgroups of ischemic stroke patients and controls were analyzed and following results were obtained. In both elderly (age > 59) and younger groups (age < 60), arylesterase activity was lower in the patients than the respective control group. In elderly group, GST activity was higher in the patients than the respective control group. On the other hand, GST activity was lower in younger patient group than that of control group. In both gender group, GST activity of stroke patients was higher than those of controls. However in males group ARE activity of patients was higher than the respective control group and in females group ARE activity of patients was lower than those of control. In hypertensives, ARE activity of stroke patients and controls were very close. In normotensives, however, patients had slightly lower arylesterase activities than controls. In addition, GST activity of stroke patients and controls were very close in both hypertensive and normotensive groups. Stroke patients had lower arylesterase activity than controls in non-diabetics group. However non-diabetic stroke patients had higher glutathione S-transferase activity than those of controls. ARE activities of stroke patients were lower when compared to those of controls, both in smokers and non-smokers. Smoker stroke patients had higher GST activity than smoker control group. On the other hand, the GST activities of non-smoker patients was equal to GST activities of non-smoker controls.

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

Hastanın Beyanı

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

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

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

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

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

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

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

Katılımcı

Adı, soyadı:

Adres:

Tel:

İmza:

Görüşme tanığı

Adı, soyadı:

Adres:

Tel:

İmza:

Katılımcı ile görüşen hekim

Adı soyadı, unvanı:

Adres:

Tel:

İmza:

APPENDIX 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 3

ETHICAL COMMITTEE APPROVAL FORM

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

OTURUM NO	: 43
OTURUM TARİHİ	: 20 Eylül 2005
OTURUM BAŞKANI	: Prof. Tbp. Kd. Alb. Hamdullah AYDIN
OTURUM SEKRETERİ	: Doç. Dr. Ecz. Kd. Alb. Adnan ATAÇ

GATA Etik Kurulu'nun 20 Eylül 2005 günü yapılan 43. oturumunda, GATA Nöroloji AD'dan Prof. Dr. Okay Vural'ın sorumlu araştırıcılığını yaptığı **"Paraoksonaz 1'in Aktivite ve Gen Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** başlıklı, çok merkezli, risk faktörü ve yatkınlık çalışması olan araştırma dosyası değerlendirildi. Araştırma dosyasının amaç, yöntem ve yaklaşım bakımından etik ilkelere UYGUN olduğuna karar verildi.

BAŞKAN	ÜYE	ÜYE	ÜYE
Hamdullah AYDIN Prof.Tbp.Kd. Alb.	İsmail ARSLAN Prof. Hv. Tbp. Kd. Alb.	Okay KOZAK Prof.Tbp.Kd.Alb.	Ali Uğur URAL Prof.Tbp.Kd.Alb.
ÜYE	ÜYE	ÜYE	ÜYE
Adnan ATAÇ Doç.Dr.Ecz.Kd.Alb.	Turgay ÇELİK Doç. Tbp. Alb.	Mükerrem SAFALI Doç. Tbp. Alb.	Emin ÖZTAŞ Doç. Hv. Tbp. Bnb.

ONAY

Derviş ŞEN
Prof. Tbp. Tümgeneral
Askeri Tıp Fakültesi Dekanı
ve Eğitim Hastanesi Baştabibi

APPENDIX 4

LIST OF STUDY POPULATION

List of study population composed of 172 stroke patients and 105 controls including demographic characteristics, lipid parameters, ARE and GST activities of the study population composed of. M: male; F: female; Y: yes; N: no; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; ARE: arylesterase activity; GST: glutathione S-transferase activity.

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
1	Patient	75	M	Y	Y	Y	72	24	108	11.5
2	Patient	57	F	Y	Y	N	132	51	60	13
3	Patient	41	M	N	N	Y	104	34	74.5	10.5
4	Patient	73	M	Y	N	N	74	57	49	8.9
5	Patient	53	M	Y	Y	N	296	59	108.7	6.8
6	Patient	66	M	Y	Y	Y	85	20	80	10.9
7	Patient	84	F	Y	N	N	70	63	77	6.3
8	Patient	56	F	Y	Y	N	68	38	137.4	8.3
9	Patient	54	M	Y	N	Y	115	42	129	7.8
10	Patient	67	F	Y	N	N	139	49	103	11.8
11	Patient	76	M	Y	N	Y	91	50	123	10.4
12	Patient	78	F	Y	Y	N	79	36	94.5	11.5

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
13	Patient	75	F	Y	N	N	127	34	106	6.3
14	Patient	74	F	Y	Y	N	107	48	60	9.9
15	Patient	68	F	Y	N	Y	75	25	74	11.5
16	Patient	72	F	N	N	N	165	40	134.5	9.4
17	Patient	84	M	Y	N	N	93	31	128.5	9.4
18	Patient	81	F	Y	N	N	115	42	91.5	7.3
19	Patient	73	F	Y	Y	N	125	42	160	5.7
20	Patient	73	F	Y	N	N	107	55	156.7	7.8
21	Patient	67	F	Y	N	N	143	40	106	10.9
22	Control	69	F	N	N	N	91	74	57.3	10.4
23	Control	71	M	Y	N	N	82	42	152	9.8
24	Patient	61	M	Y	Y	N	108	37	129	9.8
25	Patient	40	M	N	N	Y	78	47	74	10.5
26	Control	61	F	Y	N	N	46	68	100	12.5
27	Patient	60	F	Y	Y	N	119	43	74	8.4
28	Patient	86	M	Y	N	Y	113	46	100	9.4
29	Patient	75	M	N	N	N	71	85	68.5	10.4
30	Patient	76	F	Y	N	N	50	46	172	9.4
31	Control	76	M	Y	Y	N	74	39	68.5	11.4
32	Control	51	M	N	N	Y	80	35	169	11.4

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
33	Control	50	F	N	N	N	73	30	133	11
34	Control	42	F	N	N	N	57	24	177.5	11.5
35	Control	45	F	N	N	Y	73	57	123	8.8
36	Patient	70	M	Y	N	N	59	42	118	8.3
37	Patient	76	M	N	N	Y	73	30	77	8.3
38	Control	63	M	Y	Y	Y	60	37	106	8.8
39	Patient	83	F	N	N	N	182	55	80	8.8
40	Control	63	F	N	N	N	123	34	112	13
41	Patient	86	F	N	N	N	150	58	130	10
42	Control	75	M	Y	Y	N	100	51	74	11.5
43	Control	58	F	Y	N	N	43	42	86	11
44	Control	78	F	Y	N	N	71	49	91.5	11
46	Control	74	M	N	N	N	100	51	43	12
47	Patient	71	M	Y	N	N	126	35	80	10.4
50	Control	61	M	N	N	N	155	40	91.5	12.5
51	Control	85	M	Y	N	N	24	63	69	11
52	Control	65	F	N	Y	N	126	84	218	7.8
53	Control	65	M	N	N	N	128	52	100	11
54	Control	58	M	N	Y	Y	169	39	100	14.5
55	Control	61	F	N	N	N	170	59	166	11

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
56	Control	80	F	Y	N	N	83	24	89	12
57	Control	80	F	N	N	N	46	71	94	12
58	Control	67	F	Y	Y	N	85	41	77	12
59	Control	76	M	N	N	N	143	47	112	14
61	Patient	61	M	N	Y	N	76	43	66	6.3
62	Control	69	F	Y	N	N	74	46	166	13.5
63	Control	66	M	N	Y	Y	160	38	94	0
64	Control	60	F	Y	N	N	121	45	140	10.4
66	Patient	64	F	N	N	Y	99	48	77	18.7
67	Patient	58	F	Y	Y	N	228	50	120	5.2
68	Patient	74	F	Y	N	N	162	48	154.5	11.4
69	Control	71	M	N	N	N	80	55	109	12
71	Patient	80	F	Y	N	N	90	38	103	15
72	Patient	62	M	Y	Y	N	128	39	115	12.5
73	Control	68	M	N	N	N	123	64	120	13.5
74	Control	65	F	N	N	N	123	46	146	11
76	Control	72	F	Y	N	N	158	46	114.5	5.8
77	Control	65	M	N	N	N	64	37	83	7.3
78	Control	63	F	Y	Y	N	66	43	138	7.3
79	Control	70	F	N	N	N	118	43	132	7.8

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List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
80	Control	70	F	Y	N	N	130	51	86	11.4
81	Control	65	F	Y	N	N	147	51	140	11.4
82	Patient	63	M	Y	N	N	72	41	169	12
83	Control	65	F	Y	N	N	85	66	163	9.4
84	Control	78	F	Y	N	N	93	54	177	10.4
85	Patient	68	M	Y	N	N	95	60	100	4.2
86	Control	47	M	N	N	N	130	57	140	11.4
87	Patient	77	F	Y	N	N	137	39	183	14.5
88	Control	77	F	Y	Y	N	103	36	114.5	10.4
89	Patient	80	F	Y	N	N	120	62	166	11
90	Control	71	M	N	N	N	90	52	140	4.2
91	Patient	55	M	Y	N	N	95	37	89	6.3
92	Control	73	M	Y	N	N	76	20	137	11
93	Control	61	M	N	N	Y	114	59	117	0
94	Control	37	M	N	Y	N	110	50	123	11.4
95	Patient	62	M	N	N	N	54	43	95	5.7
96	Patient	77	M	N	N	N	68	68	97	9.4
97	Patient	24	M	N	N	N	143	41	152	7.8
98	Patient	53	F	N	N	Y	23	35	114.5	7.8
99	Patient	61	M	N	N	N	121	37	155	12

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
101	Control	52	M	N	N	N	183	63	103	4.7
102	Patient	78	M	Y	N	Y	120	44	77	11.5
103	Control	65	M	N	N	N	113	32	123	13
105	Patient	81	M	Y	N	N	37	42	69	0
106	Patient	80	F	Y	N	N	57	25	172	11
107	Control	50	F	Y	N	N	84	24	118	4.7
108	Control	87	F	Y	N	N	54	34	175	13
109	Control	67	M	N	N	N	82	6	143	11.4
110	Patient	84	M	Y	N	N	74	49	120	11.4
111	Control	38	M	N	N	Y	111	63	137	11
112	Control	50	M	N	N	N	79	57	152	6
113	Patient	75	F	Y	N	N	52	46	66	12
114	Patient	26	M	N	N	N	80	63	126	12
115	Patient	55	M	Y	N	N	123	54	112	9
116	Patient	26	M	N	N	N	114	29	149	0
117	Patient	73	F	Y	N	N	99	35	106	14.5
119	Control	80	F	N	Y	N	77	51	206	12.5
120	Patient	36	M	N	N	N	116	43	106	5.2
121	Patient	56	M	N	N	N	100	45	169	7.3
122	Patient	47	F	N	N	N	123	36	114.5	10

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List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
123	Patient	21	M	N	N	Y	46	47	135	5.8
124	Patient	81	F	Y	Y	N	69	31	72	0
125	Patient	84	M	Y	Y	N	118	48	148	8.3
126	Patient	73	M	Y	Y	N	118	35	149	7
127	Patient	73	F	Y	N	N	99	35	106	9.4
128	Patient	66	F	N	N	N	58	21	72	8.4
129	Patient	74	F	Y	N	N	154	51	108.7	12
130	Control	44	M	N	N	Y	96	62	134.5	7.3
131	Control	51	F	N	N	N	110	48	83	7.8
132	Control	67	M	Y	N	N	101	41	140	7.8
133	Control	73	F	Y	Y	N	143	43	132	7.3
134	Control	88	F	Y	N	N	100	40	77	15
135	Patient	88	F	Y	N	N	87	44	126	10.4
136	Patient	66	M	Y	Y	N	49	29	126	12.5
137	Patient	89	M	N	N	N	113	41	117	13.5
138	Patient	61	F	Y	Y	N	63	25	134.5	13.5
139	Patient	78	M	N	N	Y	70	40	63	14.5
140	Control	59	M	N	N	N	90	45	149	17.7
141	Control	69	F	N	Y	N	157	60	77	15.5
142	Patient	80	M	Y	N	N	239	50	126	17.2

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
143	Patient	76	F	Y	Y	N	98	38	137.5	10.4
144	Patient	79	F	Y	N	N	129	46	174.5	12.5
146	Control	51	F	N	N	N	54	38	158	15
147	Patient	21	M	N	N	N	100	32	135	11.5
148	Patient	76	M	N	N	N	132	40	114.5	17.7
149	Patient	68	F	Y	N	N	110	45	155	19.2
151	Patient	28	M	N	N	Y	78	44	109	9.4
152	Patient	20	M	N	N	Y	100	50	97	12
153	Patient	80	F	Y	N	N	86	57	100	11
154	Patient	64	F	Y	N	N	77	39	52	17.2
156	Patient	67	F	Y	Y	N	297	49	195	8
157	Patient	58	F	Y	Y	N	169	54	163	12.5
158	Control	77	M	N	N	N	80	46	126	11.4
159	Patient	49	M	N	N	Y	139	46	200	12.5
161	Patient	78	F	N	N	N	103	57	181	18.2
162	Patient	65	M	N	N	N	79	42	154.5	16.6
163	Patient	75	M	Y	N	N	130	37	106	14.5
164	Patient	79	M	N	N	Y	54	35	86	17.7
165	Patient	73	F	Y	Y	N	205	23	86	14
166	Patient	84	F	Y	N	N	170	72	117	17.2

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List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
167	Patient	25	M	N	N	N	107	43	69	12
168	Patient	73	M	Y	N	N	119	60	146	15.1
169	Patient	74	M	Y	N	N	70	34	140	7.3
170	Patient	78	F	Y	Y	N	109	44	89	16.6
172	Patient	56	M	Y	Y	N	105	41	114.5	10
173	Patient	67	M	Y	N	N	89	43	123	17.2
174	Patient	74	M	Y	N	N	79	50	94.5	15.6
175	Patient	64	M	Y	Y	N	98	41	134.5	6.8
176	Patient	73	M	Y	Y	N	100	43	123	5.2
177	Control	52	F	N	N	N	73	54	140	8.8
179	Patient	57	M	N	N	Y	116	41	106	11.4
180	Patient	76	M	N	N	N	94	33	123	19.2
181	Patient	61	M	N	N	Y	116	46	129	14
182	Patient	85	M	N	N	Y	31	41	92	13
184	Control	77	F	Y	N	N	79	47	92	10
185	Patient	62	F	N	Y	N	91	37	83	13.5
186	Patient	73	M	N	N	Y	204	47	112	22.3
187	Patient	63	M	N	N	Y	66	46	109	15.5
188	Patient	52	M	N	N	Y	108	31	92	0
189	Control	79	M	N	N	N	150	37	89	0

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List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
190	Patient	61	F	Y	N	N	62	34	74.4	190
191	Control	46	M	Y	N	Y	207	47	112	191
193	Patient	45	M	N	N	Y	51	33	114.5	0
194	Patient	64	M	N	N	Y	103	36	100	0
195	Patient	56	F	N	Y	N	169	44	152	0
196	Patient	67	M	Y	Y	N	171	44	137.5	12
197	Patient	53	M	Y	N	N	176	48	129	13
200	Control	36	M	N	N	N	75	32	106	16.1
201	Control	38	M	N	N	Y	100	52	109	13
202	Patient	80	M	N	N	N	146	50	75	0
203	Patient	62	M	Y	Y	N	143	46	74.4	9.4
204	Patient	83	F	Y	N	N	77	43	80	11
207	Control	41	F	N	N	N	80	40	86	16
209	Patient	80	M	N	N	N	123	50	175	6.3
210	Patient	61	M	N	N	Y	75	34	109	13
211	Patient	67	F	N	Y	N	125	38	51.5	13.5
212	Patient	64	M	N	N	Y	103	44	92	11
213	Control	48	F	N	N	N	55	28	72	5.7
214	Patient	65	F	N	N	Y	149	52	132	13
216	Patient	79	F	Y	Y	N	103	31	97	15.5

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
217	Patient	61	M	Y	N	Y	119	41	83	15.5
218	Patient	36	F	N	N	Y	148	63	166	10.4
219	Patient	80	M	Y	Y	N	173	41	109	10.4
221	Patient	61	M	N	Y	Y	84	39	51.5	6.8
222	Patient	69	M	Y	N	N	105	36	117	8.3
223	Patient	76	F	Y	Y	N	104	40	120	6.8
224	Patient	69	F	N	N	N	70	33	83	8.8
225	Control	43	F	N	N	Y	137	52	112	15.1
226	Patient	77	M	Y	Y	Y	102	31	83	8.8
227	Patient	82	M	N	N	N	80	35	66	10.4
229	Control	45	F	N	N	N	101	51	144	13
231	Control	38	F	N	N	N	139	58	112	9.4
232	Patient	78	F	Y	N	N	124	52	94.5	12.5
233	Control	64	F	Y	N	N	186	56	149	9.4
234	Patient	58	F	N	N	N	94	52	77.5	13.5
235	Control	66	M	N	N	N	135	57	63	6.8
239	Patient	53	M	N	N	Y	86	46	89	11
240	Control	42	M	N	N	Y	96	33	129	0
241	Control	65	M	Y	N	N	76	49	69	10.4
242	Control	87	M	Y	Y	N	38	44	66	10.4

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
243	Patient	87	F	Y	N	N	110	22	66	14.5
244	Control	75	F	N	N	Y	113	55	112	15.1
245	Patient	54	M	N	N	N	121	37	103	8.8
246	Control	77	M	Y	N	N	95	32	83	15.1
247	Control	69	F	N	N	N	106	49	126	8.8
248	Patient	75	F	N	Y	N	118	54	106	9.4
249	Patient	87	F	Y	N	Y	138	70	106	10.4
250	Patient	84	F	N	N	N	102	59	89	12.5
251	Patient	78	M	Y	Y	N	100	36	74.5	10.4
252	Patient	40	F	N	N	Y	127	46	97	12.5
253	Patient	48	M	Y	N	N	147	50	126	13.5
254	Patient	80	F	Y	Y	N	112	54	106	15.5
255	Patient	41	M	N	N	N	88	33	134.5	11.5
256	Patient	77	F	Y	Y	N	123	43	129	11.4
257	Patient	63	F	Y	Y	N	100	37	154.5	11
258	Patient	55	M	Y	N	Y	207	37	117	16.1
259	Patient	79	M	Y	Y	N	61	45	75	11
260	Patient	67	F	Y	Y	N	114	58	103	14
261	Patient	63	M	Y	Y	N	83	31	74.5	11.4
262	Patient	75	F	Y	N	N	152	42	160	15.6

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
263	Control	65	M	N	N	N	103	44	74.5	13.5
264	Control	78	M	N	N	N	87	55	83	11
265	Control	81	M	Y	N	N	48	49	131.5	13
266	Control	56	M	N	Y	N	110	43	103	11
267	Control	64	F	Y	Y	N	86	31	120	0
268	Control	79	F	Y	N	N	110	49	92	14
269	Control	67	F	N	N	N	228	50	132	11.5
270	Control	76	F	Y	N	N	85	59	106	7.3
271	Control	64	M	N	N	Y	86	26	57	16.6
272	Control	77	M	N	Y	N	243	63	120	12.5
273	Control	75	M	N	N	N	109	52	120	16
274	Patient	54	F	N	N	N	114	27	100	0
275	Patient	61	F	Y	N	N	126	35	143	0
276	Patient	71	M	Y	N	N	73	38	71.5	18.2
277	Control	68	F	Y	N	N	97	55	123	5.7
278	Patient	74	F	Y	N	N	123	55	126	6.3
279	Patient	59	M	N	N	N	60	23	103	16.6
280	Patient	62	M	N	N	Y	103	37	108	6.2
281	Patient	82	F	Y	Y	N	120	38	74	16.6
282	Control	71	F	Y	N	N	90	40	80	9.4

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
283	Control	52	F	N	N	N	100	40	154.5	6.2
284	Control	78	M	Y	N	N	97	37	80	17.7
285	Patient	70	M	Y	N	N	134	49	97	17.7
286	Patient	69	M	Y	N	N	111	42	69	17.7
287	Control	80	M	Y	N	N	126	54	80	10.4
288	Patient	57	F	N	Y	N	135	44	86	10.4
289	Patient	58	F	Y	N	N	60	41	86	14.5
292	Patient	80	M	Y	N	N	78	46	132	13.5
294	Control	78	F	N	N	N	98	41	92	17.7
295	Patient	62	M	Y	Y	Y	57	36	80	13.5
296	Control	57	F	N	N	N	19	56	92	0
297	Control	60	F	Y	N	N	112	54	114.5	16.6
298	Control	63	F	Y	N	N	160	48	92	11.4
299	Patient	74	M	N	Y	N	65	52	80	13.5
300	Patient	50	M	Y	N	N	24	38	80	14.5
301	Control	58	M	Y	N	N	80	30	108	15.6
302	Control	54	M	Y	N	N	94	51	108	16.6
303	Patient	86	F	Y	N	N	53	79	120	10.4
304	Control	78	M	N	N	N	80	50	69	13.5
305	Patient	57	M	N	Y	N	139	29	114.5	8.3

List of study population (continued).

		Demographic Characteristics					Lipid Parameters		Enzyme Activities	
No	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	LDL-C(mmol/L)	HDL-C(mmol/L)	ARE (U/ml)	GST (U/L)
306	Control	75	M	Y	Y	N	115	56	114.5	14.5
307	Control	77	M	Y	N	N	64	36	74	12.5
308	Patient	85	M	N	N	N	118	44	92	15.6
309	Patient	62	M	Y	N	N	119	29	63	8.3
310	Patient	81	F	Y	Y	N	96	48	57.3	7.3