INVESTIGATION OF THE ASSOCIATION BETWEEN GENETIC AND ACTIVITY POLYMORPHISMS OF PARAOXONASE 1 AND ISCHEMIC STROKE RISK

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ABSTRACT

INVESTIGATION OF THE ASSOCIATION BETWEEN GENETIC AND ACTIVITY POLYMORPHISMS OF PARAOXONASE 1 AND ISCHEMIC STROKE RISK

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Stroke is the third leading cause of death. Atherosclerosis in the carotid arteries is a risk factor for ischemic stroke. Oxidized low density lipoprotein (LDL) plays a central role in the progression of atherosclerosis. Human paraoxonase 1 (PON1), a high-density lipoprotein (HDL) associated serum esterase/lactonase, protects HDL and LDL from oxidative modifications. Thus, PON1 is protective against the development of atherosclerosis. PON1 gene has two functional coding region (192Q/R and 55L/M) and one promoter region (−107T/C) polymorphism that affect the catalytic efficiency and levels of the enzyme, respectively. In this study, the aim was to determine the importance of PON1 genetic polymorphisms and activity as risk factors for ischemic stroke.

The study population was comprised of 172 unrelated adult Caucasian patients with acute hemispheric ischemic stroke and 105 symptom-free controls. Serum and total blood samples were obtained from Gülhane Military Medical Academy Hospital Neurology Department, Ankara. Hypertension and diabetes were 2 times more common and HDL-C was significantly lower among patients compared to controls. Logistic regression analysis revealed hypertension and smoking to be

significant predictors of stroke. Serum PON1 activities towards three substrates, paraoxon (paraoxonase activity; PON), phenyl acetate (arylesterase activity; ARE) and diazoxon (diazoxonase activity), which were measured by spectrophotometric methods, were found to be lower in stroke patients compared to controls. PON and PON/ARE were negatively associated with ischemic stroke by use of logistic regression analysis. PON/ARE was 1.26 times protective against stroke. The frequencies of the risky alleles 192*R*, 55*L* and −107*T* were increased in the patient group. Frequency of the 55*L* allele of PON1 was significantly increased among patients (0.690) compared to controls (0.628; *P*=0.003). Logistic regression analysis revealed PON1 55*LL* genotype to be associated with a 1.8-fold increase in the risk of ischemic stroke versus control status. Prevalence of triple combined haplotype *QRLMTC* was significantly lower in stroke patients (4.1%) when compared to controls (11.4%; *P*=0.019). The combined heterozygote haplotype had around 7 times increased protective effect against stroke in the overall population and 10 times protective effect in the elderly population. The low expressor genotype −107*TT* was associated with almost 2 times increased risk for stroke in elderly. 192*R* allele of PON1 represented 1.554 times increased risk for ischemic stroke in hypertensives relative to normotensives. Furthermore, the risk of hypertensive individuals having ischemic stroke was highest in the 192*RR* group (Odds Ratio; OR=7), followed by 192*QR* heterozygotes (OR=2.18), and the risk decreased to insignificant levels in 192*QQ* individuals. 192*R* allele constituted a 1.55 times increased risk in diabetics. 55*L* allele was associated with a 1.66 times increased risk of stroke in hypertensives and a 2.6 times increased risk for stroke in diabetics relative to non-diabetics. PON1 −107*T* allele also represented a 1.35 times risk for stroke in hypertensives.

Keywords: Paraoxonase, PON1, Arylesterase, Diazoxonase, Stroke, 192Q/R, 55L/M, −107T/C, Genotype, Polymorphism

ÖZ

PARAOKSONAZ 1 AKTİVİTE VE GENETİK POLİMORFİZMLERİ İLE İNME RİSKİ ARASINDAKİ İLİŞKİNİN ARAŞTIRILMASI

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İnme ölüm nedenleri arasında üçüncü sırada gelmektedir. Karotisdeki (şahdamarı) ateroskleroz iskemik inmeye yol açan risk faktörlerinden birisidir. Okside olmuş düşük dansiteli lipoprotein (LDL), aterosklerozun ilerlemesinde önemli bir rol oynamaktadır. Serumda yüksek dansiteli lipoproteine (HDL) bağlı olarak bulunan bir esteraz/laktonaz olan insan paraoksonaz 1 (PON1), HDL ve LDL'yi oksidatif değişimlere karşı korur. Bu yüzden PON1 aterosklerozun ortaya çıkmasına karşı koruyucu etkiye sahiptir. PON1 geninde bulunan iki fonksiyonel kodlayan bölge (192Q/R ve 55L/M) ve bir promotor bölge (−107T/C) polimorfizmi sırasıyla enzimin katalitik verimini ve kandaki seviyesini belirlemektedir. Bu çalışmada amaç iskemik inme için PON1 genetik polimorfizmleri ve aktivitesinin risk faktörü olarak önemini belirlemektir.

Çalışma popülasyonu, aralarında akrabalık olmayan Kafkas ırkına mensup 172 erişkin akut hemisferik iskemik inme hastası ve bu semptomları göstermeyen 105 kontrolden oluşmuştur. Serum ve tam kan örnekleri Gülhane Askeri Tıp Akademisi Hastanesi Nöroloji Bölümünce temin edilmiştir. Hipertansiyon ve diyabet, inme hastalarında kontrollerden 2 kat fazla görülmüştür. HDL-kolesterol hastalarda ciddi biçimde düşük bulunmuştur Lojistik regresyon analizi, hipertansiyon ve sigaranın

inme için önemli tahmin unsurları olduğunu ortaya koymuştur. Serum PON1'in aktivitesi üç substrata karşı spektrofotometrik yöntemlerle belirlenmiş ve paraokson (paraoksonaz aktivitesi; PON), fenil asetat (arilesteraz aktivitesi; ARE) ve diazoxon (diazoxonaz aktivitesi) hidroliz aktivitesi which were measured by spectrophotometric methods, inme geçirmiş hastalarda kontrollere göre düşük bulunmuştur. Düşük PON ve PON1 aktivite oranının (PON/ARE) iskemik inme için risk faktörü olduğu lojistik regresyon analizi ile bulunmuştur. PON/ARE'nin inmeye karşı 1,26 kat koruyucu olduğu tespit edilmiştir. Riskli alellerin (192*R*, 55*L* ve −107*T*) frekansı hastalarda yüksek bulunmuştur. PON1'in 55*L* alelinin frekansı hastalarda (0.690) kontrollerden (0.628; *P*=0.003) ciddi biçimde yüksek bulunmuştur. Lojistik regresyon analizi PON1 55*LL* genotipinin inme için 1,8 kat risk oluşturduğunu ortaya koymuştur. Üçlü kombine haplotip *QRLMTC*'nin yaygınlığı inme hastalarında (4.1%) kontrollere (11.4%) göre anlamlı şekilde düşük bulunmuştur (*P*=0.019). Kombine heterozigot haplotip inmeye karşı yaklaşık 7 kat koruyucu etkiye sahiptir. The low expressor genotype −107*TT* was associated with almost 2 times increased risk for stroke in elderly. PON1'in 192*R* alelinin hipertansiflerde, normotensiflere göre 1.55 kat fazla iskemik inme riski teşkil ettiği bulundu. Üstelik, hipertansiyonlu insanların iskemik inme riski 192*RR* grubunda en yüksek (eşitsizlik oranı [OR; Odds Ratio]=7) bulunmuş, bunu 192*QR* heterozigotlar (OR=2,18) takip etmiştir ve risk 192*QQ* genotipli bireylerde önemsiz seviyelere düşmüştür. 192*R* aleli diyabetlilerde 1,55 kat yüksek risk teşkil etmektedir. 55*L* aleli hipertansiyonlularda 1,66 kat ve diyabetiklerde diyabetik olmayanlara göre 2,6 kat yüksek iskemik inme riski ile ilişkili bulunmuştur. PON1 −107*T* aleli de hipertansiyonlularda inme için 1,35 kat risk teşkil etmektedir.

Anahtar kelimeler: Paraoksonaz, PON1, Arilesteraz, Diazoksonaz, İnme, 192Q/R, 55L/M, −107T/C, Genotip, Polimorfizm.

to my parents

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TABLE OF CONTENTS

LIST OF TABLES

Tables

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

Å	Angstrom
ACE	Angiotensin I converting enzyme
ANOVA	Analysis of variance
APO	Apolipoprotein
ARE	Arylesterase activity
CAAD	Carotid artery atherosclerotic disease
CAPN-10	Calpain-10
CHD	Coronary heart disease
CI	Confidence interval
CT	Computed tomography
CVD	Cardiovascular disease
CYP	Cytochrome P450
Cys	Cysteine
DIA	Diazoxonase activity
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
FLAP	5-lipoxygenase activating protein
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-Coenzyme A
ICA	Internal carotid artery
IDDM	Insulin dependent diabetes mellitus
IMHP	2-isopropyl-4-methyl-6-hydroxy pyrimidine
IMT	Intima media thickness
IP	Intraperitoneal
LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoprotein
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
Met	Methionine
MCP-1	Monocyte chemoattractant protein
M-CSF	Macrophage-colony stimulating factor
MI	Myocardial infarction
MM-LDL	Minimally oxidized – low density lipoprotein
mRNA	Messenger ribonucleic acid
MTHFR	Methylene tetrahydrofolate reductases
NIDDM	Non-insulin dependent diabetes mellitus
PAFAH	Platelet-activating factor acetyl hydrolase
PCR	Polymerase chain reaction
PDE4D	Phosphodiesterase 4D

PON	Paraoxonase or paraoxonase activity
PON/ARE	Paraoxonase activity divided by arylesterase activity
PON/DIA	Paraoxonase activity divided by diazoxonase activity
RE	Restriction Endonuclease
RNA	Ribonucleic acid
ROS	Reactive oxygen species
OP	Organophosphate
OR	Odds ratio
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
TIA	Transient ischemic attack
UTR	Untranslated region
X-CAM	Monocyte binding molecule

CHAPTER I

INTRODUCTION

1.1 Stroke

A stroke, or a cerebrovascular accident, occurs when blood vessels that deliver oxygen to the brain, either rupture or become clogged, causing brain/nerve cells to die. Symptoms of stroke include:

- Sudden numbness or weakness of the face, arm or leg, especially on one side of the body
- Sudden confusion, trouble speaking or understanding
- Sudden trouble seeing in one or both eyes
- Sudden trouble walking, dizziness, loss of balance or coordination
- Sudden, severe headache with no known cause (http://www.americanheart.org).

Stroke is a major public health problem that ranks in the top four causes of death in most countries and is responsible for a large proportion of the burden of neorologic disorders. More often disabling than fatal, stroke is the leading cause of severe neurologic disability and results in enormous costs measured in both lost productivity and money spent for health-care (Sacco, 2005).

1.1.1 Definition and Nosology

In the broadest sense, the World Health Organization has defined stroke as "rapidly developing clinical signs of focal (at times global) disturbance of cerebral function, lasting more than 24 hours or leading to death with no apparent cause other than that of vascular origin". By conventional clinical definitions, if the neorologic symptoms continue more than 24 hours, a person is diagnosed with stroke; otherwise, a focal neurologic deficit lasting less than 24 hours is defined as a transient ischemic attack (TIA). Such terms defined by the duration of neurologic symptoms are being redefined with the more widespread use of sensitive brain imaging, such as diffusion-weighed magnetic resonance imaging (MRI). Patients with symptoms lasting less than 24 hours, but with an infarction imaged by MRI, have been reclassified as having stroke instead of TIA. The most recent definition of stroke for clinical trials has required either symptoms lasting more than 24 hours or imaging of an acute clinically relevant brain lesion in patients with rapidly vanishing symptoms. The duration and severity of the syndrome may than be used to classify patients as those with minor or major stroke (Sacco, 2005).

1.1.2 Stroke Epidemiology: Incidence, Prevalence, and Mortality

Stroke incidence is determined by the number of first cases of stroke, over a defined time interval in a defined population, whereas stroke prevalence measures the total number of cases (new and old), at a particular time also in a defined population. Stroke incidence may be viewed as the sum of hospitalized, sudden fatal and nonhospitalized stroke. The American Heart Association estimates that in the United States of America, there are almost 4.7 million stroke survivors (prevalence) and approximately 700,000 new or recurrent strokes occur per year (American Heart Association, 2004). Overall, age-adjusted incidence rates range between 100 and 300 cases per 100,000 population per year, and depend on the study methodology, country of origin, and population demographics. In the United States of America, the age-adjusted stroke incidence rates (per 100,000) are 167 for white men and 138 for white women, while black patients have nearly twice the risk, with incidence rates

(per 100,000) at 323 for men and 260 for women. The reported incidences of stroke are 0.1 % in Europe (Robinson and Toole, 1992), 0.2 % in Denmark (Boysen *et al.*, 1993), 0.198 % in Poland (Wender *et al.*, 1990), and 0.22 % in Italy (Ricci *et al.*, 1991). In Turkey, stroke incidence was found as 0.137 % (Akhan *et al.*, 2000). Overall, stroke accounts for about 10 % of all deaths in most industrialized countries and most of these deaths are among persons over the age of 65. Stroke-related death rates are greatest in Japan and China (Sacco, 2005).

1.1.3 Classification and Frequency of Stroke Subtypes

Stroke is a heterogeneous disease with three main pathological subtypes: ischemic stroke, intracerebral hemorrhage, and subarachnoid hemorrhage (Warlow *et al.*, 2003). Ischemic stroke is 3 to 4 times as frequent as hemorrhagic stroke, accounting for 70 % to 80 % of all strokes. Intracerebral hemorrhage usually accounts for 10 % to 30 % of the cases, depending on the geographic origin of the patients, with greater relative frequencies reported in Chinese and Japanese series (Sacco, 2005). Among Turkish stroke patients, 77 % were found to have ischemic stroke, 19 % had primary intracerebral hemorrhage, and 4 % had subarachnoid hemorrhage (Kumral *et al.*, 1998).

1.1.4 Pathogenesis and Classification of Ischemic Stroke

Ischemic stroke can be classified into two main types: *thrombotic* and *embolic*. The most common problem leading to ischemic stroke is narrowing of the arteries in the neck or head. This is most often caused by build up of fatty and inflammatory tissue on the inside surface of an artery, forming a plaque (Figure 1.1). Platelets, fibrin and other blood products can stick to this part of a clot, which is called a thrombus. These blood clots can block the artery where they are formed (*thrombosis*). This leads to some degree of blockage of flow through the artery, which is known as carotid stenosis. If the blockage is measured to be 40-69 %, it is called moderate blockage, i.e., moderate stenosis. Mild to moderate carotid stenosis may or may not cause any symptoms. However, sometimes, a fragment of the plaque

(*embolus*) can break off and become trapped in arteries closer to the brain (*embolism*), leading to a transient ischemic attack (TIA), or a full stroke. If the plaque build up involves blockage of 70 % or more of the inner opening (luminal diameter) of the internal carotid artery (ICA), the stenosis is referred to as "high-grade". High grade stenosis may result in only a trickle of flow (or at least impaired flow) in the ICA more distant to the blockage. The brain may recognize this via stroke-like symptoms (vision loss, sensory and muscle function loss, speaking difficulty, etc.) (http://www.strokecenter.org, http://www.brain-aneurysm.com).

Plaque formation starts with fatty streak development. Details of the basic mechanisms that induce the sequence of events, starting from fatty streak formation, and leading to clinical event are given under the heading of "pathogenesis of atherosclerosis" in section 1.1.5.2. Before that, definition of atherosclerosis is given.

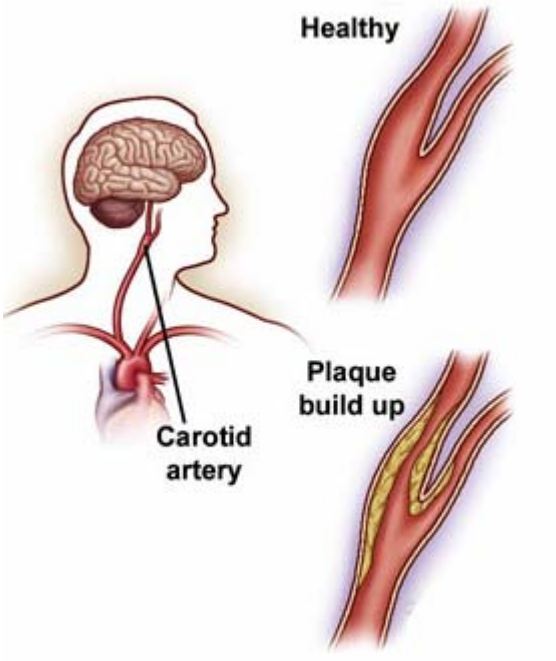


Figure 1.1 Healthy carotid artery and carotid artery with plaque build up (taken from http://www.vascularweb.org).

1.1.5 Atherosclerosis

1.1.5.1 Definition

Atherosclerosis, which means literally "hardening of the arteries," is a disease of the blood vessels. Atherosclerosis is a class of arteriosclerosis, the latter being a general term denoting thickening and hardening of the arteries. Atherosclerosis is derived from the Greek words *athero* (meaning gruel or paste) and *sclerosis* (meaning hardness) (Tegos *et al.*, 2001).

1.1.5.2 Pathogenesis of Atherosclerosis

The process of atherogenesis generally begins during childhood, with a preclinical phase that can last for decades. The consequences of atherosclerosis, however, generally manifest in middle-aged or elderly persons. Components of the earliest lesion, the fatty streak, which itself is not clinically significant, are also responsible for the latter events that lead to clinically significant disease (Berliner *et al.*, 1995). A model of the sequence of changes in the artery wall that leads to a clinical event is given in Figure 1.2 and explained in detail below.

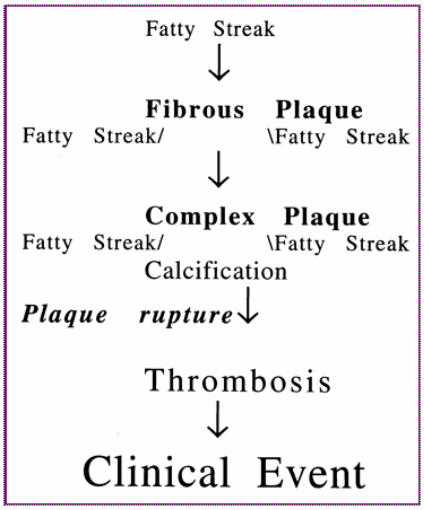


Figure 1.2 Model showing the sequence of events starting from fatty streak to clinical event (taken from Berliner *et al.*, 1995).

Fatty Streak

Table 1.1 and Figure 1.3 depict some of the steps in fatty streak development. The first step is lipoprotein transport into the artery wall. This concentration-dependent process does not require receptor-mediated endocytosis, so that at high concentrations of low density lipoprotein (LDL; step 1 in Figure 1.3), the particles accumulate in the subendothelial space (Steinberg *et al.*, 1989; Young and Parthasarathy, 1994; Shih *et al.*, 2002).

Table 1.1 Development of the fatty streak (taken from Berliner *et al.*, 1995).

Step	
1	Lipoprotein transport
2	Lipoprotein retention
3	Lipoprotein modification
4	Monocyte adherence
5	Monocyte migration (chemotaxis)
6	Monocyte differentiation
7	Foam cell formation

LDL that becomes trapped in the extracellular matrix of the subendothelial space (step 2 in Table 1.1 and in Figure 1.3) is subject to modification (step 3 in Table 1.1) (Berliner *et al.*, 1995; Shih *et al.*, 2002). Modification may be in the form of non-enzymatic modifications (proteoglycans, glycosylation, immune complexes) and enzymatic modifications (lipases, oxygenases), which were shown to affect the physicochemical (size, charge) as well as the biological (cellular uptake, secretion) properties of the lipoprotein (Aviram, 1993). Of special interest is the oxidative modification of LDL which was demonstrated to occur *in vivo*. The trapped LDL becomes oxidized (step 3 in Figure 1.3) probably as a result of interactions with reactive oxygen species (ROS) (step 4 in Figure 1.3).

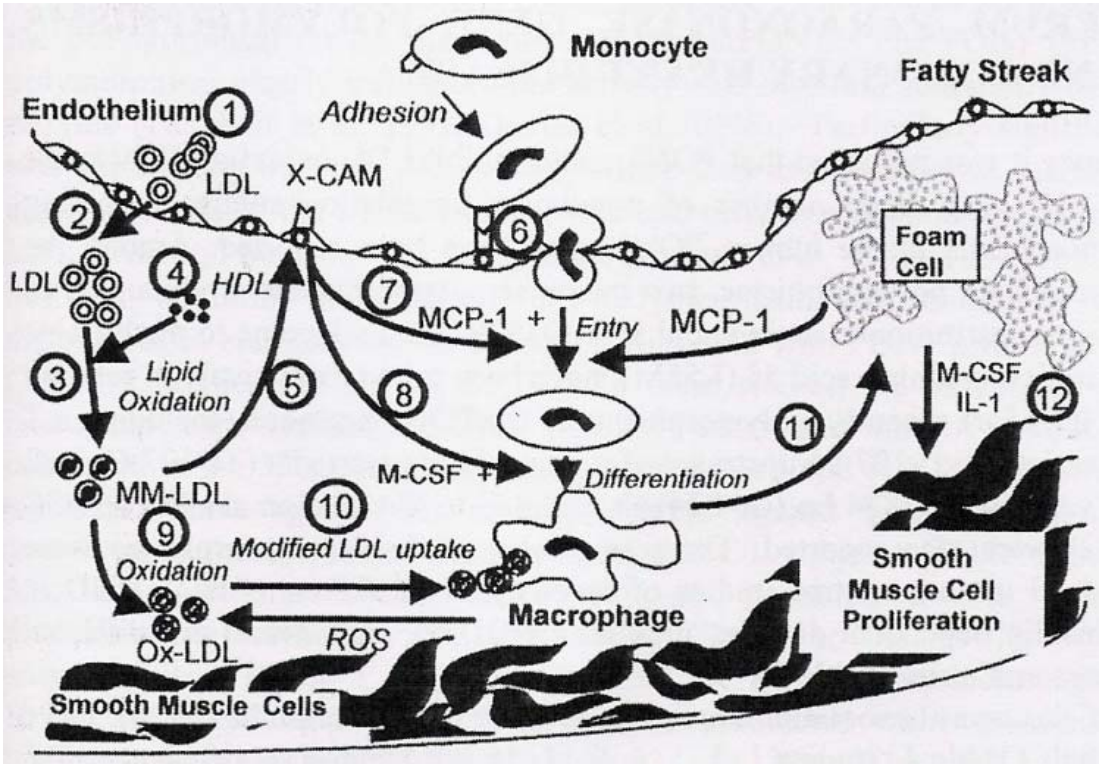


Figure 1.3 A model for early steps in the development of the atherosclerotic lesion. See text for details (taken from Shih *et al.*, 2002).

The mechanism of this process involves cellular lipid peroxidation (Aviram, 1993) and takes place mainly in the intima of the arterial wall, because the plasma compartment has an effective antioxidant defense system (Esterbauer *et al.*, 1992). In recent years increasing evidence suggests that the oxidative modification of LDL is the key step in the sequence of events leading to atherosclerosis (Steinberg *et al.*, 1989; Parthasarathy *et al.*, 1992). Inhibition of such LDL modifications may arrest the development of the atherosclerotic lesion (Aviram, 1993). Oxidation of LDL is inhibited by HDL (step 4 in Figure 1.3). In the next section (1.1.5.3), critical role of HDL in preventing oxidation of LDL is discussed. *In vitro* and *in vivo* studies suggest that such minimally oxidized LDL (MM-LDL) exhibits a potent biological activity capable of inducing endothelial cells to express adhesion molecules for monocytes (X-CAM) (step 5 in Figure 1.3), monocyte chemoattractant protein (MCP-1) (step 7 in Figure 1.3), and macrophage-colony stimulating factor (M-CSF)

(step 8 in Figure 1.3). The expression of these and other molecules results in the recruitment of blood monocytes to the artery wall (step 6 in Figure 1.3), where the monocytes differentiate into macrophages. With time, the LDL particles become highly oxidized (Ox-LDL) (step 9 in Figure 1.3), perhaps as a result of the high levels of ROS produced by macrophages. Such highly oxidized LDL particles are recognized by scavenger receptors on macrophages, resulting in rapid endocytosis (step 10 in Figure 1.3). Unlike the LDL receptor, the scavenger receptors are not downregulated by high levels of cellular cholesterol, and the macrophages continue to accumulate cholesterol until they give rise to cholesterol engorged foam cells (step 11 in Figure 1.3) (Aviram, 1993; Steinberg, 1993; Shih *et al.*, 2002). Infiltration and deposition of the foam cells in the arterial wall are considered the initiating steps to develop atherosclerotic plaque (atheroma) (Livrea *et al.*, 1998). Such foam cells, the hallmark of fatty streaks, may contribute to the development of advanced lesions by production of cytokines and growth factors (step 12 in Figure 1.3) (Aviram, 1993; Steinberg, 1993; Shih *et al.*, 2002).

Fibrous plaque

The fibrous plaque is representative of various forms of advanced atherosclerosis and is widely accepted as the most common atherosclerotic precursor to occlusive lesions. As such, it is the major determinant of clinically significant disease. This lesion is whitish in gross appearance and protrudes into the vessel lumen (Gerrity and Antonov, 1997).

Complex plaque

The progression of the lesion is associated with the activation of genes that induce arterial calcification. At this stage plaque is made up of fat, cholesterol, calcium, and other substances found in the blood. The lipid content of the atherosclerotic plaque mainly consists of oxidized lipids (Rosenfeld *et al.*, 1990). As it grows, the buildup of plaque narrows the inside of the artery and, in time, may restrict blood flow (Berliner *et al.*, 1995).

Plaque rupture and thrombosis

Calcification of the artery changes the mechanical characteristics of the artery wall and predisposes to plaque rupture at sites of monocytic infiltration (Beadenkopf *et al.*, 1964). When atherosclerotic plaques rupture, a thrombus forms, which can interrupt blood flow or break off and embolize to another part of the body (Berliner *et al.*, 1995). Plaques may become symptomatic when they are large enough to restrict blood flow, leading to tissue ischemia (Scheuner, 2004). As plaque builds up, it can cause serious diseases and complications. These include:

- Coronary artery disease
- Cerebrovascular disease
- Peripheral arterial disease (Tegos *et al.*, 2001).

1.1.5.3 Antioxidant Role of HDL

High-density lipoprotein (HDL) is today regarded as one of the most important protective factors against arteriosclerosis. Numerous cohort studies and clinical trials have confirmed the association between a low HDL-cholesterol concentration and risk of coronary heart disease (Miller and Miller, 1975; Rhoads *et al.*, 1976; Gordon *et al.*, 1977). In experimental animals, an inverse correlation between HDL concentration and the development of arteriosclerosis has been demonstrated (Tomás *et al.*, 2004). It has been observed that arteriosclerotic lesions tend to regress *in vivo* as the concentration of HDL or its apolipoproteins increases (Badimon *et al.*, 1990; Miyazaki *et al.*, 1995; Nissen *et al.*, 2003; Tomás *et al.*, 2004). Certain genetically inherited diseases that are characterized by an abnormally low HDL level, such as Tangier disease, fish-eye disease and diseases linked to *apo A1* gene mutations, are frequently associated with arteriosclerosis and premature ischemic heart disease (Ordovas *et al.*, 1989; Matsunaga *et al.*, 1991; Kuivenhoven *et al.*, 1997; Srivastava, 2002; Tomás *et al.*, 2004). Evidence is strong that low HDL cholesterol is a marker for the presence of a small, dense, cholesterol-depleted LDL in the circulation, which itself increases the risk of atherosclerosis, probably because of its susceptibility to oxidation (Chait *et al.*, 1993; Durrington *et al.*, 2001).

There does not appear to be any single explanation for the inverse relationship between serum HDL and risk of atherosclerosis. Traditionally, HDL's protective function has been attributed to its active participation in the reverse transport of cholesterol (Durrington *et al.*, 2001). Other potentially anti-atherogenic properties of HDL, such as reducing blood viscosity, regulation of prostaglandin and thromboxane synthesis and the activation of fibrinolysis have also been reported and are detailed in Table 1.2 (Mackness *et al.*, 2002a).

Table 1.2 Antiatherogenic effects of HDL (taken from Mackness *et al.*, 2002a).

1	Inhibition of lipid-peroxidation
2	Inhibition of inflammatory processes
3	Promotion of reverse cholesterol transport
4	Reduction of blood viscosity
5	Maintenance of red-blood cell shape
6	Regulation of prostaglandin and thromboxane synthesis
7	Activation of fibrinolysis
8	Stimulation of nitric oxide synthase
9	Inhibition of apoptosis

However, the greatest interest is into the capacity of HDL to protect LDL against lipid peroxidation (Figure 1.4; Mackness *et al.*, 2000a). HDL was found to protect against LDL oxidation by metal ions in vitro (Hessler *et al.*, 1979; Parthasarathy *et al.*, 1990) and to prevent the production of mildly oxidized LDL by the artery wall cells in a coculture model (Navab *et al.*, 1991; Berliner *et al.*, 1995). Mackness and coworkers (2000a) hypothesized that HDL has a ubiquitous role in protecting cell membranes against lipid-peroxide-induced damage and that LDL behaves like a cell membrane in this context. The most likely mechanism by which

HDL diminished lipid peroxide accumulation was an enzymatic hydrolysis of phospholipid hydroperoxides (Mackness *et al*., 1993a; Durrington *et al.*, 2001). In section 1.2.2.4.2 sufficient experimental evidences are given to support the view that paraoxonase 1 (PON1), an HDL-associated ester hydrolase, is the enzyme which is mainly responsible for prevention of LDL lipid peroxidation.

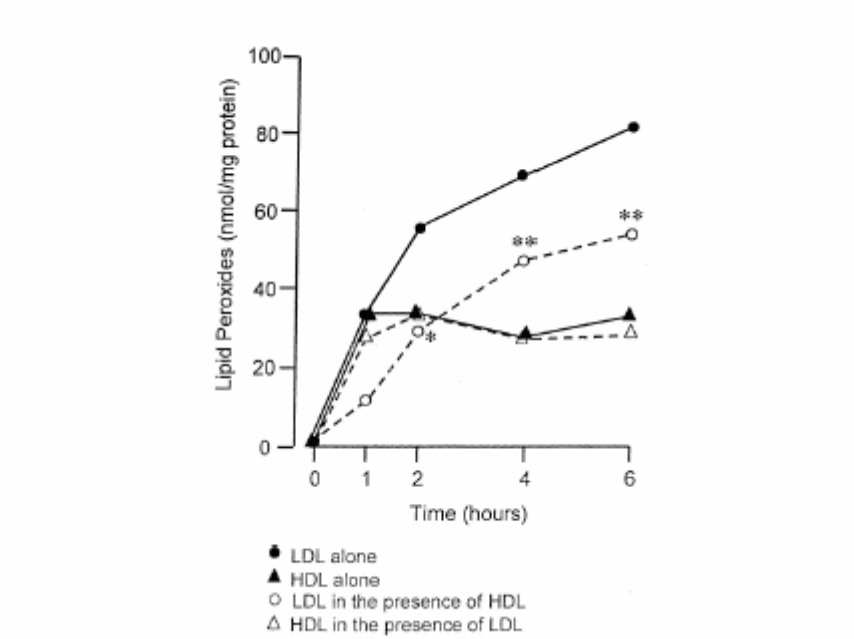


Figure 1.4 Lipid peroxide accumulation on LDL and HDL incubated under oxidizing conditions singly and together. *P<0.05 and **P<0.001 vs. LDL incubated alone (taken from Durrington *et al.*, 2001).

1.1.6 Determinants of Stroke

1.1.6.1 Nonmodifiable Risk Factors

Although cerebrovascular disorders may occur at any age, at any time, in either sex, in all families, and in all races, each of these non-modifiable factors affects the incidence of stroke (Sacco, 2005). The strongest determinant of stroke is age (Petitti

et al., 1997). Although stroke is less common before the age of 40 years, stroke in young adults is of growing concern because of the impact of early disability (Bogousslavsky and Pierre, 1992). As the population ages, the prevalence and public health impact of stroke will undoubtedly increase (Sacco, 2005).

Stroke incidence is greater among men (Sacco *et al.*, 1998; Kumral *et al.*, 1998), among those with a family history of stroke, and among certain race-ethnic groups (Sacco *et al.*, 1998). In a hospital- and community-based cohort study of all cases of first stroke in northern Manhattan, black patients had an overall age-adjusted annual stroke incidence rate 2.4 times that of white patients; Hispanic patients had an incidence rate 1.6 times that of white patients (Figure 1.5; Sacco *et al.*, 1998).

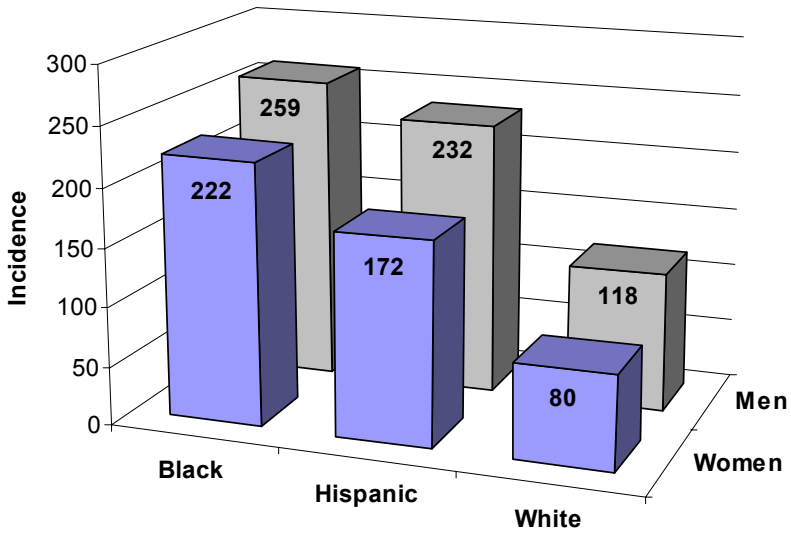


Figure 1.5 Average age-adjusted incidence rates of stroke (per 100,000 population) among persons aged 20 years in northern Manhattan, as occur in white, black, and Hispanic women and men (taken from Sacco, 2005).

1.1.6.2 Modifiable Risk Factors

Current modifiable components of the stroke-prone profile include hypertension (Lawes *et al.*, 2004), cardiac disease (particularly atrial fibrillation), diabetes, hypercholesterolemia, physical inactivity, cigarette use, alcohol abuse, asymptomatic carotid stenosis, and a history of TIAs (Table 1.3; Sacco, 2005).

The major risk factor for stroke is hypertension. Current trial data, although limited, suggest that lowering blood pressure by 5-6 mmHg diastolic and 10-12 mmHg systolic for two or three years should reduce annual risk of stroke from 7 % to 4.8 % (Progress Collaborative Group, 2001; Sacco, 2005).

Table 1.3 Modifiable risk factors that may increase the probability of stroke (taken from Sacco, 2005).

Risk Factors	***Modification***
Hypertension	Antihypertensives, diet
Heart disease Atrial fibrillation	Antiplatelets Anticoagulants, antiarrhytmics
Diabetes mellitus	Glucose and blood pressure control
Hypercholesterolemia	Diet, lipid lowering medication
Physical inactivity	Routine exercise
Smoking	Cessation
Heavy alcohol use	Quantity reduction
Asymptomatic carotid stenosis	Antiplatelets, endarterectomy, angioplasty
Transient ischemic attack	Antiplatelets, endarterectomy

Diabetes mellitus also has been associated with increased stroke risk, with relative risks ranging from 1.5 to 3.0, depending on the type and severity. The effect was found in both men and women, did not diminish with age, and was independent of coexisting hypertension (Wolf *et al.*, 1991; Kuller *et al.*, 1985; Sacco, 2005).

Cigarette smoking has been established clearly as a biologically plausible independent determinant of stroke. Stroke risk was greatest in heavy smokers and reduced within 5 years among those who quit. It was an independent determinant of carotid-artery plaque thickness. Smoking increases the risk of stroke by around 50 % (Rudd *et al.*, 1997; Sacco, 2005).

1.1.6.3 Potential Risk Factors

Other potential stroke risk factors identified by some studies need to be confirmed and clarified in further epidemiological investigations. Migraine, oral contraceptive use, drug abuse, and snoring have been associated with a higher stroke risk. Various laboratory test result abnormalities, often reflecting an underlying metabolic-, coagulation-, or inflammatory disturbance, have been associated with stroke and identified as possible stroke precursors. These include hematocrit, polycythemia, sickle cell anemia, white blood count, C-reactive protein, fibrinogen, hyperuricemia, hyperhomocysteinemia, protein C and free protein S deficiencies, lupus anticoagulant, and anticardiolipin antibodies. Some are clear stroke risk factors, whereas others require further epidemiological investigations (Sacco, 2005).

1.1.7 Genetics of Stroke

Numerous single-gene mutations of autosomal or mitochondrial DNA cause ischemic or hemorrhagic stroke. Twin and family studies suggest a genetic contribution to ischemic stroke. Both paternal and maternal history of stroke have been associated with an increased stroke risk (Welin *et al.*, 1987; Kiely *et al.*, 1993). Linkage studies indicate that two genes, the phosphodiesterase 4D (PDE4D) and 5-lipoxygenase activating protein (FLAP) genes, may increase the risk of ischemic

stroke by approximately two-fold (Gretarsdottir *et al.*, 2003; Helgadottir *et al.*, 2004). In addition to mapping susceptibility genes, an alternative approach is to investigate recognized stroke risk factors, such as hypertension, diabetes, or hyperlipidemia, which have both genetic and environmental/behavioral components (Nicolaou *et al.*, 2000; Rubattu *et al.*, 2001; Turner and Boerwinkle, 2003). For example, the APOE gene is related to cholesterol levels, the ACE gene is related to hypertension, and the CAPN10 gene is related to type 2 diabetes (Sacco, 2005).

Several rare genetic disorders have been associated with stroke, including vasculapathies, metabolic or connective tissue diseases and disorders of coagulation (Durlach, 2001; Desnick *et al.*, 2001; Sacco, 2005). Clinical symptoms of patients with homocystinuria may indicate a progression to severe neurologic deterioration and stroke. Some patients with homocystinuria show severe deficiency of methylene-tetrahydrofolate reductase (MTHFR), caused by rare mutations in the MTHFR gene (Scott and Sutton, 1999; Fodinger *et al.*, 2000; Hassan *et al.*, 2004; Sacco, 2005). Many coagulopathies, which include protein C and S deficiencies, factor V Leiden mutations, and various other factor deficiencies, can lead to an increased risk of venous thrombosis (Bertina *et al.*, 1994; Ridker *et al.*, 1995; Deschiens *et al.*, 1996; Hillier *et al.*, 1998). However there has not been a strong association between several of these disorders and arterial events, such as MI and stroke (Bertina *et al.*, 1994; Hankey *et al.*, 2001; Juul *et al.*, 2002; Sacco, 2005).

1.1.8 Stroke Prevention

Stroke is a highly preventable disease in the majority of patients. Gorelick (1999) estimated that up to 80% of strokes could be prevented with currently available treatments such as antihypertensive, lipid-lowering, and antithrombotic therapy. The goal of stroke prevention strategies is to identify high-risk patients through the presence of modifiable and nonmodifiable risk factors and to target these modifiable risk factors through the use of appropriate pharmacologic and nonpharmacologic interventions (Brass, 2006). In primary prevention, smoking cessation, exercise, blood pressure management (Psaty *et al.*, 1997; Gueyffier *et al.*,

1997; Neal *et al.*, 2000; Zhang *et al.*, 2006), and correction of hyperlipidemia with diet and use of HMG-CoA reductase inhibitors (statins) (Welch, 2004; Castilla-Guerra *et al.*, 2006) reduce the risk of stroke. Carotid endarterectomy in patients with high-grade, asymptomatic carotid stenosis prevents stroke, although the absolute risk reduction is small (Goldstein *et al.*, 2001). Patients with atrial fibrillation benefit from warfarin therapy (Atrial Fibrillation Investigators, 1994). Antiplatelet agents have shown limited evidence of primary stroke prevention, although the recent Women's Health Initiative report (Ridker *et al.*, 2005) did suggest a role for aspirin in stroke prevention in middle aged and elderly women (Kirshner *et al.*, 2005).

Stroke prevention also requires the active involvement of the physician to help patients develop motivational drivers to control or stop stroke risk factors, which include hypertension, smoking, diabetes mellitus, elevated LDL, obesity, sedentary life, and negative stress levels (Yatsu, 2005; Sacco, 2005). Refer to Kirshner *et al.*, 2005 and Goldstein *et al.*, 2006 for two excellent reviews on stroke prevention.

1.2 Paraoxonases

Esterases are classified into two groups based on their interaction with organophosphorus compounds. 'A'-esterases hydrolyze organophosphorus compounds, whereas 'B'-esterases are inhibited by them. 'A'-esterases include the aryldialkylphosphatases (paraoxonases) and diisopropylfluorophosphatases (DFPases). 'B'-esterases comprise carboxylesterases and cholinesterases (Aldridge, 1953a, b). The most commonly used substrate for the study of aryldialkylphosphatase activity is paraoxon (*O, O*-diethyl-*O*-*p*-nitrophenyl phosphate), which has led to the enzyme being named paraoxonase (PON; EC 3.1.8.1). Paraoxon is hydrolyzed by PON as shown in Figure 1.6 (Furlong *et al.*, 1988).

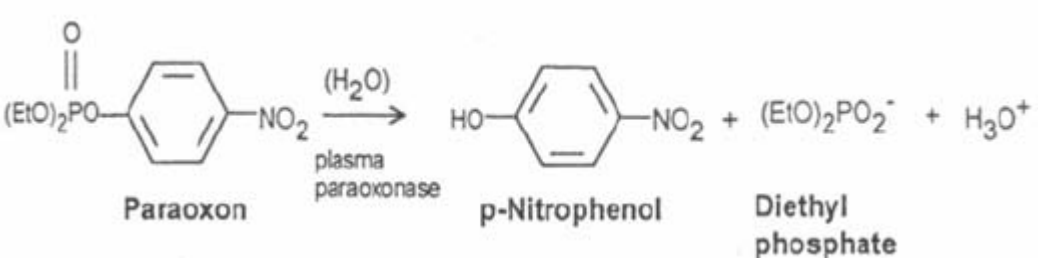


Figure 1.6 Hydrolysis of paraoxon by paraoxonase (taken from Furlong *et al.*, 1988).

PON activity is found in a variety of tissues, such as liver, kidney, intestine, and blood, although liver and blood generally have the highest activities. Mammals have the highest activity in all organs. Other animal groups, e.g. birds, fish and insects, have little or no activity (Draganov and La Du, 2004).

1.2.1 Paraoxonase Family

In 1996, it was established that the gene responsible for paraoxonase/arylesterase activities is a member of a multigene family. The human paraoxonase (PON) gene family consists of three members, *PON1*, *PON2*, and *PON3*, aligned next to each other on chromosome 7q21.3-22.1 (Figure 1.7). Based on their respective cDNA structures and the deduced amino acid sequences, there is over 80 % identity in amino acid residues in human, mouse and rabbit PON1 proteins, and at least 60 % identity between the PON 1, 2, and 3 within each of these species (Primo-Parmo *et al.*, 1996). The three PON genes contain nine exons of approximately the same length (Primo-Parmo *et al.*, 1996). The human liver PON1 cDNA has been cloned and sequenced independently by two laboratories (Hassett *et al.*, 1991; Adkins *et al.*, 1993). The gene for PON1 has nine exons that span 26 kb. Polymorphic variants are common in at least the human and rabbit PONs (Watson *et al.*, 2001).

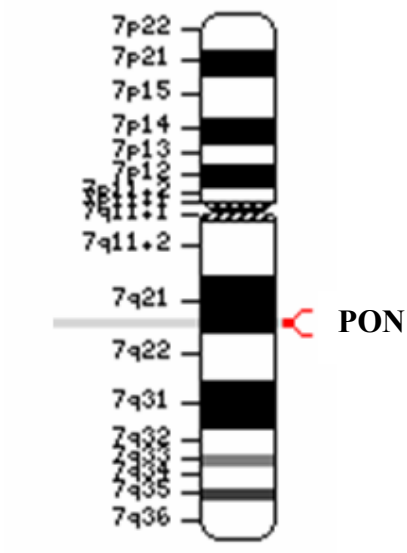


Figure 1.7 View of human PON gene in genomic location on chromosome 7 (taken from http://www.ncbi.nlm.nih.gov).

PON1 and PON3 reside in high density lipoprotein (HDL) cholesterol, whereas PON2 is found in many tissues (Harel *et al.*, 2004). PON1 is also present in human liver microsomes (Gonzalvo *et al.*, 1997). Primo-Parmo and coworkers (1996) have shown that mRNA for PON1 is present in a number of tissues apart from the liver, namely kidney, heart, brain, small intestine and lung. In rat, PON1 protein was detected in the endothelial lining of liver, kidney, lung and brain (Rodrigo *et al.*, 2001b).

The name, paraoxonase, is purely historical, as the PON family is a hydrolase family with one of the broadest specificities known. PON1 is a proficient esterase toward several synthetic substrates, whereas PON2 and PON3 exhibit high lactonase activity. But the paraoxonase activity of PON1 is rather weak, and PON2 and PON3 exhibit almost no paraoxonase activity. However, all of these activities toward man-made chemicals are promiscuous activities of PONs rather then their primary functions. A variety of physiological roles have been proposed for PONs, including phospholipase A2 action (Rodrigo *et al*., 2001), and hydrolysis and inactivation of homocysteine thiolactone, a risk factor for atherosclerotic vascular disease

(Jakubowski, 2000; Harel *et al.*, 2004). A reasonable conclusion from these observations is that this family of enzymes arose by gene duplication and the PONs have some important physiological roles that are insured by the redundancy and polymorphic forms of the proteins (Draganov and La Du, 2004).

By far the most-studied member of the family is the serum paraoxonase 1 (PON1) (Draganov and La Du, 2004). Due to its well documented role in preventing oxidation of LDL and HDL, and hence protecting against atherosclerosis (see section 1.2.2.4.2), paraoxonase 1 was the target PON family member which was chosen in this study to seek its relation to stroke. For this reason, properties of PON1 will be reviewed for the rest of this chapter.

1.2.2 Paraoxonase 1 (PON1)

1.2.2.1 Enzymatic Characteristics and Tissue Distribution of PON1

Paraoxonase/arylesterase 1 (PON1; EC 3.1.8.1/3.1.1.2) is a calcium dependent glycoprotein of 43-45-kDa (Gan *et al.*, 1991). Human PON1 is synthesized mostly in the liver as a 355 amino acid peptide, from which only the amino-terminal methionine residue is removed during secretion and maturation (Figure 1.8; Hasset *et al.*, 1991). PON1 is released from liver by a docking process whereby HDL particles transiently associate with the cell membrane and remove PON1 from the membrane (Deakin *et al.*, 2002). The retained leader sequence (*N*-terminal signal peptide) is a structural requirement for PON1's association with HDL particles (Sorenson *et al.*, 1999), and indeed PON1 is entirely associated with HDL in human serum (Blatter *et al.*, 1993). PON1 is extracted from HDL with deoxycholate and Nonidet NP-10 or Triton X-100 (Gan *et al.*, 1991). The amount of PON1 in human blood is 50 mg L^{-1} (Sorenson *et al.*, 1995).

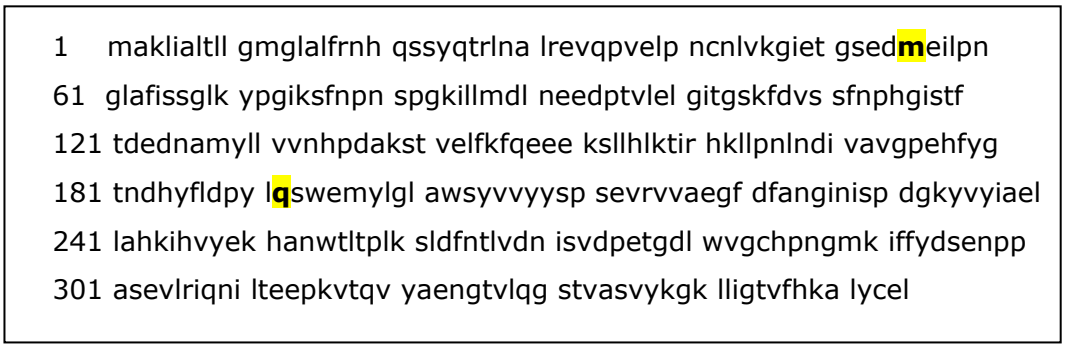


Figure 1.8 Human serum paraoxonase amino acid sequence, with polymorphic amino acids at positions 55 and 192 highlighted yellow (taken from Hassett *et al.*, 1991; http://www.ncbi.nlm.nih.gov).

1.2.2.2 PON1 Structure; Physicochemical Properties and Overall Architecture of PON1

Physicochemical properties of PON1 are summarized in Table 1.4. As calculated from the sequence, the molecular mass of deglycosylated PON1 is 40 kDa. On 0.1 % SDS-PAGE, purified PON1 was found to exhibit 2 bands at 45 and 48 kDa, which are glycosylation isoforms, the 48 kDa band containing 2 carbohydrate chains and the 45 kDa band containing one carbohydrate chain (Gan *et al.*, 1991; Furlong *et al.*, 1991).

A schematic representation of many of the features of human PON1 is shown in Figure 1.9, where the two polymorphic sites at positions 55 and 192, the internal disulfide bond between cysteine residues 42 and 353, the free cysteine at position 284, the location of potential sugar chains, and the hydrophobic retained leader sequence at the amino terminal end are illustrated (La Du *et al.*, 1999).

Table 1.4 Physicochemical properties of the human PON1 (taken from Josse *et al.*, 2002).

Unprocessed protein	355 amino acids
Mature protein	354 amino acids (deletion of Met-1)
M_{app} (0.1 % SDS-PAGE)	37-48 kDa
Molecular mass of the PON1 fraction: Detergent- or phospholipid-free associated to detergent micelles	 ~ 70 kDa ~ 85 kDa
pI	~ 5
Glycan Chains	Two: linked to Asn-253 and 324
Cys residues	1 disulfide bond linking Cys-42 and Cys-353 Cys-284 free
Calcium-binding sites: Catalytic Structural	 Kd_1=6.6± 1.2 10^{-6} M Kd_2=3.6± 0.9 10^{-7} M
N-terminal signal peptide	Not cleaved, hydrophobic helix

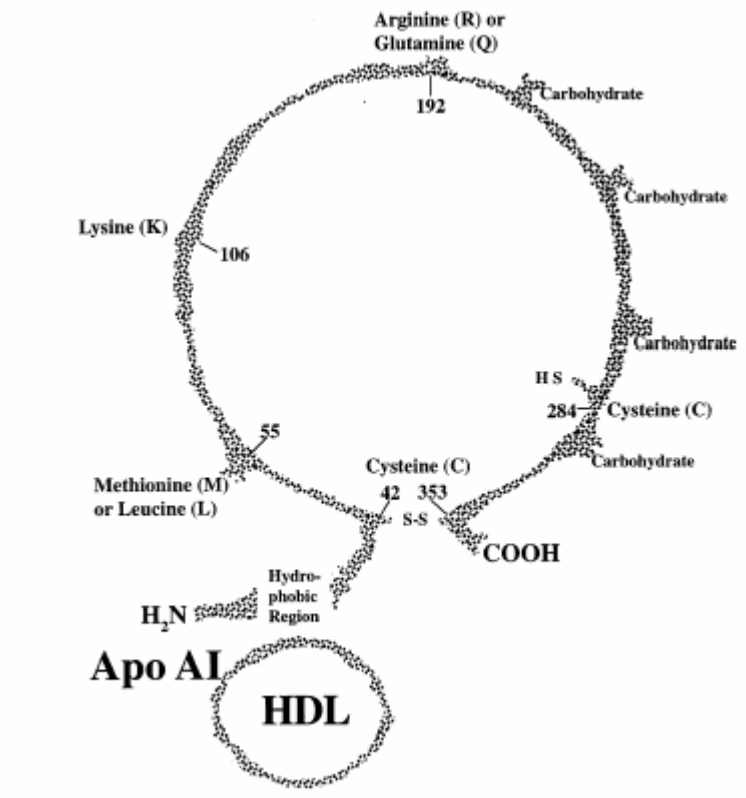


Figure 1.9 Representation of the human PON1 structure (taken from La Du *et al.*, 1999).

The overall architecture of PON1 was dissolved in 2004 by Harel and colleagues. PON1 is a six-bladed β-propeller, and each blade contains four strands (Figure 1.10) (Harel *et al.*, 2004). Two calcium ions, 7.4 Å apart, are seen in the central tunnel of the propeller, one at the top (Ca1) and one in the central section (Ca2). Ca2 is most probably a 'structural calcium' whose dissociation leads to irreversible denaturation. Ca1 is assigned as the 'catalytic calcium' (Kuo and La Du, 1998; Harel *et al.*, 2004). The two calcium ions exhibit markedly different affinities; Ca2 is the higher-affinity calcium (Kuo and La Du, 1995; Harel *et al.*, 2004).

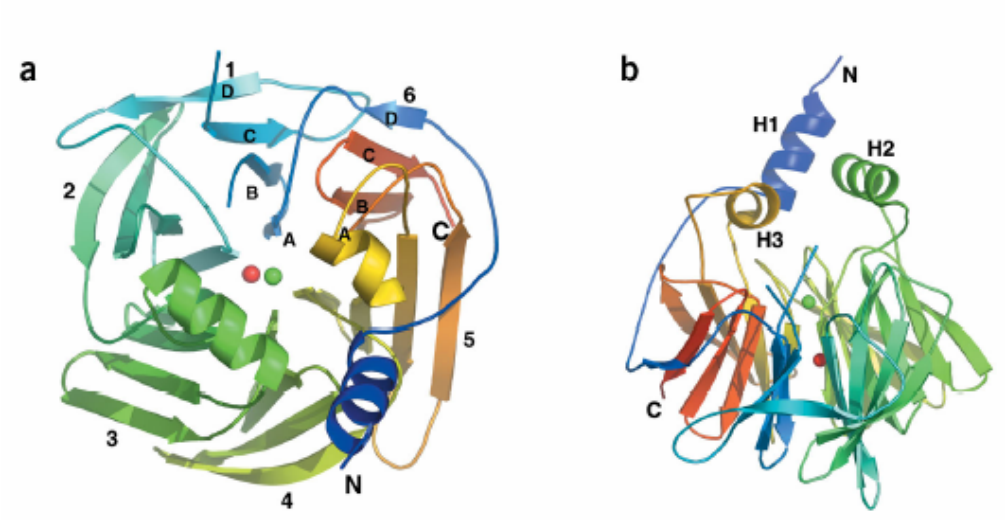


Figure 1.10 Overall structure of PON1. a) View of the six-bladed β-propeller from above. Shown are the N and C termini, and the two calcium atoms in the central tunnel of the propeller (Ca1, green; Ca2, red). b) A side view of the propeller, including the three helices at the top of the propeller (H1–H3) (taken from Harel *et al.*, 2004).

PON1 has 3 Cys residues in positions 42, 284, and 353. Purified native PON1 contains a disulfide bond between Cys-42 and Cys-353, Cys-284 is free (Kuo and La Du, 1995; Harel *et al.*, 2004). PON1 has a carbohydrate content of 15.8 % (w/v) (Gan *et al.*, 1991). Glycosylation is not essential for the hydrolytic activities of PONs (Aharoni *et al.*, 2004; Josse *et al.*, 1999) but may be important in increasing their solubility and stability, or in preventing nonspecific binding to cell membranes, as proposed for other HDL-associated enzymes (Jonas, 2000, Harel *et al.*, 2004).

1.2.2.3 Structure-Activity Relationships

PON1 has a broad substrate specificity since it hydrolyzes organophosphates, arylesters, carbamates, cyclic carbonate esters (Draganov and La Du, 2004), lactones (Jakubowski, 2000), and possibly phospholipids (Josse *et al.*, 2002). The three-

dimensional structure does provide a hint regarding the origins of PON1's notably wide substrate range (Harel *et al.*, 2004). Hydrophobicity is common to almost all of PON1's effective substrates. The hydrophobicity and depth of PON1's active site explain this preference, and account for the fact that PON1's substrates, whether poor or effective, have *K*m values in the millimolar range, but markedly different *k*cat values (Draganov and La Du, 2004; Aharoni *et al.*, 2004; Harel *et al.*, 2004). PON1's multispecificity is, therefore, driven primarily by nonspecific hydrophobic forces, as has been observed for other enzymes with deep hydrophobic active sites, such as acetylcholinesterase (Greenblatt *et al.*, 2003). Harel and colleagues (2004) postulated that poor and effective substrates bind at the active site with similar affinity; yet the mode of binding differs, as the poor substrates are inadequately positioned relative to Ca1 and to the catalytic base.

Catalysis of both C-O and P-O hydrolyses at one site is unusual but not unprecedented (Millard *et al.*, 1998; Bencharit *et al.*, 2003; Harel *et al.*, 2004). The structure, the directed evolution results, the pH-rate profiles and previous biochemical data (Draganov and La Du, 2004; Aharoni *et al.*, 2004) show that both these activities take place at the same site (Harel *et al.*, 2004). At this stage, however, a possibility remains that certain PON1 activities, for example, as a homocysteine thiolactonase (Jakubowski, 2000), make use of a different subset of residues of this site (for details see section 1.2.2.4) (Harel *et al.*, 2004).

It is clearly established that chelation of calcium ions and mutations or deletion of residues essential for the PON1 hydrolase activity do not abolish the antioxidative action of PON1 (Aviram *et al.*, 1998a). Conversely, the free cysteine (Cys284) was reported to be essential for the antioxidative activity (Aviram *et al.*, 1998a), but could be substituted with Ala without dramatically changing the hydrolase activity (Sorenson *et al.*, 1995). Harel and colleagues (2004) suggested that Cys284 is part of a highly conserved stretch that includes active site His285, and is packed against four highly conserved residues from the adjacent strands. Because it is buried, it is unlikely to have a functional role. Its mutation, however, is likely to destabilize the

core structure, thereby affecting function indirectly. Indeed, it was found that Cys284 mutants of rePON1 are poorly expressed and relatively unstable (Harel *et al.*, 2004).

Polymorphism of the PON1 gene and its effects on susceptibility to organophosphate poisoning and to atherosclerosis are the subject of intensive research. The two most common PON1 forms are 192Q/R and 55L/M. The structure of a variant of PON1 obtained by directed evolution by Harel and coworkers (2004) reveals that Lys192 is part of the active site wall. In human PON1, this position is either glutamine or arginine due to the commonly observed 192Q/R polymorphism. The 55L/M polymorphism may considerably affect PON1's stability and thereby account for the lower enzymatic activity (Leviev *et al.*, 2001a). This is due to the key role of Leu55 in packing the propeller's central tunnel, and of its neighboring residues, which ligate both Ca1 and Ca2 (Harel *et al.*, 2004).

1.2.2.4 The Catalytic Mechanism

PON1 has a unique active site lid that is also involved in HDL binding. At the very bottom of the active site cavity, the upper calcium (Ca1), and a phosphate ion are found (Figure 1.11b).

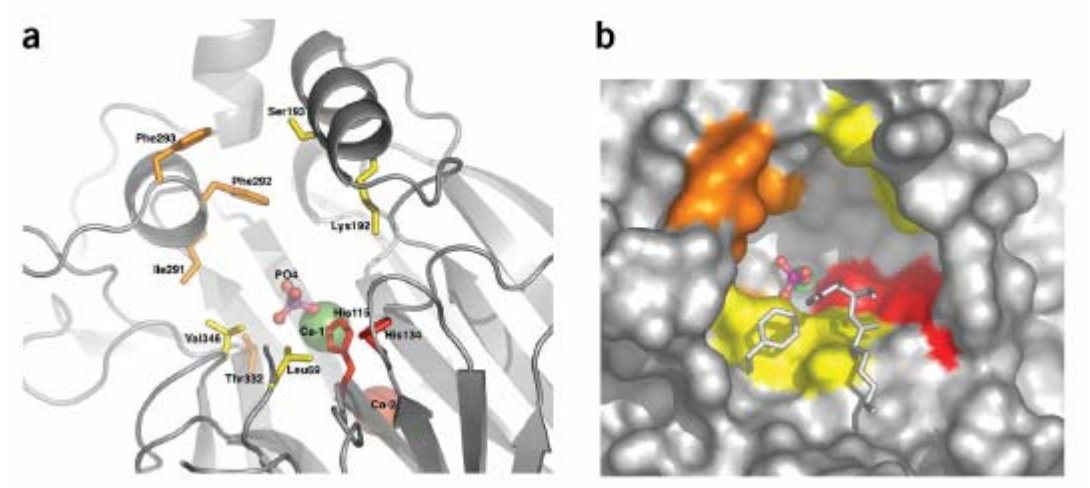


Figure 1.11 PON1's active site viewed from above the propeller. a) Central tunnel of the propeller with the two calcium atoms, and the side chains of the residues found to be mutated in the newly evolved PON1 variants for esterase and lactonase (orange) or for phosphotriesterase activity (yellow), including the 192Q/R human polymorphism (in the rePON1-G2E6 variant, this position is a lysine). The putative catalytic His-His dyad is red. b) A surface view of the active site. Lys70, Tyr71 and Phe347 are shown as sticks to permit a better view of the active site. At the deepest point of the cavity lies the upper calcium atom (Ca1, green) to which a phosphate ion is bound (taken from Harel *et al.*, 2004).

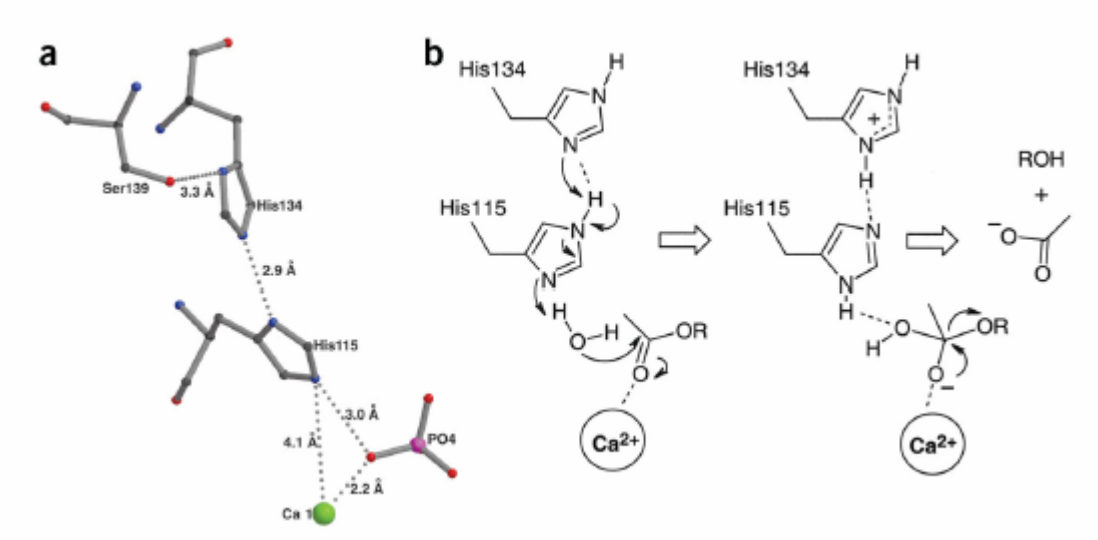


Figure 1.12 The postulated catalytic site and mechanism of PON1. a) The catalytic site includes the upper calcium atom (Ca1), the phosphate ion at the bottom of the active site and the postulated His-His dyad. b) Schematic representation of the proposed mechanism of action of PON1 on ester substrates such as phenyl and 2-naphthylacetate. The first step involves deprotonation of a water molecule by the His-His dyad to generate a hydroxide anion that attacks the ester carbonyl, producing an oxyanionic tetrahedral intermediate. This intermediate breaks down (second step) to an acetate ion and either phenol or 2-naphthol (taken from Harel *et al.*, 2004).

One of the phosphate's oxygens is only 2.2 Å from Ca1. This phosphate ion may be bound in a mode similar to that of the intermediates in the hydrolytic reactions catalyzed by PON. One of its negatively charged oxygens, the one nearest to Ca1, may mimic the oxyanionic moiety of these intermediates, which is stabilized by the positively charged calcium (Harel *et al.*, 2004). Harel and coworkers (2004) identified a His-His dyad near both Ca1 and the phosphate ion (Figure 1.12). They (Harel *et al.*, 2004) hypothesized that His115 (the closer nitrogen of which is only 4.1 Å from Ca1) acts as a general base to deprotonate a single water molecule and generate the attacking hydroxide, whereas His134 acts in a proton shuttle mechanism to increase His115's basicity. In support of the postulated mechanism are the H115Q mutation, which caused a marked decrease (~2×10^4-fold) in activity, and the H134Q mutation, which produced a milder, yet substantial, decrease (6–150-fold) (Harel *et al.*, 2004).

It is possible that certain PON1 activities, for example, homocysteine thiolactonase (Jakubowski, 2000) activity, make use of a different subset of residues of this site, including His285, whose side chain also points toward the center of the cavity and to the phosphate ion (Harel *et al.*, 2004).

1.2.2.5 Enzymatic Activities of PON1

PON1 hydrolyzes a variety of substrates (see Table 1.5), including the toxic oxon metabolites of a number of insecticides such as parathion, diazinon and chlorpyrifos, nerve agents such as sarin and soman, aromatic esters such as phenyl acetate, lactones, cyclic carbonates and phospholipid hydroperoxides (see Fig. 1.13a, b; Draganov and La Du, 2004).

Most organophosphate insecticides are biotransformed by the cytochrome P450 system to produce their toxicities (see section 1.2.2.5.1). Among the aromatic ester substrates for PON1 are phenylacetate, thiophenylacetate, and 2-naphthylacetate (Figure 1.13c; Draganov and La Du, 2004).

Table 1.5 Substrates of human PON1.

Oxon metabolites of organophosphates	**Aryl (aromatic) esters**
paraoxon	phenyl acetate
methyl paraoxon	thiophenylacetate
pirimiphos-methyl oxon	2-naphtylacetate
chlorpyrifos oxon	**Aromatic lactones**
diazoxon	**Aliphatic lactones**
chlorthion oxon	dihydrocoumarin
EPN oxon	γ-butyrolactone
fenitroxon	homocysteine thiolactone
	Cyclic carbonates
Nerve Agents (gases)	prulifloxacin
soman	**Phospholipid hydroperoxides**
sarin	
armin	

A variety of aromatic and aliphatic lactones as well as cyclic carbonates are also hydrolyzed by PON1, e.g. homogentisic acid lactone, dihydrocoumarin, γ-butyrolactone, and homocysteine thiolactone (Figure 1.13d). PON1 also catalyzes the reverse reaction, lactonization, of γ- and δ-hydroxycarboxylic acids (Draganov and La Du, 2004; Draganov *et al.*, 2005). Since lactones are common constituents of plants, and are natural flavoring agents in many food products, lactonase activity may represent an important common feature of the PON enzymes. Protection against dietary and environmental lactones could even be a selective force responsible for maintaining the balanced polymorphisms found in the mammalian PON enzymes (Draganov and La Du, 2004). Jakubowski (2000) determined that PON1 is an L-homocysteine thiolactone hydrolase. However, it has a very low affinity (Km ~23 mM) for L-homocysteine thiolactone (Jakubowski, 2000) when compared to phenylacetate and paraoxon (Km ~0.5 mM) (Gan *et al.*, 1991; Smolen *et al.*, 1991).

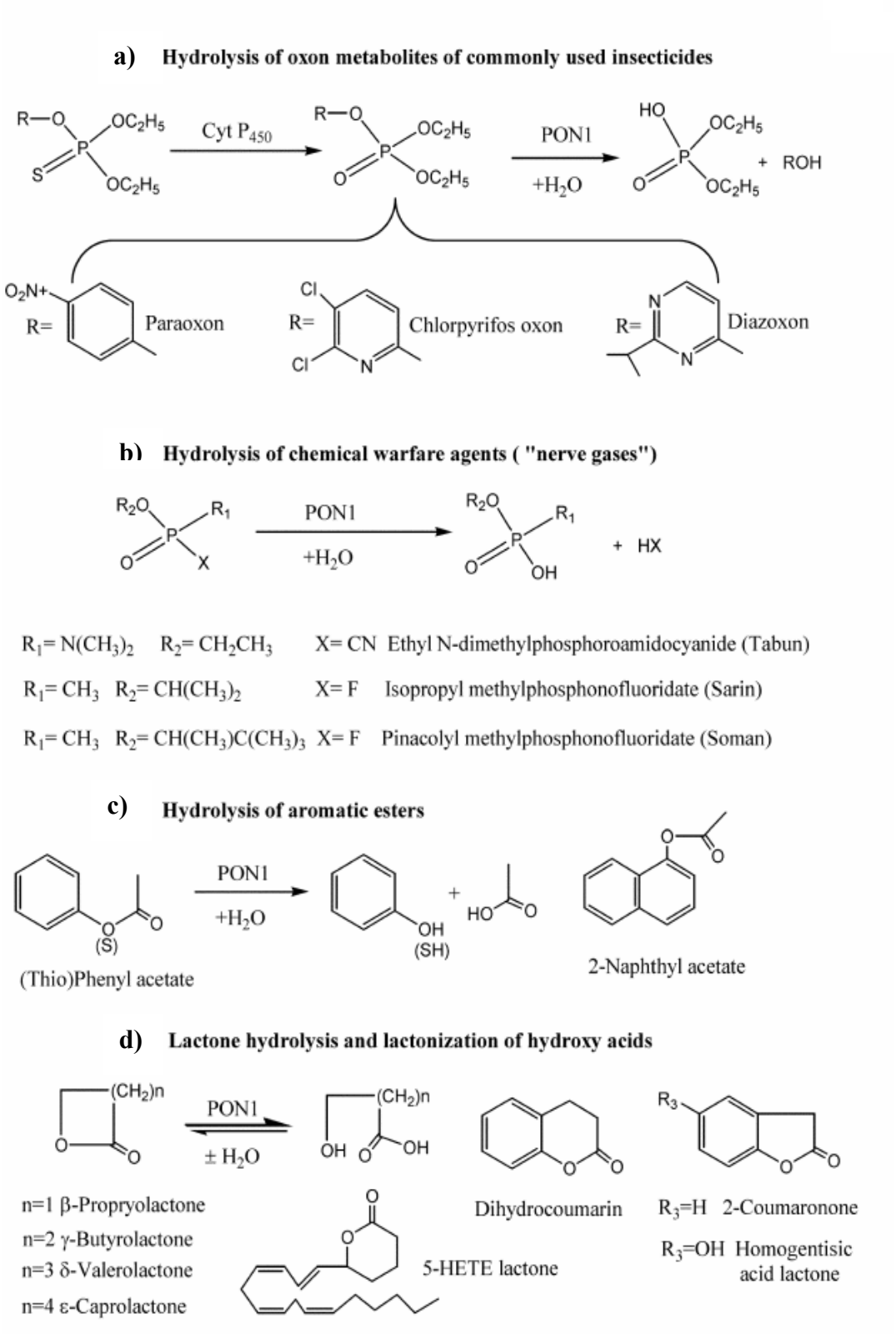


Figure 1.13 Enzymatic activities and representative substrates for human PON1 (taken from Draganov and La Du, 2004).

PON1 also hydrolyzes the unsaturated cyclic carbonate prodrug prulifloxacin to the active quinolone antibiotic NM394 (Tougou *et al.*, 1998). PON1's lactonase activity has been utilized in the development of locally acting glucocorticoid drugs, which undergo rapid hydrolysis and inactivation when they reach the circulation, and thus serum PON1 prevents their unwanted systemic effects (Biggadike *et al.*, 2000). Lactone hydrolysis of lovastatin, simvastatin and spironolactone reported for purified human serum PON1 (Billecke *et al.*, 2000) was due to PON3, present in small amounts in the PON1 preparations (Draganov *et al.*, 2005). PON1 is also known to hydrolyze phospholipid hydroperoxides and in this way protect LDL and HDL against oxidative modifications (Costa *et al.*, 2003; see section 1.2.2.5.2).

1.2.2.5.1 Role of PON1 in Organophosphate Selective Toxicity

Many organophosphorus (OP) compounds are triesters of phosphoric acid. Their major source is from pesticides, although some have found therapeutic applications. Acute exposure to OPs causes neurotoxicity, in the form of a cholinergic syndrome, i.e., an overstimulation of muscarinic and nicotinic acetylcholine receptors in the central and peripheral nervous systems, due to accumulation of acetylcholine in the synaptic cleft resulting from OPs' inhibition of acetylcholinesterase (Lotti, 2000; Costa *et al.*, 2003). Only OPs with a P=O moiety can interact with acetylcholinesterase. Commonly used organophosphorus pesticides, such as parathion, diazinon and chlorpyrifos, are generally applied in agriculture as the relatively nontoxic sulfur (thion) derivatives. Their P=S bond need to be converted to their oxygen analogs to become toxic. They are activated *in vivo* by cytochrome-P450-dependent microsomal monooxygenases (CYP) to the highly toxic oxygen (oxon) analogue by a process known as oxidative desulfuration (Figure 1.14; La Du, 1992; Costa *et al.*, 2003).

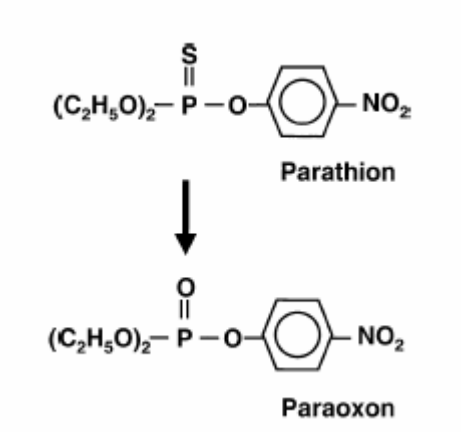


Figure 1.14 Biotransformation of parathion to paraoxon by the cytochrome P450 system (taken from Luft, 2001).

Limited *in vitro* studies have shown that CYP3A4 is the major form responsible for the oxidation of parathion, and perhaps of other OPs, to its oxygen analog (Butler and Murray, 1997). This process is believed to take place largely in the liver, where there are several enzymes capable of metabolizing both thions and oxons such as glutathione-*S*-transferases (Baars and Breimer, 1980), monooxygenases (Armstrong, 1987; Can Demirdöğen and Adali, 2005) and paraoxonases (La Du, 1992; Mackness *et al.*, 1998a). Thus the balance of hepatic OP metabolism is toward detoxication (Mackness *et al.*, 1998a).

In mammals, any oxon that escapes hepatic detoxification can be hydrolyzed in the blood by serum paraoxonase before it reaches the brain, which is the site of OP action. With some OPs—for example, pirimiphos-methyloxon—hydrolysis by serum paraoxonase is so rapid that it has been estimated that no active OP actually reaches the brain by this route (Brealey *et al.*, 1980; Mackness *et al.*, 1998a). Many studies have shown that serum paraoxonase plays a significant role in the detoxication of a variety of OPs in mammals (Li *et al.*, 1995; Pond *et al.*, 1995; Mackness *et al.*, 1998a).

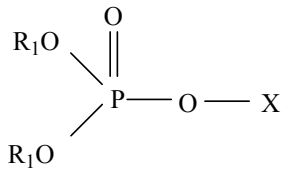
Evidence for the physiological importance of PON1 in modulating exposures to chlorpyrifos oxon and diazoxon comes from several different studies. Early studies noted that species with high levels of PON1 were much more resistant to certain organophosphorus (OP) insecticides than were species with low levels (Furlong *et al.*, 2005). In detailed studies of the selective toxicity of OPs in birds compared with mammals (Brealey *et al.*, 1980), birds were found to be much more susceptible to OP poisoning than were mammals; this finding was due to the almost complete absence of serum paraoxonase in birds (Mackness *et al.*, 1998a). Injecting purified rabbit PON1 into rats or mice significantly increased resistance to paraoxon, chlorpyrifos and chlorpyrifos oxon, thus demonstrating that high PON1 levels were protective against exposure (Furlong *et al.*, 2005). The consequences of low levels of plasma PON1 were examined in PON1 knockout mice generated by Shih and co-workers (Shih *et al.*, 1998). These mice were found to be highly sensitive to exposures to either diazoxon or chlorpyrifos oxon, but surprisingly not to paraoxon (Furlong *et al.*, 2005).

Although purified human serum paraoxonase has been shown to hydrolyze several OP compounds (Smolen *et al.*, 1991; Mackness *et al.*, 1991a), the extent to which it contributes to human metabolism of OPs is not known (Mackness *et al.*, 1998a). The molecular polymorphisms of human serum paraoxonase, which will be explained in section 1.2.2.7.1, do, however, have the potential to be determinants of the variable toxicity of OPs in different people (Weber, 1995; Mackness *et al.*, 1998a). An additional issue related to the role of PON1 in determining sensitivity to OP toxicity stems from the possibility that young children may be more sensitive than adults to the toxic effects of certain pesticides (Eskenazi *et al.*, 1999; Costa *et al.*, 2003). The acute toxicity of OPs appears to be influenced by age, with young animals being more sensitive (Benke and Murphy, 1975; Pope and Liu, 1997; Costa *et al.*, 2003).

1.2.2.5.2 Role of PON1 in Lipid Metabolism and Atherosclerosis

In the 1980's Mackness and coworkers first suggested a role for serum PON1 in the metabolism of phospholipids based on similarities in the structures of organophosphates and phospholipids (Figure 1.15; Mackness, 1989; Mackness *et al.*, 2002a).

a)



b)

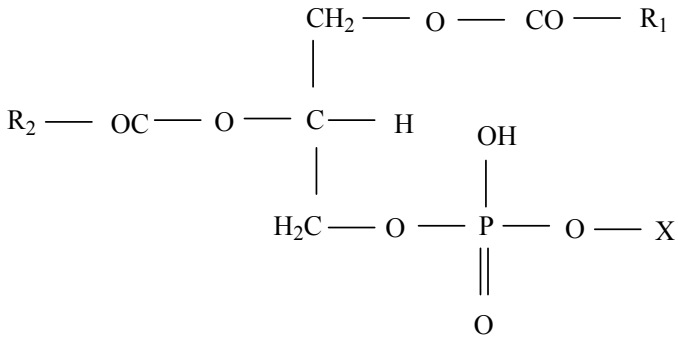


Figure 1.15 General structures of organophosphates and phospholipids. a) R_1= alkyl groups, X= "leaving group", usually an aromatic group but sometimes a halide b) R_1 and R_2=fatty acid residues, X=an organic base, amino acid or alcohol such as choline or ethanolamine (taken from Mackness *et al.*, 2002a).

Of the proteins present on HDL that possess enzymatic (usually hydrolytic) activity (Table 1.6), it was hypothesized that PON1 in the human was principally responsible for the breakdown of lipid peroxides before they could accumulate on LDL (Mackness *et al.*, 1991b; Mackness *et al.*, 1993a and b).

Table 1.6 Proteins present in human HDL that possess enzymatic activity (taken from Durrington *et al.*, 2001). PAFAH; platelet activating factor acetyl hydrolase. LCAT; lecithin cholesterol acetyl transferase.

Proteins in HDL with enzymatic activity
PON1
LCAT
PAFAH
Proteinase (elastase-like)
Phospholipase D
Albumin
Apo A-I

This hypothesis was originally based on findings by Mackness and coworkers (1991b) that purified human PON1 was highly effective in preventing lipid peroxidation of LDL (Mackness *et al.*, 1991b; Mackness *et al.*, 1995) which has since been confirmed (Watson *et al.*, 1995a; Arrol *et al.*, 1996; Aviram *et al.*, 1998b; Shih *et al.*, 1998; Durrington *et al.*, 2001). In the experiments of Arrol and colleagues, PON1 was substantially more effective than was LCAT or apoA-I in protecting LDL against oxidation, although the combination of all 3 did slightly enhance the effect of PON1 alone (Arrol *et al.*, 1996; Durrington *et al.*, 2001) (Figure 1.16).

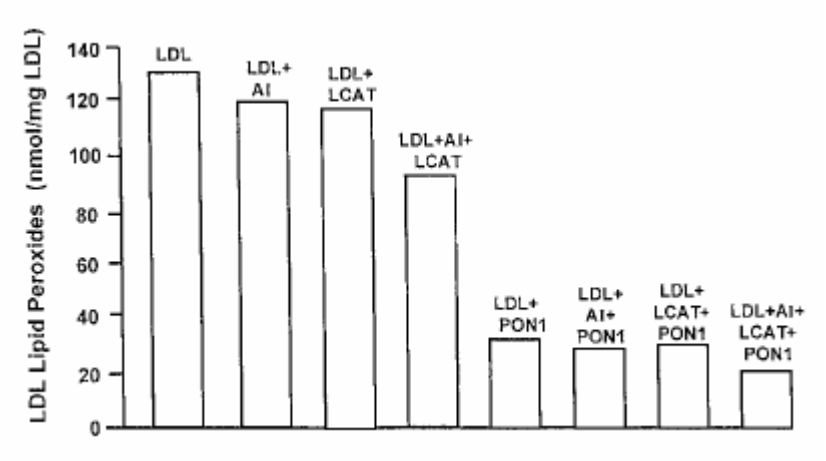


Figure 1.16 Inhibition of LDL lipid peroxidation by PON1, ApoA-I (AI), and LCAT (taken from Durrington *et al.*, 2001).

Platelet-activating factor (PAF) acetyl hydrolase (PAFAH) has an action resembling that postulated for PON1. Although PAFAH is undoubtedly present in HDL, it is not established that the PAFAH activity of HDL is due to anything other than PON1 (Rodrigo *et al.*, 2001a; Durrington *et al.*, 2001). Avian HDL has no PON1 activity and fails to protect human LDL against lipid peroxidation (Mackness *et al.*, 1998b; Durrington *et al.*, 2001). Experiments with inhibitors of PON1 also suggest that it is responsible for the antioxidant effect of HDL (Aviram *et al.*, 1998b; Durrington *et al.*, 2001). Experiments with PON1 knockout mice were also unequivocal: serum PAFAH activity was unaltered in the PON1 knockout mice, yet their HDL failed to protect LDL against oxidation. Moreover, they were susceptible to diet-induced atherosclerosis (Shih *et al.*, 1998; Durrington *et al.*, 2001). In mice overexpressing PON1, decreased atherosclerotic lesion formation was observed (Tward *et al.*, 2002; Oda *et al.*, 2002; Mackness *et al.*, 2006).

HDL-associated PON efficiently protects HDL from oxidative damage. PON-mediated hydrolysis of lipid peroxides and of cholesteryl linoleate hydroperoxides in oxidized HDL are two mechanisms for PON-induced inhibition of HDL oxidation. A hypothesized mechanism for the hydrolysis of oxidized lipids in macrophages by

PON1 to yield lysophosphatidylcholine (LPC) is given in Figure 1.17. Oxidized lipids with hydroxyl groups at the 5-position or related derivatives could be lactonized by PON1 to yield LPC and the respective –valerolactone products. The latter can be hydrolyzed by PON1 to yield the corresponding 5-hydroxycarboxylic acid or remain intact, depending on the pH and water content of the environment (Rosenblat *et al.*, 2006). Cellular LPC formation by PON1 enhances HDL binding to macrophages and HDL-mediated macrophage cholesterol efflux (Rosenblat *et al.*, 2005; Table 1.7). PON1 was also found to be able to substantially hydrolyze hydrogen peroxide (H_2O_2), a major reactive oxygen species produced under oxidative stress during atherogenesis. Thus, PON1 preserves antiatherogenic functions of HDL in reverse cholesterol transport, as well as its protection of LDL from oxidation (Aviram *et al.*, 1998b).

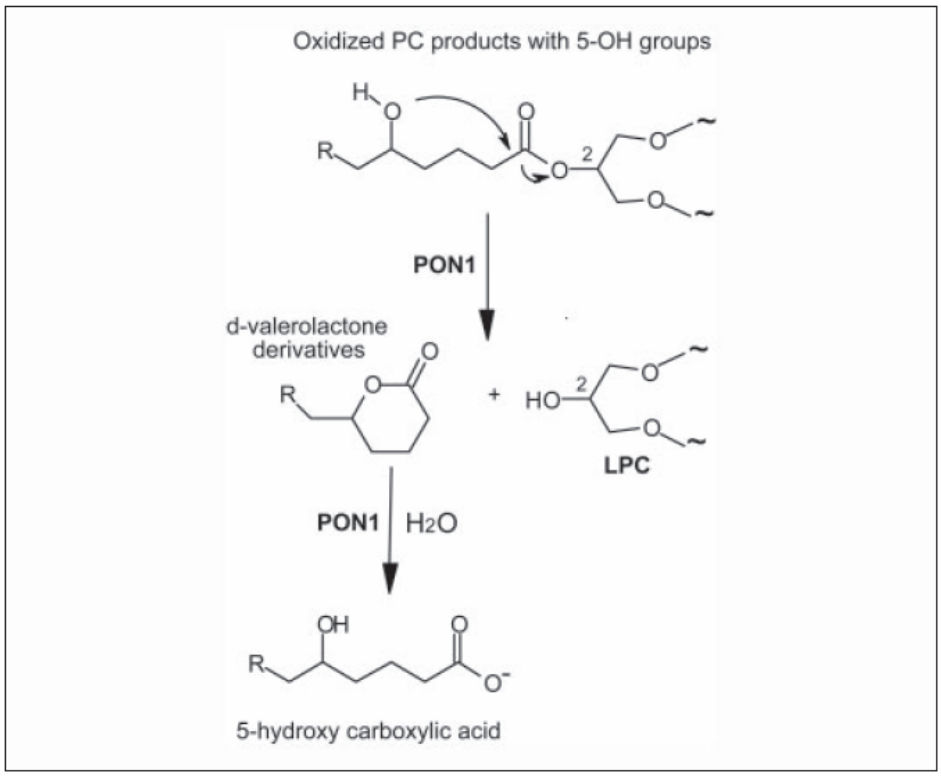


Figure 1.17 Hypothesized mechanism for hydrolysis of oxidized lipids in macrophages by PON1 to yield LPC (taken from Rosenblat *et al.*, 2006).

Evidences for human PON1 antiatherogenicity are summarized in Table 1.7. As stated before, macrophage foam cell formation is the hallmark of early atherogenesis and cellular cholesterol accumulation is dependent on the balance between cholesterol influx and efflux. PON1 contibutes to a decrement in macrophage cholesterol accumulation by inhibiting cholesterol biosynthesis (Rozenberg *et al.*, 2003) and uptake of oxidized LDL via the scavenger receptor (Fuhrman *et al.*, 2002). PON1 increases HDL binding to macrophages and thereby stimulates HDL-mediated macrophage cholesterol efflux by its hydrolytic action on macrophage phospholipids, to form lysophosphatidylcholine, which increases HDL binding to the cells (Rosenblat *et al.*, 2005; see Figure 1.18).

Table 1.7 Evidence for human PON1 antiatherogenicity (taken from Draganov and La Du, 2004).

1. Protects LDL against oxidation ↓ lipid peroxides
2. Protects HDL against oxidation and preserves its functions ↑ cellular cholesterol efflux from macrophages
3. Ameliorates effects of oxidized LDL ↓ inflammatory and cytotoxic oxidized phospholipids ↓ LDL uptake by macrophages ↓ monocyte transmigration induced by oxidized LDL
4. Decreases lipid peroxides in atherosclerotic lesions

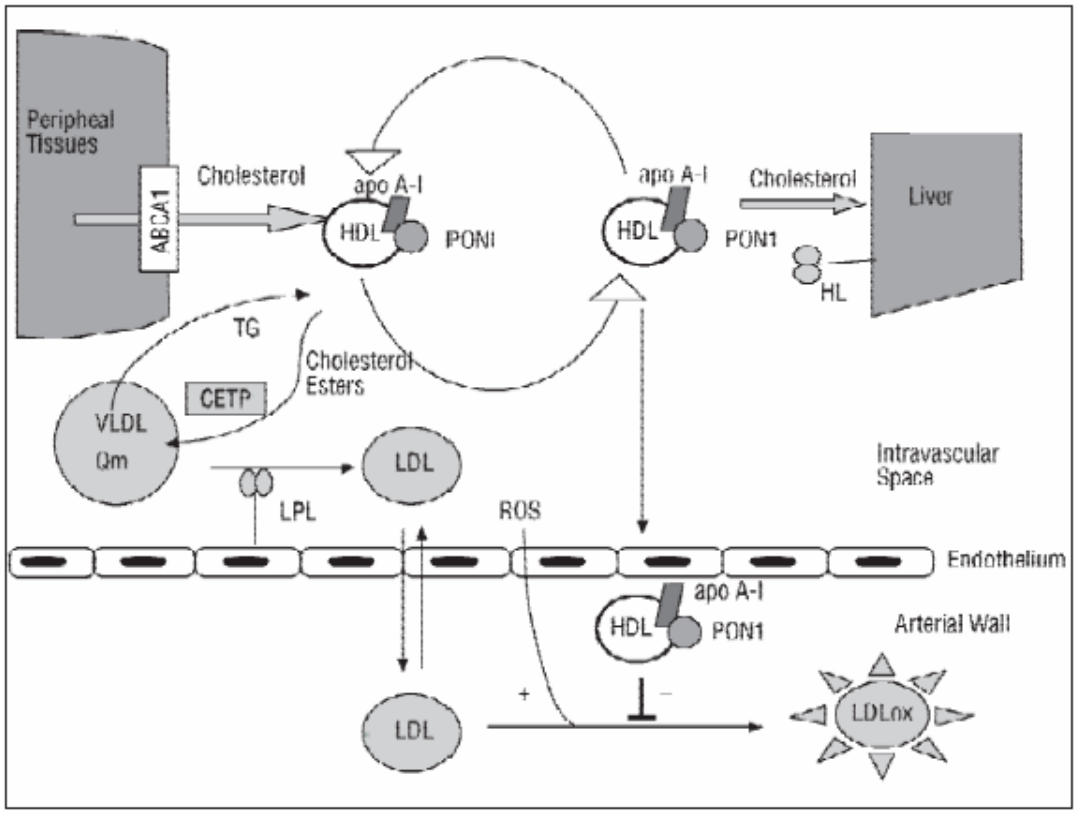


Figure 1.18 The antioxidant role of paraoxonase-1 in arteriosclerotic plaque formation. ABCA1 indicates ATP-binding cassette A1; apo A-I, apolipoprotein A-I; CETP, cholesterol ester transfer protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLox, oxidized LDL; HL, hepatic lipase; LPL, lipoprotein lipase; PON1, paraoxonase-1; Cm, chylomicron; ROS, reactive oxygen species; TG, triglycerides; VLDL, very-low-density lipoprotein (taken from Tomás *et al.*, 2004).

PON1 is located in a subfraction of HDL that contains apoA-I and clusterin (apoJ) (Mackness *et al.*, 1981; Kelso *et al.*, 1994; Blatter *et al.*, 1993). It has been suggested that this subfraction of HDL may function to protect cell membranes generally against lipid peroxidation and other toxic effects (Durrington *et al.*, 2001; Mackness *et al.*, 1995). Clusterin has likewise been proposed as a protein protecting cell membranes (Jordan-Starck *et al.*, 1992; Durrington *et al.*, 2001). HDL is the most abundant protein in the tissue fluid and, indeed, the only lipoprotein in the central nervous system (Mackness *et al.*, 1997a; Durrington *et al.*, 2001). It is unlikely that its antioxidant function has evolved to protect humans against atheroma, a disease that appears to have been prevalent for less than a century (Herrick, 1912; Durrington *et al.*, 2001). Therefore, its antioxidant capacity is probably part of a much older protective role, and LDL shares in this protection because of its resemblance to a cell membrane (Durrington *et al.*, 2001).

Lipid substrates of PON1

The exact substrate specificity of PON1 has yet to be determined. PON1 exhibits high activity toward short chain esters, and lower activity for long chain esters, which are typical substrates of lipases (Harel *et al.*, 2004). It is known that hydroperoxides of linoleate or arachidonate-containing phospholipids can act as substrates for PON1 (Aviram *et al.*, 1998b; Mackness *et al.*, 2002a). It has been suggested that PON1 hydrolyzes the fatty acid hydroperoxide to produce a short chain fatty acid aldehyde or ketone derivative still attached to the phospholipid backbone, which in turn becomes a substrate for PAFAH (Watson *et al.*, 1995a; Mackness *et al.*, 2002a) to produce lysophospholipid which is potentially proatherogenic. Arachidonate and linoleate hydroperoxide derivatives of cholesteryl esters are also substrates for PON1 (Aviram *et al.*, 1998a, b). Aviram and coworkers (2000b) showed that cholesteryl lineolate hydroperoxides and cholesteryl lineolate hydroxides in human coronary and carotid artery atherosclerotic lesion homogenates were hydrolyzed by PON1 to produce cholesterol and linoleic acid hydroperoxides and linoleic acid hydroxides (Mackness *et al.*, 2002a). Purified PON1 could also reduce linoleic acid hydroperoxides to linoleic acid hydroxides. This finding indicates that PON1 has both esterase and peroxidase type activities (Mackness *et al.*, 2002a).

1.2.2.5.3 Protection against Toxicity from Bacterial Endotoxins

For some years, the plasma high density lipoprotein complex (HDL) has been known to be protective against endotoxin toxicity (Levine *et al.*, 1993; La Du *et al.*, 1999). Only recently has it been possible to obtain conclusive evidence that the protein component of HDL responsible for these protective effects is PON1 (La Du *et al.*, 1999). The intraperitoneal (IP) injection of purified human PON1 (type *Q*) 2 h before an IP injection of lipopolysaccharide (LPS), which mimics gram negative infections, to adult mice resulted in the survival of 60 % of the animals. In contrast, giving the PON1 about 2 h after the LPS reduced the survival rate to about 30 %. Without the PON1 injection all the mice died from the LPS treatment (La Du *et al.*, 1999). These and other experiments have led to the proposal by La Du and colleagues (1999) that PON1 is able to protect cells from LPS and prevent, or greatly reduce the release of cytokines (La Du *et al.*, 1999).

1.2.2.5.4 Possible Physiological Roles for PON1

Several studies in recent years suggest that PON1's organophosphatase and arylesterase activities, which utilize mostly human-made chemicals, seem to be ancillary rather than primary function of the enzyme (Harel *et al.*, 2004): natural organophosphates such as anatoxin-a, for example, are not hydrolyzed by PON1 (La Du *et al.*, 1999; Draganov and La Du, 2004). In contrast, lactonase/lactonizing activities utilize natural substrates and are shared by all three PONs and their homologues (Draganov and La Du, 2004). Homocysteinylation is a potential contributing factor to atherosclerosis. By detoxifying homocysteine thiolactone, paraoxonase would protect proteins against homocysteinylation, and thereby against atherosclerosis (Jakubowski, 2000). Thus, possible physiological roles of PON1 are related to lactonase activity and lipid metabolism (Draganov *et al.*, 2005).

1.2.2.6 PON1 Activity is Variable between Individuals

Early studies on rates of paraoxon hydrolysis indicated that there is a 10-40-fold difference in serum paraoxonase activity between individuals (Humbert *et al.*, 1993) and serum paraoxonase activity was polymorphically distributed in human populations (Krisch, 1968; Playfer *et al.*, 1976; Zech and Zurcher, 1974). There was considerable controversy as to whether the activity was bi- or trimodally distributed. A number of different assay conditions were used to explore the polymorphism (reviewed in Ortigoza-Ferado *et al.*, 1984). Since single substrate assays were not capable of distinguishing the three activity phenotypes, Eckerson and colleagues (1983a) introduced a two substrate analysis where rates of phenylacetate hydrolysis were plotted against rates of paraoxon hydrolysis for individuals of a sampled population. This analysis provided a much better resolution of three phenotypes and also clearly demonstrated that a single substrate assay analysis did not have the capability to resolve the three paraoxonase phenotypes. They postulated that a single enzyme with two isozymic forms hydrolyzed both phenylacetate and paraoxon. Paraoxon was termed a discriminating substrate with a polymorphic distribution of activities while phenylacetate was termed a non-discriminating substrate (Brophy *et al.*, 2002).

1.2.2.7 Sources of Variation in PON1 Activity

1.2.2.7.1 PON1 Polymorphisms

Genetic polymorphisms are natural variations in the genomic DNA sequence present in greater than 1% of the population, with single nucleotide polymorphisms (SNPs) representing DNA variations in a single nucleotide. All together, around 200 SNPs have been identified in the human PON1 gene (see Table 1.8 and http://www.ncbi.nlm.nih.gov), many of which are in strong linkage disequilibrium and form certain haplotypes within the gene (Jarvik *et al.*, 2003).

Table 1.8 Current number of SNPs identified in human PON1 gene (taken from La Du, 2003).

Gene region	No. of SNPs
Nontranslated 5'-end	7
Exons	5
55L/M (Exon 3)	
102I/V (Exon 4)	
160R/G (Exon 5)	
192Q/R (Exon 6)	
194W/stop (Exon 6)	
Introns	171
Non-translated 3'-end	15
Total	198

Presence of linkage disequilibrium limits the usefulness of association studies with particular PON1 genotypes as emphasized in the "PON1 status" section below (Draganov and La Du, 2004). Figure 1.19 gives a schematic representation of PON1 polymorphisms in the 5'-flanking region, coding region and the 3' UTR.

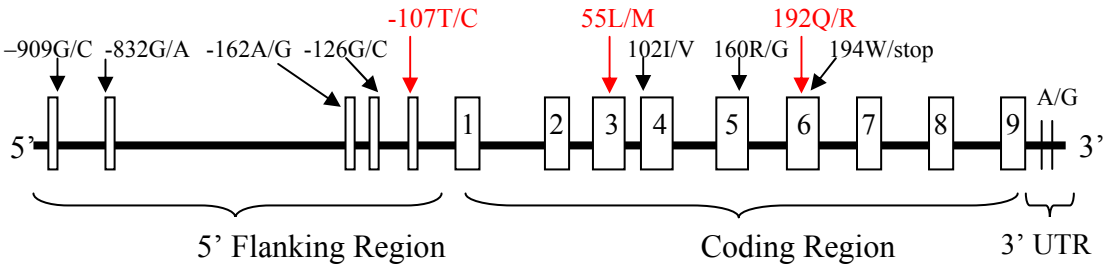


Figure 1.19 Schematic representation of human PON1 gene with associated polymorphisms. The numbered boxes represent the exons; polymorphisms occuring throughout the gene are indicated by arrows; the polymorphisms investigated in this study are indicated in red arrows (adapted from Brophy *et al.*, 2002).

1.2.2.7.1.1 PON1 Coding Region Polymorphisms

PON1 coding region contains 5 SNPs (Table 1.8). In the earlier studies, two polymorphisms were observed in the PON1 coding sequence: a leucine (L) to methionine (M) substitution at position 55 (55L/M) and a glutamine (Q) to arginine (R) substitution at position 192 (192Q/R). Subsequent research demonstrated that the 192Q/R polymorphism was responsible for the activity polymorphism (Adkins *et al.*, 1993; Humbert *et al.*, 1993). PON1 192*Q* hydrolyzed paraoxon slower than PON1 192*R*. The two polymorphisms should result in the following genotypes:

Homozygotes	Heterozygotes
55*MM*/192*QQ*	55*LM*/192*QQ*
55*LL*/192*QQ*	55*LM*/192*RR*
55*MM*/192*RR*	55*LM*/192*QR*
55*LL*/192*RR*	55*LL*/192*QR*
	55*MM*/192*QR*

However, Adkins and coworkers (Adkins *et al.*, 1993) noted that the 55*L* and 192*R* alleles were in strong disequilibrium. Other studies have confirmed this strong disequilibrium with approximately 98% of the 192*R* alleles having *L* at position 55 (Blatter-Garin *et al.*, 1997; Mackness *et al.*, 1998c; Schmidt *et al.*, 1998; Brophy *et al.*, 2000; 2001a, b).

Recently, two other polymorphisms in the PON1 coding region have been reported (Figure 1.19): isoleucine to valine at position 102 in Finns (Marchesani *et al.*, 2003) and arginine to glycine at position 160 in the Chinese Han population (Wang *et al.*, 2003). Their presence in other populations remains unknown. At present it is also not known whether these polymorphisms have any effect on PON1's hydrolytic and/or protective activities (Draganov and La Du, 2004).

1.2.2.7.1.1.1 Effect of the Coding Region Polymorphisms on PON1 Activity

The initial observations of large differences in rates of paraoxon hydrolysis that were the basis of the early studies on the PON1 polymorphism were explained by the position 192Q/R polymorphism (Adkins *et al.*, 1993; Humbert *et al.*, 1993; Brophy *et al.*, 2002). Population distribution studies of PON1 activities with the substrates paraoxon, chlorpyrifos oxon, diazoxon, soman, and sarin further indicated that the position 192Q/R polymorphism was responsible for the differences in rates of hydrolysis of these substrates in a substrate dependent manner (Davies *et al.*, 1996; Richter and Furlong 1999; Brophy *et al.*, 2002). The 192*Q* and 192*R* allozymes have different affinities and catalytic activities towards a number of substrates (Table 1.9; Deakin and James, 2004). The PON1 192*Q* isoform was found to hydrolyze paraoxon more slowly, but diazoxon, sarin, soman (Davies *et al.*, 1996) and phospholipid hydroperoxides (Mackness *et al.*, 1997b) more rapidly than PON1 192*R*. Conversely, PON1 192*R* hydrolyzes paraoxon and chlorpyrifos rapidly, and diazoxon more slowly *in vitro*. Phenylacetate hydrolysis rates are not polymorphic and both isoforms hydrolyze this substrate with equal rates (Eckerson *et al.*, 1983b; Davies *et al.*, 1996; Mackness *et al.*, 1997b; Brophy *et al.*, 2002). Table 1.9 summarizes the substrate dependence of the PON1 activity polymorphism.

The L/M polymorphism at position 55, on the other hand, has not been found to affect catalytic efficiency (Adkins *et al.*, 1993; Humbert *et al.*, 1993) but has been associated with the variability of PON1 levels present in plasma (Brophy *et al.*, 2002). PON1 55*M* individuals were found to have lower levels of PON1 activity (Blatter-Garin *et al.*, 1997; Brophy *et al.*, 2000, 2001a, b), of circulating PON1 (Blatter-Garin *et al.*, 1997; Mackness *et al.*, 1998c), and lower levels of PON1 mRNA (Leviev *et al.*, 1997). It was shown that the 55*L* isoform is more stable and resistant to proteolysis (Leviev *et al.*, 2001a). However, Brophy and coworkers (2002) stated that the 55L/M effect of the lowered activity is not due to the amino acid change but is, rather, largely due to linkage disequilibrium with the −107 regulatory-region polymorphism (Brophy *et al.*, 2000; 2001b; Suehiro *et al.*, 2000).

Leviev and James (2000) found that this link did not completely explain the effect of 55L/M, which remained significant when the −107 site was kept constant. These data were supported by the recent publication of the PON1 crystal structure which shows a key role of 55*L* in the correct packing of the protein (Deakin and James, 2004; Harel *et al.*, 2004).

Table 1.9 PON1 substrate activity polymorphism (taken from Mackness *et al.*, 1998d).

192*R* more active with :	Paraoxon Methylparaoxon Fenitroxon Chlorthion oxon EPN oxon Armin
Similar activity with:	Phenyl acetate Chlorpyrifos oxon 2-Naphthyl acetate
192*Q* more active with:	Diazoxon Sarin Soman Phospholipid hydroperoxides

The capacity of PON1 allozymes to protect LDL from oxidation is the complete reverse of that of paraoxon hydrolytic activity. Paraoxon hydrolytic activity is greatest with purified PON1 from PON1 192*RR* and PON1 55*LL* individuals and least with PON1 192*QQ* and PON1 55*MM* individuals (Adkins *et al.*, 1993; Davies *et al.*, 1996; Mackness *et al.*, 1997b). On the other hand, 55*MM*/192*QQ* individuals have PON1 associated with the greatest protective capacity against LDL oxidation (Mackness *et al.*, 1997c; Aviram *et al.*, 1998a; Mackness *et al.*, 1998d; Durrington *et al.*, 2001).

1.2.2.7.1.1.2 Structural Basis of the PON1 Activity Polymorphism

Structural basis for the observed differences in enzyme activity for different 192Q/R isoforms of PON1 was explained by Harel and coworkers (2004) by the presence of number 192 amino acid (either glutamine or arginine) on the active site wall. This single nucleotide change probably reshapes the active site walls and perimeter, thereby improving the positioning of some substrates (and of their respective catalytic intermediates and transition states) and worsening that of others (Harel *et al.*, 2004). The 55L/M polymorphism's effect on the stability of PON1 is at least in part due to the key role of Leu55 in the correct packing of the protein (Harel *et al.*, 2004).

1.2.2.7.1.1.3 Ethnic Distribution of PON1 Coding Region Polymorphisms

As predicted by earlier studies based on enzyme activity distributions (Playfer *et al.*, 1976), differences in gene frequencies between different ethnic groups were evident. There is a wide variability in the world distribution of PON1 allele frequencies (Table 1.10). The polymorphism at position 192 has been the most studied, with gene frequencies for PON1 192*R* ranging from 0.248 for Caucasians of Northern European origin (Draganov and La Du, 2004) to 0.789 for an Ecuador population (Cayapa Indians; Scacchi *et al.*, 2003) (Table 1.10). As given in Table 1.11, in particular, the 192*R* allele is the most frequent in the Cayapa Indians (Scacchi *et al.*, 2003). Due to their same origin, the Japanese and Chinese also show the same characteristic pattern of a higher 192*R* allele frequency (Table 1.10 and 1.11).

European populations are substantially homogeneous and have the lowest 192*R* allele frequencies, ranging from 0.248 to 0.380. PON1 192*R* allele frequencies of Turkish (Table 1.11) and Indians (Table 1.10) are quite low, which agrees with their conspicuous component of European descent (Scacchi *et al.*, 2003). The gene

frequency for 55*L* ranges from 0.57 in Caucasian populations of Europe to 0.99 in an Oji-Cree population of Canada (Fanella *et al.*, 2000; Costa *et al.*, 2003).

Table 1.10 Geographic and ethnic distribution of human PON1 polymorphisms (taken from Draganov and La Du, 2004).

Geographic region		**192*R* allele frequency**	**55*L* allele frequency**
America	Caucasian	0.280-0.300	0.640
	Afro-American	0.622	
	Cayapa Indians (Ecuador)	0.789	
	Oji-Cree	0.240	0.990
Europe	Caucasian	0.248-0.380	0.570-0.640
Africa	Beninese	0.610	
	Ethiopians	0.410	
Asia	Chinese	0.570-0.640	0.960
	Japanese	0.590-0.630	0.910-0.940
	Indians	0.276	
Australia	Caucasian	0.630	0.600

Table 1.11 PON1 allele frequencies of the coding region polymorphisms in different populations. NIDDM; non-insulin dependent diabetes mellitus, IDDM; insulin dependent diabetes mellitus (taken partly from Brophy *et al.*, 2002).

Ethnicity	N	192*Q*	192*R*	55*L*	55*M*	Comments	Reference
Caucasian/ European							
Caucasian	263	0.74	0.26			NIDDM	Ruiz *et al.*, 1995
Caucasian	247	0.69	0.31				Serrato and Marian, 1995
Caucasian	527	0.70	0.30				Rice *et al.*, 1997
European	282	0.74	0.26	0.64	0.36	20 % E. Indians	Mackness *et al.*, 1998b
Caucasian/ French	197	0.70	0.30			NIDDM	Cao *et al.*, 1998
Caucasian	118	0.75	0.25			NIDDM	Pfohl *et al.*, 1999
Caucasian	106	0.70	0.30	0.64	0.36		Jarvik *et al.*, 2000
Swiss	100	0.75	0.25				Blatter-Garin *et al.*, 1994
Swiss	368	0.69	0.32	0.65	0.34		Leviev and James, 2000a
Swiss	273	0.73	0.27	0.60	0.40		James *et al.*, 2000a
British	100	0.67	0.33				Blatter-Garin *et al.*, 1994
British?	279	0.74	0.26	0.64	0.36		Mackness *et al.*, 1997a
British?	36	0.62	0.38	0.57	0.43		Mackness *et al.*, 1998d
British	93	0.64	0.36	0.61	0.39	NIDDM	Mackness *et al.*, 2000b
British	152	0.75	0.25	0.63	0.37		Mackness *et al.*, 2000c
Hutterite	840	0.71	0.29				Hegele *et al.*, 1995
Finnish	169	0.74	0.26				Antikainen *et al.*, 1996
Finnish	115			0.61	0.39		Malin *et al.*, 1998
Finnish	106			0.62	0.39		Malin *et al.*, 1999
Finnish	110	0.65	0.35				Salonen *et al.*, 1999
N. Irish	388	0.69	0.31	0.65	0.36	Renal transplant	Hasselwander *et al.*, 1999
N. Irish	165	0.67	0.33				Mackness *et al.*, 2000d
Irish	170	0.71	0.29				Herrmann *et al.*, 1996
French	186	0.76	0.24				Mackness *et al.*, 2000d
French	531	0.70	0.30				Herrmann *et al.*, 1996
French	70	0.72	0.28				Helbecque *et al.*, 1999
Australian	156	0.63	0.37	0.60	0.40		Kao *et al.*, 1998
Italian	196	0.68	0.32				Dessi *et al.*, 1999
Italian	162	0.70	0.30				Ombres *et al.*, 1998
Austrian	144	0.75	0.25	0.59	0.41		Schmidt *et al.*, 1998
Russian	117	0.74	0.26				Akhmedova *et al.*, 1999
German	971	0.73	0.27	0.67	0.33		Cascorbi *et al.*, 1999
Dutch	250	0.72	0.28	0.63	0.37	Young	Heijmans *et al.*, 2000
Dutch	364	0.68	0.32	0.65	0.35	Elderly	Heijmans *et al.*, 2000
Dutch	201	0.68	0.32	0.63	0.37		Leus *et al.*, 2000
Spanish	310	0.70	0.30				Senti *et al.*, 2000
Spanish ?	116	0.58	0.42				Tomas *et al.*, 2000
Spanish ?	215	0.75	0.25	0.61	0.39	Male	Ferré *et al.*, 2002a

Table 1.11 (continued)

Ethnicity	N	192*Q*	192*R*	55*L*	55*M*	Comments	Reference
Turkish	381	0.69	0.31	0.72	0.28		Aynacioglu *et al.*, 1999
Turkish	105	0.69	0.31				Aynacioglu and Kepekci, 2000
Turkish	109	0.63	0.37	0.70	0.30		Agachan *et al.*, 2005
Turkish	51	0.63	0.37				Karakaya *et al.*, 1999
Turkish	30	0.83	0.17			Children	Karaaslan-Biyikli *et al.*, 2005
East Indian							
	165	0.67	0.33				Sanghera *et al.*, 1997
	80	0.82	0.17				Pati and Pati, 1998
	183	0.69	0.31	0.80	0.20		Sanghera *et al.*, 1998a
	189	0.68	0.32				Sanghera *et al.*, 1998b
	166	0.88	0.12				Singh *et al.*, 1998
Chinese							
	244	0.42	0.58				Sanghera *et al.*, 1997
	218	0.36	0.64				Ko *et al.*, 1998
	181	0.43	0.57	0.96	0.04		Sanghera *et al.*, 1998b
	141	0.38	0.62				Padungtod *et al.*, 1999
Japanese							
	252	0.38	0.62				Suehiro *et al.*, 1996
	122	0.42	0.58			NIDDM	Odawara *et al.*, 1997
	326	0.66	0.34			Healthy, NIDDM, IDDM	Yamasaki *et al.*, 1997
	115	0.41	0.59	0.91	0.09		Zama *et al.*, 1997
	108	0.40	0.60	0.94	0.06		Ikeda *et al.*, 1998
	252	0.38	0.62				Kondo and Yamato, 1998
	45	0.53	0.47				Murata *et al.*, 1998
	240	0.31	0.69				Sakai *et al.*, 1998
	90	0.48	0.52				Sodeyama *et al.*, 1999
	431	0.35	0.65	0.92	0.08		Imai *et al.*, 2000
	87	0.66	0.34				Kujiraoka *et al.*, 2000
	132	0.40	0.60	0.94	0.07		Suehiro *et al.*, 2000
Other							
Oji-Cree	478	0.76	0.24	0.99	0.01		Fanella *et al.*, 2000
Inuit	243	0.70	0.30	0.96	0.04		Fanella *et al.*, 2000
Cayapa Indians	83	0.21	0.79				Scacchi *et al.*, 2003
Costa Rican	518	0.76	0.24	0.74	0.26		Sen-Banerjee *et al.*, 2000
Thai	202	0.71	0.29	0.95	0.05		Phuntuwate *et al.*, 2005

1.2.2.7.1.2 Regulatory Region and Other Non-coding Region Polymorphisms

At least five polymorphisms have been identified in the 5′-regulatory region of PON1: −107(108)T/C, −126G/C, −162A/G, −832(824)G/A and −909(907)C/G with somewhat different effects on PON1 expression (Leviev and James, 2000a; Suehiro *et al.*, 2000; Brophy *et al.*, 2001a, b; Draganov and La Du, 2004). Of these only the –107, stimulating protein-1 (Sp1) binding site, and the –162, a potential nuclear factor-1 (NF-1) binding site, lie within consensus sequences for known transcription factors (Brophy *et al.*, 2001a). T at position –107 disrupts the GGCGGG recognition sequence for Sp1 and results in decreased affinity for hepatocyte nuclear extracts and Sp1 (Deakin *et al.*, 2003a; Draganov and La Du, 2004). Brophy and coworkers (2001b) reported that the –107C/T polymorphism accounted for 22.8 % of the observed variability in PON1 expression levels, which was much greater than that attributable to other PON1 polymorphisms. The –107*CC* genotype is associated with the highest serum PON1 levels, –107*TT* with the lowest, and the heterozygotes with intermediate levels. The polymorphism at position –162 also contributes a small (2.4%) amount (Brophy *et al.*, 2001b; Costa *et al.*, 2003). The –909 polymorphism, which is in linkage disequilibrium with the other sites, appeared to have little or no independent effect on PON1-activity level *in vivo*. In addition, fifteen polymorphic sites have been identified in the 3′-untranslated regions of PON1 and the introns contain 171 SNPs (see Table 1.8; La Du, 2003). The observed allele frequencies for the PON1 promoter polymorphisms are given in Table 1.12.

Table 1.12 Allele frequencies of the PON1 promoter polymorphisms in different populations (partly taken from Brophy *et al.*, 2002).

		Position and allele									
		–107 (–108)		–126		–162		–832		–909 (–907)	
Ethnicity	**Reference**	C	T	C	G	A	G	G	A	C	G
Caucasian (North America)	Brophy *et al.*, 2001b	0.50	0.50			0.23	0.77			0.46	0.54
Swiss	Leviev and James, 2000a	0.46	0.54					0.75	0.25	0.59	0.41
	James *et al.*, 2000a	0.44	0.56								
Japanese	Suehiro *et al.*, 2000	0.48	0.52	0.09	0.91	0.10	0.90				
N.Irish	Leviev *et al.*, 2002									0.54	0.46
French	Leviev *et al.*, 2002									0.56	0.44
Scottish	Leviev *et al.*, 2002									0.52	0.48
Thai	Phuntuwate *et al.*, 2005	0.25	0.75							0.73	0.27
Brazil	Voetsch *et al.*, 2004	0.59	0.41								
Italian	Campo *et al.*, 2004	0.41	0.59								

1.2.2.7.1.3 PON1 Status

The existence of PON1 coding-region polymorphisms, which affect catalytic activity toward organophosphates, and of promoter–region polymorphisms, which affect the levels of PON1 expression, led to the proposal of determining the "PON1 status" of an individual (Li *et al.*, 1993; Richter and Furlong 1999; Costa *et al.*, 2003). PON1 serum levels differ significantly between individuals (up to 13-fold) and are modulated by disease state, dietary, life-style, and environmental factors (Draganov and La Du, 2004). These influences are likely to interact with the PON1 promoter polymorphisms to produce further variation (Costa *et al.*, 2003). With the technological advancements available today it is very easy to determine particular PON1 genotypes directly by DNA sequencing, PCR amplification/restriction enzyme analysis, real time fluorescent PCR with melting point analysis etc. However, the phenotyping ratio methods provide a direct quantitative measure of the functional effects of the usual or variant forms of the PON1 with different substrates. This additional information referred as the PON1 status supplies a broader basis than the genotype, alone, for correlation with disease susceptibility, or responsiveness to environmental agents (Furlong, 2000; La Du *et al.*, 2001; Brophy *et al.*, 2002; Draganov and La Du, 2004). Thus the catalytic efficiency of each PON1 192 allozyme must be considered, as well as the *level* of that particular allozyme. PON1 192QR heterozygous individuals have a mixture of the two isoforms, and in some heterozygotes, there will be a higher level of one of the two allelic types than is present in some individuals homozygous for that allozyme (it is incorrect to assume that all heterozygotes have one-half of the average level of each allozyme). If the two allelic forms of the enzyme differ greatly in their catalytic efficiencies, the contribution of each allelic form should be evaluated with respect to toxicity from environmental chemicals (La Du *et al.*, 2001; Draganov and La Du, 2004).

A two-substrate assay/analysis was developed by Furlong and coworkers (2005) that provides both PON1 phenotype and functional genotype. Plotting the rates of diazoxon hydrolysis (at high salt) vs. paraoxon hydrolysis by plasma from a given population breaks the data into three clear groups, individuals homozygous for

PON1 192*Q*, heterozygotes, and individuals homozygous for PON1 192*R* (Figure 1.20; Furlong *et al.*, 2005).

In addition to providing the functional position 192 alloform(s), this analysis also provides the levels of the individuals' plasma PON1 which are at least as important, if not more so, than the position 192 amino acid (Q or R). This functional analysis has been referred to as PON1 status, i.e., it provides both functional position 192 genotype as well as phenotype. It also provides an excellent means of examining risk associated with an individual's PON1 status (Furlong *et al.*, 2005).

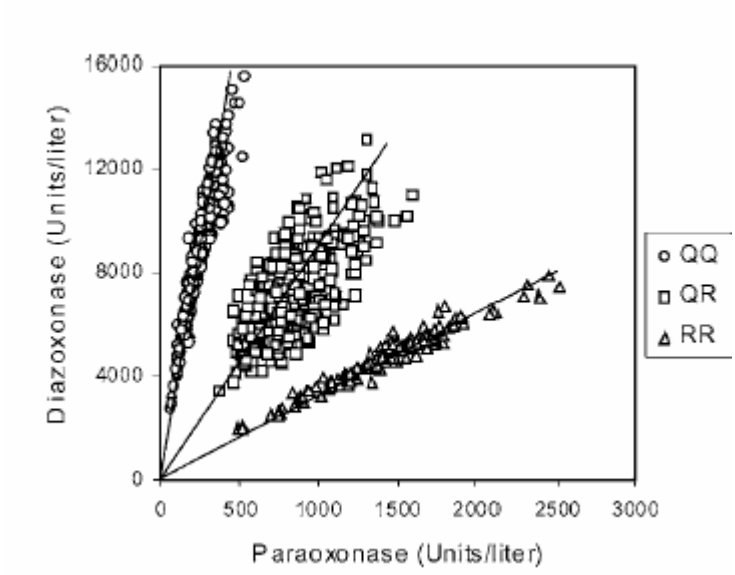


Figure 1.20 Determination of PON1 status. Each data point represents the initial rates of hydrolysis of diazoxon and paraoxon by citrate plasma from a single individual. Note that this two-dimensional analysis divides the population clearly into three groups, individuals homozygous for PON1 192*Q*, heterozygotes, and individuals homozygous for PON1 192*R*. In addition to providing the functional position 192 genotype, the levels of PON1 in the individuals' plasma are also revealed. Note also that the data points for both classes of homozygotes fall tightly along the trend lines, while heterozygote data points vary considerably from the trend line (taken from Furlong *et al.*, 2005).

1.2.2.7.2 Modulation of PON1 by Physiological and Pathological States, Dietary and Lifestyle Factors, and Environmental Chemicals

Serum PON1 activity in a given population can vary by 40-fold. Though most of this variation can be explained by polymorphisms in the coding region (192Q/R; see section 1.2.2.7.1.1) and the 5' regulatory region (–107T/C; see section 1.2.2.7.1.2), modulation of PON1 by a variety of other factors should be taken into account, including age, physiological and pathological states, dietary and lifestyle factors (such as smoking, alcohol) and environmental chemicals and drugs, that have been shown to modulate PON1 activity in either direction. Factors that decrease and increase PON1 activity are explained in detail below, and summarized in Tables 1.13 and 1.14, respectively.

1.2.2.7.2.1 Effects of Development and Ageing on PON1 Activity

Studies in humans have shown that serum PON1 activity is very low at birth and increases over time, reaching a plateau between 6 and 15 months of age (Augustinsson and Barr, 1963; Ecobichon and Stephens, 1973; Mueller *et al.*, 1983; Cole *et al.*, 2003). PON1 activity in the fetus may be even lower, as suggested by data indicating a 24 % lower activity in premature babies (33–36 weeks of gestation) compared to term babies (Table 1.13; Ecobichon and Stephens, 1973). In humans, PON1 serum arylesterase activity also increases from birth to 15–25 months of age, when it seems to reach a plateau whose level is determined by the 5' regulatory-region polymorphisms and the genetic background of the individual (Costa *et al.*, 2002; Costa *et al.*, 2003). However, efficient PON1 regulatory regions do not alone guarantee a high PON1 activity level (Brophy *et al.* 2001a; Costa *et al.*, 2003). Enzyme inducers, environmental chemicals, pathological states, and dietary and lifestyle factors have demonstrated effects on PON1 activity (Table 1.13 and Table 1.14; Costa *et al.*, 2003). PON1 activity is quite constant over time, once it reaches adult values. Recent investigations have reported, however, a progressive decrease in PON1 activity in elderly subjects (Leviev *et al.*, 2001b; Milochevitch and Khalil, 2001; Jarvik *et al.*, 2002; Seres *et al.*, 2004; Costa *et al.*, 2005).

Table 1.13 Physiological and pathological states, dietary and life style factors and environmental chemicals which decrease PON1 activity.

	Effectors	Note
Development and ageing	Pregnancy	PON1 activity decreased from 145.8 U/L at preconception to 111.1 U/L ($p<0.01$) at 32 weeks and 100.4 U/L ($p<0.001$) at labour (Ferré *et al.*, 2006).
	Development	24 % lower PON1 activity in premature babies (Ecobichon and Stephens, 1973).
	Age	Lower PON1 activity in elderly (Leviev *et al.*, 2001b; Milochevitch and Khalil, 2001; Jarvik *et al.*, 2002; Seres *et al.*, 2004)
Physiological and pathological conditions	Menopause	
	Cardiovascular disease	(McElveen *et al.*, 1986; Ayub *et al.*, 1999; Ferré *et al.*, 2002a; Mackness *et al.*, 2003; Graner *et al.*, 2006)
	Cerebrovascular disease	(Jarvik *et al.*, 2000 and 2003; Aydin *et al.*, 2006; Kim *et al.*, 2007)
	Diabetes	36 % decrease in streptozotocin-treated diabetic rats (Patel *et al.*, 1990).
	Diabetes	Both PON1 activity and concentration were significantly lower by 16.7% and 19.2% in the type 1 diabetes group (Mackness *et al.*, 2002b).
	Familial hypercholesterolemia (FH)	Mean paraoxonase activity was 47.6 % lower in FH than in controls (Mackness *et al.*, 1991c)
	Renal disease	Paraoxonase activity was 35.2 U/L in renal failure population and 54.8 U/L in control group (Hasselwander *et al.*, 1998).
	Hyperthyroidism	~40 % decrease (Raiszadeh *et al.*, 2004).
	HDL deficiency	Paraoxonase activity of two patients with fish-eye disease was only 11% of the mean value for control subjects (Mackness *et al.*, 1987).

Table 1.13 (continued).

	Effectors	Note
Physiological and pathological conditions	Alzheimer's disease and vascular dementia	30–40% decrease (Paragh *et al.*, 2002).
	Liver cirrhosis	>80 % decrease experimental cirrhosis in rats due to carbon tetrachloride administration (Ferré *et al.*, 2001).
	Chronic liver disease (chronic hepatitis and cirrhosis)	PON1 activities were significantly decreased in patients with chronic hepatitis and liver cirrhosis (Ferré *et al.*, 2002b)
	Acute phase response (due to lipopolysaccharide administration)	50 % transient decrease in serum and liver PON1 activity and hepatic mRNA levels (Feingold *et al.* 1998).
Life style factors	Smoking	paraoxonase activities and concentrations were significantly lower in current than in never smokers. (James *et al.*, 2000b)
	Ethanol and other aliphatic alcohols	45 % reduction in heavy drinkers (Rao *et al.*, 2003).
Diet	High fat diet	60 % decrease in sensitive strain of mice (B6) (Shih *et al.*, 1996).
	Fish oil	24-39 % reduction (Kudchodkar *et al.*, 2000).
	Degraded cooking oil	serum paraoxonase activity decreased significantly (17%) after the used fat meal (Sutherland *et al.*, 1999)
	Lipid peroxides	(Aviram *et al.*, 1998a and 1999).
Exogenous compounds (drugs)	<u>Statins</u>	
	• Pravastatin, simvastatin and fluvastatin	in vitro exposure of HuH7 human hepatoma cells 25–50% decrease in PON1 activity (Gouedard *et al.*, 2003).
	• fluvastatin	20 mg/kg/day for 3 weeks to rats reduced both plasma and liver PON1 activity (Beltowski *et al.*, 2004).

Table 1.13 (continued).

	Effectors	Note
Exogenous compounds	Organophospates	acute exposure (Sözmen *et al.*, 2002)
	Ionizing radiation	exposure for more than 5 years (Serhatlioglu *et al.*, 2003).
	EDTA	in vitro
	Barium, lanthanum, copper, zinc, and mercurials	in vitro rat or human liver PON1 (Gonzalvo *et al.*, 1997).

1.2.2.7.2.2 PON1 in Certain Physiological and Pathological Conditions

PON1 activity can vary depending on physiological conditions or pathological states (Table 1.13). For example, serum PON1 activity is significantly decreased during pregnancy (Weitman *et al.*, 1983; Geldmacher-Von Mallinckrodt and Diepgen, 1988; Costa *et al.*, 2003; Ferré *et al.*, 2006) and with the beginning of menopause (Senti *et al.*, 2001; Costa *et al.*, 2003). As also given in Figure 1.21; low PON1 activity has been found in cardiovascular disease (McElveen *et al.*, 1986; Ayub *et al.*, 1999; Ferré *et al.*, 2002a; Mackness *et al.*, 2003; Graner *et al.*, 2006), cerebrovascular disease (Jarvik *et al.*, 2000 and 2003; Aydin *et al.*, 2006; Kim *et al.*, 2007), diabetes mellitus (Patel *et al.*, 1990; Mackness *et al*., 1991c; Abbott *et al.*, 1995; Mackness *et al.*, 1998c; Mackness *et al.*, 2002b), familial hypercholesterolemia (Mackness *et al*., 1991c); renal disease (Hasselwander *et al.*, 1998), rheumatoid arthritis (Tanimoto *et al.*, 2003), liver cirrhosis (Ferré *et al.*, 2002b) and various HDL deficiencies such as fish-eye disease (Mackness *et al.*, 1987; Costa *et al.*, 2003). Patients with Alzheimer's disease and vascular dementia displayed lower (30–40%) serum PON1 activity (Paragh *et al.*, 2002). Hyperthyroidism was also associated with lower (~40%) serum PON1 activity; after

treatment with methimazole, patients who became euthyroid had only a 14% lower serum PON1 activity than controls (Raiszadeh *et al.*, 2004; Costa *et al.*, 2005).

Animal studies have indicated that PON1 activity can be altered during the acute phase response (Table 1.13; Feingold *et al.* 1998; Costa *et al.*, 2005). Administration of lipopolysaccharide (LPS), which mimics gram negative infections, to mice, caused a 50% decrease of serum and liver PON1 activity and a similar change in hepatic mRNA levels; this decrease was transient, as PON1 returned to control values within 48 h (Costa *et al.*, 2002; Costa *et al.*, 2005).

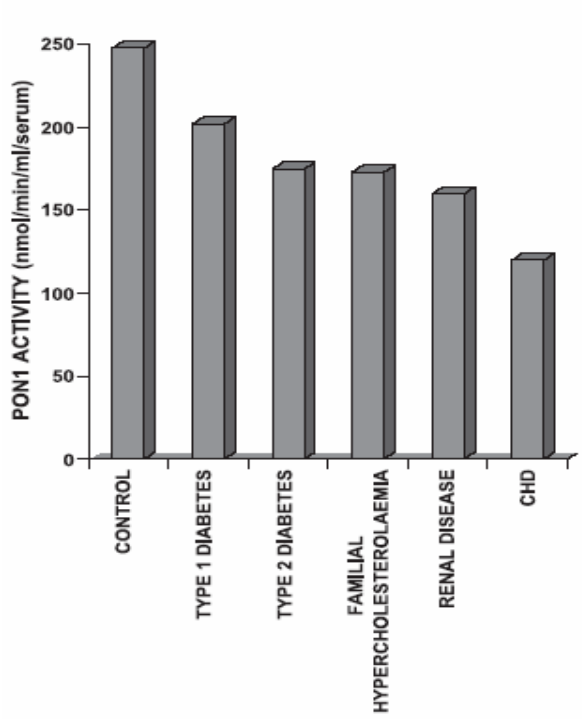


Figure 1.21 Relative PON1 activities in populations at risk of developing atherosclerosis compared to healthy controls and those with coronary heart disease (CHD). All populations are significantly different from controls, $P<0.05$ or better (taken from Mackness and Mackness, 2004).

1.2.2.7.2.3 Modulation of PON1 by Life-style Factors

As given in Tables 1.13 and 1.14, lifestyle factors can also affect PON1 activity. Cigarette smoke extract was found to inhibit human plasma PON1 activity (Nishio and Watanabe, 1997). Studies in humans have confirmed that smoking is associated with reduced serum PON1 levels and activity (Table 1.13; Jarvik *et al.*, 2000; James *et al.*, 2000b; Jarvik *et al.*, 2002; Boemi *et al.*, 2004), both of which appear to normalize relatively soon (3–24 months) after cessation (Costa *et al.*, 2003).

Ethanol and other aliphatic alcohols have been shown to inhibit serum PON1 activity (Table 1.13; Debord *et al.*, 1998); however, a study in middle-aged men indicated that daily moderate alcohol consumption increased serum PON1 activity, with no differences between wine, beer, and spirits (Table 1.14; Van der Gaag *et al.*, 1999). This increase may be due to the consumption of alcohol itself or to that of antioxidants, as similar results were obtained after consumption of red wine (Hayek *et al.*, 1997) or pomegranate juice (Table 1.14; Aviram *et al.*, 2000a; Kaplan *et al.*, 2001). However, in another study, light drinkers had a 395% higher, whereas heavy drinkers had a 45% lower serum plasma PON1 activity compared to non-drinkers (Rao *et al.*, 2003; Costa *et al.*, 2005).

1.2.2.7.2.4 Modulation of PON1 Activity by Diet

A high-fat diet was shown to reduce serum PON1 levels in mice (Table 1.13; Shih *et al.*, 1996; Hedrick *et al.*, 2000a). In rats, dietary supplementation with fish oil also decreased serum PON1, whereas supplementation with triolein increased PON1 activity (Kudchodkar *et al.*, 2000; Costa *et al.*, 2003). Degraded cooking oil has also been reported to decrease PON1 activity (Table 1.13; Sutherland *et al.*, 1999; Mackness and Mackness, 2004). Lipid peroxides inhibit the paraoxonase, arylesterase and antioxidant activities of PON1, probably via interactions with a sulfur group on the enzyme (Aviram *et al.*, 1998a and 1999). One important consequence of this phenomenon is that, if HDL is oxidized, there will be an

accompanying reduction in paraoxonase activity and, therefore, also a reduction in the enzyme's protective activity against LDL oxidation (Jaouad *et al.*, 2003; Tomás *et al.*, 2004).

Consumption of nutritional polyphenols, such as the pomegranate juice hydrolyzable tannin punicalagin, the red wine flavonoids quercetin and cathechin and the licorice root isoflavan glabridin, by apolipoprotein E deficient (E^0) mice preserved serum PON1 activity by reducing the oxidative stress, thereby contributing to PON1 hydrolytic activity on lipid peroxides in oxidized lipoproteins, macrophages and atherosclerotic lesions (Table 1.14). In addition, these polyphenols were all shown to increase PON1 expression, and this effect was demonstrated to be related to their antioxidant properties (Hayek *et al.*, 1997; Aviram *et al.*, 2000a; Fuhrman and Aviram, 2002; Aviram and Rosenblat, 2005). In healthy individuals, pomegranate juice consumption also increases PON1 activity, in association with a reduction in LDL oxidation (Aviram *et al.*, 2000a; Aviram and Rosenblat, 2005). Pomegranate juice administration to patients with carotid artery stenosis for 1 year resulted in a significant increase (by 83 %) in PON1 activity, paralleled by a significant 90 % reduction in basal and copper ion-induced LDL oxidation, and most importantly, a significant 35 % decreased intima media thickness (Table 1.14; Aviram *et al.*, 2004; Aviram and Rosenblat, 2005).

Table 1.14 Physiological and pathological states, dietary and life style factors and environmental chemicals which increase PON1 activity.

	Effectors	Note
Diet	Pomegranate juice	83 % increase in PON1 activity in patients with carotid artery stenosis. juice administered for 1 year (Aviram *et al.*, 2004).
	Nutritional polyphenols	
	• punicalagin (pomegranate juice hydrolyzable tannin)	refer to Aviram and Rosenblat, 2005
	• quercetin (red wine flavonoid)	refer to Aviram and Rosenblat, 2005
	• glabridin (licorice root isoflavan)	refer to Aviram and Rosenblat, 2005
	Triolein	46 % increase (Kudchodkar *et al.*, 2000).
Life style factors	Daily moderate alcohol consumption	no differences between wine, beer, and spirits (Van der Gaag *et al.*, 1999). 395 % increase in light drinkers (Rao *et al.*, 2003).
Exogenous compounds (drugs)	Statins	
	• simvastatin	simvastatin (1.5–25 mg/mL) was found to upregulate PON1 promoter activity in HepG2 cells (Deakin *et al.*, 2003b).
	• simvastatin and other statins	human PON1 activity increased in patients treated with the statin (Tomás *et al.*, 2000; Leviev and James, 2000b; Jarvik *et al.*, 2002)
	Fibrates	
	• fenofibrate and gemfibrozil	human PON1 activity increased in patients treated with the fibrate (Balogh *et al.*, 2001; Jarvik *et al.*, 2002; Deakin *et al.*, 2003b; Paragh *et al.*, 2003).
	• fenofibric acid (250 mM)	in vitro exposure of HuH7 human hepatoma cells 50 and 30% increase in PON1 activity and mRNA (Gouedard *et al.*, 2003).

Table 1.14 (continued).

	Effectors	Note
Exogenous compounds (drugs)	Aspirin	a significant increase of plasma PON1 activity and concentration (Blatter-Garin *et al.*, 2003).
Exogenous compounds (classical inducers)	Phenobarbital	20–150 % increase in liver, not in serum in rats, not in mice (Kaliste-Korhonen *et al.*, 1990 and 1998; Vitarius *et al.*, 1995).
	3-methylcholanthrene	both liver and serum in rats, not in mice (Rodrigo *et al.,* 2001b).

1.2.2.7.2.5 Modulation of PON1 by Exogenous Compounds

Statins are hypolipidemic compounds that inhibit 3-hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the intracellular biosynthesis of cholesterol. Studies with statins and fibrates to see their effects on PON1 activity have yielded somewhat conflicting results. In vitro exposure of HuH7 human hepatoma cells to pravastatin, simvastatin and fluvastatin (10–100 mM) caused a 25–50% decrease in PON1 activity in the culture medium and a similar decrease in PON1 mRNA (Table 1.13); both effects were reversed by mevalonate (Gouedard *et al.*, 2003; Costa *et al.*, 2005). In the same cells, fenofibric acid (250 mM) caused a 50 and 30% increase in PON1 activity and mRNA, respectively (Gouedard *et al.*, 2003; Costa *et al.*, 2005). Fenofibric acid was found to induce PON1 gene-promoter activity, while statins had an opposite effect (Gouedard *et al.*, 2003; Costa *et al.*, 2005). The latter finding is in contrast with results obtained in hepatic human HepG2 cells, where simvastatin (1.5–25 mg/mL) was found to upregulate PON1 promoter activity (Deakin *et al.*, 2003b; Costa *et al.*, 2005). In another in vitro study on isolated lipoproteins, two oxidized metabolites of atorvastatin (5–50 mM) and a metabolite of gemfibrozil (2–80 mM), but not the parent compounds, were found to increase PON1 activity (Aviram *et al.*, 1998c; Costa *et al.*, 2005). A study in rats

indicated that fluvastatin (20 mg/kg/day for 3 weeks) reduced both plasma and liver PON1 activity (Table 1.13), while a lower dose (2 mg/kg/day) was only effective toward liver activity. Pravastatin (4 or 40 mg/kg/day for 3 weeks), on the other hand, was devoid of significant effects (Beltowski *et al.*, 2004; Costa *et al.*, 2005).

Studies in humans indicated that the effect depends on the specific type of the statin or fibrate used. An increase in serum-PON1 activity was found in patients treated with simvastatin (Tomás *et al.*, 2000; Leviev and James, 2000b; Jarvik *et al.*, 2002) and other statins, gemfibrozil and fenofibrate (Table 1.14; Balogh *et al.*, 2001; Jarvik *et al.*, 2002; Deakin *et al.*, 2003b; Paragh *et al.*, 2003; Costa *et al.*, 2005). Factors associated with transcription may be involved in this phenomenon since it is known that simvastatin can increase the transcriptional activity of the PON1 gene promoter by 2.5 times through a mechanism that depends on mevalonate, a metabolic product of HMG-CoA reductase (Deakin *et al.*, 2003b; Tomás *et al.*, 2004). On the other hand, no changes in serum PON1 activity were reported by other studies in patients treated with ciprofibrate (Turay *et al.*, 2000), bezafibrate and gemfibrozil (Durrington *et al.*, 1998; Costa *et al.*, 2005). The mechanism of the effect of statins and polyphenols on paraoxonase gene expression is illustrated in Figure 1.22.

In a cohort of aspirin users, a significant increase of plasma PON1 activity and concentration was reported (Blatter-Garin *et al.*, 2003). Such effect may be due to the anti-inflammatory effect of aspirin, as serum PON1 levels are reduced during the inflammatory response in animal models; alternatively, aspirin may act as an anti-oxidant (Blatter-Garin *et al.*, 2003).

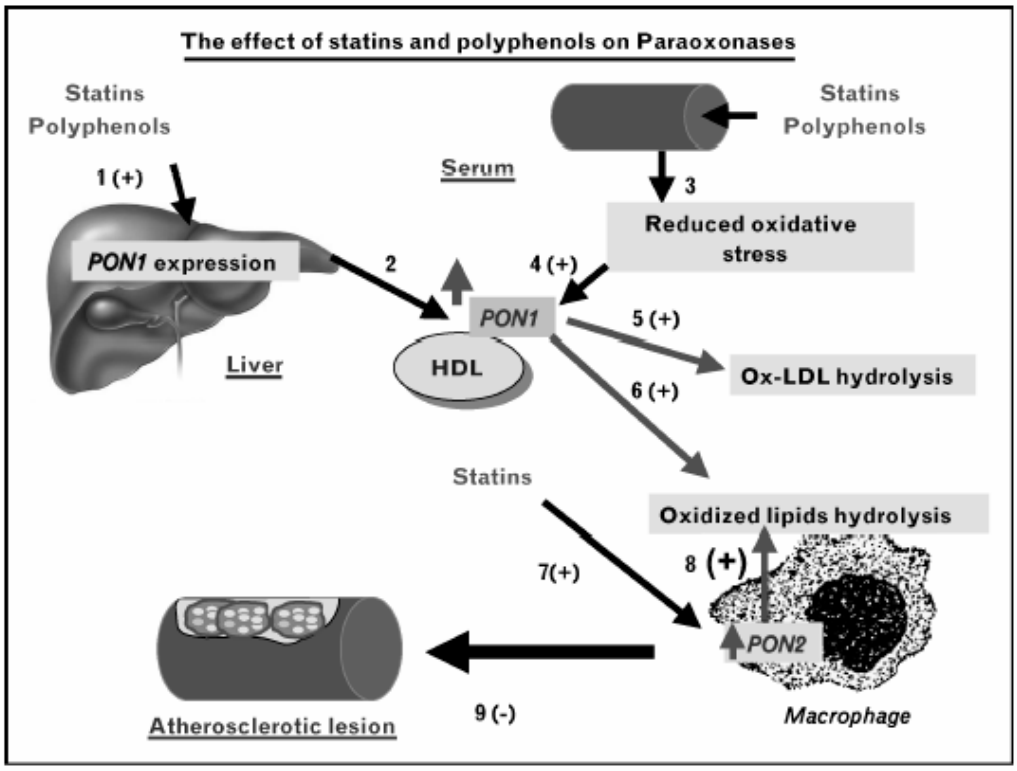


Figure 1.22 The effects of statins and polyphenols on paraoxonases. In the liver, statins upregulate paraoxonase-1 (PON1) expression (1), which results in increased serum HDL-associated PON1 activity (2). In serum, statins and polyphenols act as antioxidants and reduce serum oxidative stress (3), leading to an increase in serum HDL-associated PON1 (4). The increased serum PON1 activity enhances the hydrolysis of oxidized lipids in oxidized LDL (Ox-LDL) (5) and also in macrophages (6). Statins were also shown to upregulate PON2 expression in macrophages (7), thus stimulating the hydrolysis of cellular oxidized lipids (8). All the above effects of statins and polyphenols contribute to the attenuation of atherosclerotic lesion formation (9) (taken from Aviram and Rosenblat, 2005).

A few studies have investigated whether PON1 is an inducible enzyme. Phenobarbital, a classical enzyme inducer which is particularly effective toward certain isozymes of cytochrome P450 (e.g., CYP2B; Adali and Arinç, 1990; Adali *et al.*, 1996), caused a modest (20–150%) increase in hepatic PON1 activity (Kaliste-Korhonen *et al.*, 1990 and 1998), with a concomitant increase in liver RNA levels (Costa *et al.*, 2005). However, serum PON1 activity was decreased (by 40–50%) by phenobarbital treatment (Kaliste-Korhonen *et al.*, 1990 and 1998; Vitarius *et al.*, 1995; Costa *et al.*, 2005). β-Naphtoflavone, an inducer of CYP1A, did not change serum or liver PON1 (Kaliste-Korhonen *et al.*, 1990 and 1998), nor did 3-methylcholantrene in mice (Costa *et al.*, 2005). In rats, however, 3-methylcholanthrene was associated with increased serum and liver PON1 activity (Rodrigo *et al.,* 2001b; Costa *et al.*, 2005).

Acute exposure to organophosphates decreases PON1 activity (Sözmen *et al.*, 2002), but it is not certain yet whether chronic possible occupational low-level exposure to organophosphates or other toxins can influence PON1 activity (Durrington *et al.*, 2002). Administration of the hepatotoxicant carbon tetrachloride to rats caused liver cirrhosis and >80% decrease of hepatic PON1 (Ferré *et al.*, 2001; Costa *et al.*, 2005). A recent study also reported a decreased level of paraoxonase and arylesterase activities in radiology workers exposed for more than five years to ionizing radiation (Serhatlioglu *et al.*, 2003; Costa *et al.*, 2005).

PON1 activity depends on calcium, and the calcium chelator EDTA (0.05-0.7 mM) abolishes its activity. Other cations have also shown an inhibitory effect on PON1 activity. Barium (0.3- 1.2 mM), lanthanum (0.1-0.6 mM), copper (0.02-0.08 mM), zinc (0.25-1.5 mM), and mercurials (p-hydroxymercuribenzoate: 0.05-0.25 mM; Hg: 0.005-0.03 mM; and phenylmercuric acetate: 0.02-0.10 mM) were found to inhibit PON1 activity from rat or human liver in vitro (Gonzalvo *et al.*, 1997; Costa *et al.*, 2003). In vitro experiments with purified human enzymes showed that PON1 192*R* isozyme was more sensitive than PON1 192*Q* isozyme to inhibition by cadmium, zinc, mercury chloride, and iron, whereas PON1 192*Q* displayed a higher sensitivity to inhibition by lead. *In vivo*, however, when mice were treated with

cadmium or methylmercury to achieve environmentally relevant blood concentrations, no inhibition of PON1 activity was found (Cole *et al.*, 2002; Costa *et al.*, 2003).

1.2.2.8 PON1 Genetic Polymorphisms and Activity in Coronary Heart Disease and Atherosclerosis: Human Epidemiological Studies

Several lines of evidence link serum paraoxonase (PON1) with coronary heart disease (CHD). Mackness and colleagues (1991b) originally showed that purified human PON1 could inhibit LDL oxidation *in vitro*, suggesting an anti-atherogenic role for PON1 protein (see section 1.2.2.5.2 for details; Mackness *et al.*, 1991b). Aviram and colleagues (1998a) observed that 192*Q* allozyme decreased lipid peroxide content in human carotid and coronary atherosclerotic lesion homogenates more efficiently than the 192*R* allozyme. Thus, 55*MM*/192*QQ* individuals have PON1 associated with the greatest protective capacity against LDL oxidation (Mackness *et al.*, 1997c; Aviram *et al.*, 1998a). These observations constituted the biological basis for the numerous genetic association studies which investigated the 192*R* and 55*L* isozymes of PON1 as risk factors for coronary and carotid vascular diseases.

1.2.2.8.1 Genetic Studies

A large number of population association studies involving polymorphisms in the human PON1 gene have been reported. Among the various PON1 polymorphisms, two missense polymorphisms, a glutamine to arginine substitution at amino acid 192 (192Q/R), and a leucine to methionine substitution at amino acid 55 (55L/M), have been tested extensively in relation to CHD. More recently polymorphisms of the PON1 promoter, including a T to C variation at –107 bp upstream of the translation start site (–107T/C), a G to A variation at –824 bp (–824G/A), and a G to C variation at –907 bp (–907G/C) were also reported. The frequencies of these polymorphisms were examined in case-control studies of CHD,

CHD in non-insulin dependent diabetes mellitus (NIDDM), and several other related diseases and traits (Table 1.15; Shih *et al.*, 2002).

Of the many association studies with CHD (Table 1.15, studies 1-20), more than half (Table 1.15, studies 1, 4, 5, 8-10, 14-19) yielded significant results. Moreover, in studies of the 192Q/R polymorphism, the 192*R* allele, not the 192*Q* allele, was consistently associated with CHD. Particularly significant was the finding that the 192*R* allele, which was associated with increased atherosclerosis in the above studies, was less effective in inhibiting *in vitro* LDL oxidation than was the 192*Q* allele (Aviram *et al.*, 1998b, 2000b). The low expressor genotype of the –107T/C polymorphism (–107*TT*) was associated with increased risk for CHD (Table 1.15, study 20) and this association was independent of other risk factors, including the 192Q/R polymorphism (Shih *et al.*, 2002). High expressor –907*GG* genotype was associated with reduced risk of vascular disease (Table 1.15, study 21).

Table 1.15 Association studies with paraoxonase gene locus polymorphism and cardiovascular disease (taken from Shih *et al.*, 2002).

Study	Polymorphism	Traits studied	Experimental design	Ethnic group	Conclusion	Reference
1	192Q/R 55L/M	Coronary heart disease (CHD)	210 CHD patients and 431 control subjects	Japanese	192*R* allele associated with CHD ($p<0.001$); 55L/M not associated	Imai *et al.*, 2000
2	192Q/R	CHD	96 CHD patients and 105 control subjects	Turkish	No associations	Aynacioglu and Kepekci, 2000
3	192Q/R 55L/M	All-cause mortality and CHD	Aged (>85 yrs, n=364) and control (18-40 years, n=250) subjects compared in cross-sectional study. Aged (>85 yrs, n=666) subjects studied in prospective 10 year follow-up study.	Dutch	No associations	Heijmans *et al.*, 2000
4	192Q/R 55L/M	CHD and lipoprotein levels	233 CHD patients and 364 controls	Asian Indians and Chinese	No associations with 55L/M polymorphism but significant association of 192*R*/55*L* haplotype with CHD in Asian Indians but not Chinese	Sanghera *et al.*, 1997; 1998a
5	192Q/R 55L/M	CHD	75 patients with CHD and 115 controls	Japanese	192*R* allele associated ($p=0.006$) but 55L/M allele not associated	Zama *et al.*, 1997
6	192Q/R	CHD	218 CHD patients and 218 control subjects	Taiwanese	No associations	Ko *et al.*, 1998

Table 1.15 (continued).

Study	Polymorphism	Traits studied	Experimental design	Ethnic group	Conclusion	Reference
7	192Q/R 55L/M	CHD in renal transplant pateints	Renal transplant recipients with (n=103) and without (n=388) CHD	Irish	No associations	Hasselwander *et al.*, 1999
8	192Q/R	CHD	129 CHD patients and 189 controls	Asian Indians	Evidence of interaction with 192Q/R alleles	Sanghera *et al.*, 1998b
9	192Q/R	CHD	120 CHD patients and 80 controls	Asian Indians	192*R* allele associated with CHD (p=0.0001) in patients with or without diabetes	Pati and Pati, 1998
10	55L/M	Myocardial infarction (MI)	A prospective nested case control study of 1137 men, 55 of whom had a MI	Finnish	*MM* homozygous state associated with increased MI (p=0.025)	Salonen *et al.*, 1999
11	192Q/R	CHD	380 CHD patients and 169 controls	Finnish	Suggestive association (p=0.12)	Antikainen *et al.*, 1996
12	192Q/R	Myocardial Infarction (MI)	134 patients with MI or angina pectoris and 252 healthy subjects	Japanese	No associations	Suehiro *et al.*, 1996
13	192Q/R	Myocardial Infarction and plasma lipid levels	642 male patients and 701 controls in the ECTIM study	French	No associations	Herrmann *et al.*, 1996

Table 1.15 (continued).

Study	Polymorphism	Traits studied	Experimental design	Ethnic group	Conclusion	Reference
14	192Q/R	CHD	223 patients with angiographically assessed CHD and 247 controls	Swiss	192*R* allele associated with CHD (p=0.0003)	Serrato and Marian, 1995
15	192Q/R	Myocardial infarction in smokers	156 consecutive MI patients and 310 controls	Spanish	Smoking amount associated with MI (p<0.001) in *QQ* homozygotes but not in RR or QR individuals	Senti *et al.*, 2000
16	192Q/R	CHD in NIDDM	164 NIDDM patients with (n=42) and without (n=122) CHD	Japanese	192*R* allele associated with CHD (p=0.003)	Odawara *et al.*, 1997
17	192Q/R 55L/M	CHD in NIDDM PON1 mass and activity	408 NIDDM patients with (n=168) and without (n=240) CHD	Swiss	55*L* allele associated with CHD (p=0.031) and higher PON1 Mass (p<0.0001)	Garin *et al.*, 1997
18	192Q/R	CHD in NIDDM	434 diabetic patients with (n=171) and without (n=263) CHD	Swiss	The 192*R* allele associated with CHD (p=0.03)	Ruiz *et al.*, 1995
19	192Q/R	Myocardial infraction in patients with and without diabetes	156 consecutive myocardial infarction patients and 310 controls	Spanish	Significant association (p<0.05) between myocardial infarction and 192*R* carriers in diabetic but not in non-diabetic patients	Aubó *et al.*, 2000

Table 1.15 (continued).

Study	Polymorphism	Traits studied	Experimental design	Ethnic group	Conclusion	Reference
20	–107T/C	CHD and PON1 mass in NIDDM	NIDDM patients with (n=137) and without (n=273) CHD	Swiss	The –107*T* allele associated with low PON1 mass ($p<0.0001$) and the presence of CHD ($p<0.01$)	James *et al.,* 2000a
21	–907C/G	Myocardial Infarction	993 patients and 957 controls	N. Irish, Scottish, French	High expressor –907*GG* genotype associated with reduced risk of vascular disease	Leviev *et al.*, 2002

In 2004, Wheeler and colleagues published a meta-analysis of 43 genetic epidemiological studies published at the time. They investigated the PON1 −107, 55 and 192 polymorphisms and their association with CHD. The data set contained 11,212 CHD cases and 12,786 controls. There were no significant associations with CHD for either the PON1 −107 or 55 polymorphisms (Table 1.16). There was a weak association between the PON1 192 polymorphism and CHD; however, there was no significant effect in the five largest studies, which were less prone to selection bias. The results of the meta-analysis did not take into account ethnicity and it is therefore still possible that the PON1 gene polymorphisms may still be risk factors for CHD in particular ethnic groups (Wheeler *et al.*, 2004; Mackness and Mackness, 2004).

Table 1.16 Association of PON1 gene polymorphisms with CHD. Meta analysis of 43 studies containing 11,212 CHD cases and 12,786 controls (taken from Mackness and Mackness, 2004).

Allele		Relative risk (95 % CI)
55*M*		1.00 (0.95-1.06)
−107*T*		1.02 (0.92-1.14)
192*R*	overall	1.12 (1.07-1.16)
	largest studies	1.05 (0.98-1.13)

1.2.2.8.2 Activity Studies

Relative to genetic epidemiological studies, there have been few epidemiological studies that have included a measure of PON1 status, i.e., activity and concentration. The first of these studies, which was conducted in 1985, indicated that PON1 activity was lower in people who had a myocardial infarction (MI) compared to in controls (McElveen *et al.*, 1986). Ayub and colleagues (1999)

showed that PON1 activity and concentration were lower in people who had an MI than in age-and gender-matched controls (Ayub *et al.*, 1999). Low PON1 activity has also been found in cardiovascular disease by other researchers (see Figure 1.21; Ferré *et al.*, 2002a; Mackness *et al.*, 2003; Graner *et al.*, 2006; Mackness and Mackness, 2004).

1.2.2.8.3 Prospective Studies

The only prospective study testing the relationship of PON1 status and CHD to be reported so far is the Caerphilly Prospective Study (Mackness *et al.*, 2003). In this study, PON1 activity toward paraoxon was 30 % lower in men who had a new coronary event than in those who did not (Mackness *et al.*, 2003).

In summary, the human epidemiological studies provide strong support for the following conclusions,

i) PON1 is protective against atherosclerosis,

ii) PON1 genetic variations are important determinants of genetic susceptibility to CHD,

iii) PON1 polymorphisms have adverse effects on CHD in the presence of NIDDM.

Furthermore, the results also provide strong support for the oxidation hypothesis of atherosclerosis and suggest that PON1 polymorphisms may also affect plasma lipid levels (Shih *et al.*, 2002).

1.2.2.9 PON1 Genetic Polymorphisms and Activity in Cerebrovascular Disease (Stroke)

Cerebrovascular disease (CVD) includes all disorders in which an area of the brain is transiently or permanently affected by ischemia or bleeding and one or more of the cerebral blood vessels are involved in the pathological process. Relative to cardiovascular diseases, cerebrovascular diseases were much less studied in relation to PON1 polymorphisms and activities.

1.2.2.9.1 Genetic Studies

Carotid Atherosclerosis

Carotid artery atherosclerotic disease is a progressive disease that involves the buildup of fatty material and plaque in the carotid arteries and can lead to a stroke. Intima-media thickness (IMT) is considered to be a surrogate marker of atherosclerosis. As in the case of cardiovascular diseases, association studies of PON1 genotypes and IMT yielded mixed results. In a group of studies, no association was found between IMT and PON1 192Q/R (Schmidt *et al.*, 1998; Dessi *et al.*, 1999; Markus *et al.*, 2001; Fortunato *et al.*, 2003; Karvonen *et al.*, 2004) or 55L/M (Karvonen *et al.*, 2004) genotypes. Campo *et al.*, (2004) also reported that PON1 promoter −107T/C and coding region 192Q/R and 55L/M polymorphisms were not associated with the presence of carotid atherosclerosis in subjects with primary hypercholesterolemia.

Other studies have found significant associations between carotid artery disease or IMT and one or more of the PON1 genotypes. 55*LL* genotype was found to be significantly associated with the presence and severity of carotid disease (Schmidt *et al.*, 1998). It was also reported that familial hypercholesterolemia (FH) patients with the homozygous wild type (*LLQQ*) genotype for paraoxonase had the highest mean carotid IMTs when compared to other genotypes (Leus *et al.*, 2000). Hu and coworkers (2003) demonstrated that IMT was significantly greater in the *RR* subgroup than in both *QR* and *QQ* subgroups of type 2 diabetic subjects. −824 G/A

polymorphism was shown to be an independent predictor of carotid IMT (Roest *et al.*, 2005)**.**

Stroke

PON1 192*RR* or 192*R*+ genotypes were identified as risk factors for ischemic stroke in several studies (Imai *et al.*, 2000; Voetsch *et al.*, 2002; Ranade *et al.*, 2005; Baum *et al.*, 2006), but not all (Ueno *et al.*, 2003; Pasdar *et al.*, 2006). One recent study found 192*R* allele to be significantly more frequent in stroke patients compared to controls (Aydin *et al.*, 2006).

An association between PON1 55L/M polymorphism and stroke was not determined in most of the studies conducted so far (Voetsch *et al.*, 2002; Ranade *et al.*, 2005; Pasdar *et al.*, 2006). Surprisingly, a significant increase of the 55*M* allele in Japanese cerebral infarction subjects compared with controls was also found (Ueno *et al.*, 2003).

1.2.2.9.2 Activity Studies

Two large studies investigating the relationship of PON1 status and genotype, in the coding and promoter regions of the PON1 gene, with carotid artery atherosclerotic disease (CAAD) have been published (Jarvik *et al.*, 2000, 2003). Both of these studies found that PON1 status was associated with CAAD, whereas the PON1 genotypes were not (Jarvik *et al.*, 2000, 2003). In a recent study, PON activities were found to be significantly diminished in stroke patients compared to controls (Aydin *et al.*, 2006).

1.3 Scope and Aim of the Study

Stroke is the third leading cause of death and the leading cause of disability. There are considerable emotional and financial costs in the care of patients who have suffered a stroke (Taylor *et al.*, 1996; Gorelick *et al.*, 1999). As the population ages, the stroke rate more than doubles for each successive decade after age 55 and it is likely that morbidity will also continue to increase (Murray and Lopez, 1997; Sacco *et al.*, 1997).

Atherosclerosis of the carotid arteries is one of the causes of ischemic stroke. An enzyme called PON1, is located on HDL and is responsible for hydrolysis of lipid peroxides and thus protection against atherosclerosis. Activity of PON1 is, however, variable between individuals. Genetic polymorphisms in PON1 gene account for some of the variability between individuals.

The main objective of this study was to determine the usefulness of PON1 activity and genetic polymorphisms as biomarkers for the determination of susceptibility to ischemic stroke in Turkish population. To achieve this aim, this study was designed to follow the steps given below:

-obtaining total blood and serum samples from ischemic stroke patients and healthy controls,

- measuring PON1 enzyme activity of each individual in serum samples with three substrates; paraoxon (paraoxonase activity), phenylacetate (arylesterase activity), diazoxon (diazoxonase activity),

- isolation of genomic DNA in intact form from blood samples,

- amplification of three regions in PON1 gene; two in coding region (192Q/R and 55L/M) and one in promoter region (–107T/C) by PCR,

- digestion of the amplified fragments with restriction endonucleases to determine the genotype of each individual for 192Q/R, 55L/M and –107T/C single nucleotide polymorphisms, respectively,

- determination of the genotype and allele frequencies of 192Q/R, 55L/M and –107T/C single nucleotide polymorphisms for ischemic stroke and control groups,

- analysis of association between PON1 enzyme activities and genotypes,

- comparison of vascular risk factors, PON1 enzyme activities and genotype and allele frequencies between ischemic stroke and control groups, and between subgroups of patients and controls defined by age, gender, and presence of one of the risk factors, using statistical methods, in order to determine risk factors for ischemic stroke.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1 Population and Blood Sampling

The study population was comprised of 172 (97 males and 75 females) consecutive unrelated adult Caucasian patients with acute hemispheric ischemic stroke and 105 (52 males and 53 females) symptom-free Caucasian controls from the same geographic region (central Anatolia, Turkey). Serum and total blood samples from the participants were obtained from Gülhane Military Medical Academy Hospital Neurology Department, Ankara. Informed consent was obtained from all participants before study entry (see Appendix A). The study was approved by the ethical committee of the medical faculty (see Appendix B) and was carried out according to the principles of the Declaration of Helsinki.

Cases were selected among patients suffering atherothrombotic ischemic stroke admitted to the neurology services of Gülhane Medical Faculty, Ankara, within 24 h after onset, from October 2005 to March 2007. Patients' recruitment was performed consecutively. Stroke was defined as the new onset of rapidly developing symptoms and signs of loss of cerebral function that lasted at least 24 hours and had no apparent cause other than that of vascular origin. The cerebral infarction was initially diagnosed on the basis of neurological examination and brain CT scan. Patients were considered eligible if the following criteria were met: having anterior circulation stroke, no other major illnesses, including autoimmune diseases, neoplasms,

coagulopathies, hepatic or renal failure, no known embolic source (aortic arch, cardiac or carotid), no family history of hematological, autoimmune or chronic inflammatory diseases, no history of myocardial infarction within 3 weeks, or of transient ischemic attack or stroke at any time. Each patient underwent 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. All exclusion criteria were applied to the controls exactly plus not having carotid stenosis (lumen narrowing) >50% or ulcerated carotid plaque. All subjects underwent bilateral carotid Doppler ultrasound (CUSG) and transthoracic echocardiographic studies.

Our colleagues in Gülhane Medical Faculty Neurology Department obtained a detailed history of conventional vascular risk factors and conditions from each participant. Hypertension was defined as systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg and/or use of antihypertensive drugs. Diabetes was defined as fasting glucose ≥6.99 mmol/L and/or use of pharmacological treatment. Obesity was assigned when body mass index was 30 or higher. Smoking status of an individual was assigned “yes” if the individual is currently smoking or have quitted less than 3 months ago. Routine laboratory tests, including electrocardiogram, chest X-ray, complete blood count, leukocyte differential, erythrocyte sedimentation rate, routine biochemistry tests including fasting glucose, lipid profile (triglycerides, total cholesterol, LDL, HDL), creatinine, sodium, potassium, bilirubin, and liver function tests, routine urine tests and rheumatologic screening tests were performed for all participants in the Gülhane Medical Faculty. All laboratory measurements were done blinded to clinical characteristics.

The study population (172 patients and 105 controls) was selected from a larger population of 180 patients and 130 controls. The rationale for selection was as follows:

- 12 controls were removed from the group since they had either ischemic heart disease or carotid stenosis > %50, or both.
- 3 subjects were removed from the group, since the serum sample of these participants were hemolyzed; and thus enzyme activity determinations could not be carried out.
- 1 subject was removed from the group, because the −107T/C genotype of this individual could not be determined.
- In order to maintain mean age of patient and control groups close, 4 patients whose age was 90 years and older and 13 controls aged 35 years and younger were removed from the group.
- Thus a total of 33 subjects were removed.

2.1.2 Chemicals

The chemicals, their code numbers and suppliers used in this study are given in Appendix C. All chemicals used in this study were of molecular grade or were obtained from commercial sources at the highest grade of purity.

2.2 Methods

2.2.1 PON1 Phenotype Determination

PON1 phenotypes were determined by using three different substrates: paraoxon (paraoxonase activity), phenyl acetate (arylesterase activity) and diazoxon (diazoxonase activity).

2.2.1.1 Determination of Human Serum Paraoxonase Activity

Paraoxonase activity (PON) was determined using the method described by Furlong *et al.*, 1988, where the rate of paraoxon hydrolysis by paraoxonase 1 is followed by the formation of hydrolysis product *p*-nitrophenol (Figure 2.1).

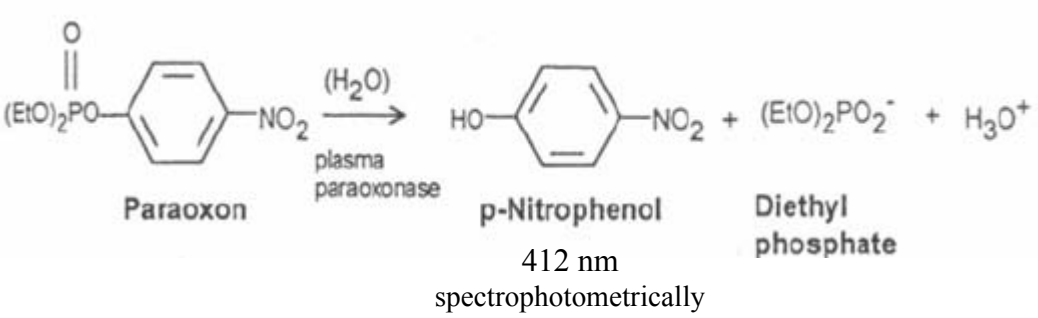


Figure 2.1 Hydrolysis of paraoxon by plasma paraoxonase into p-nitrophenol and dietylphosphate (taken from Richter and Furlong, 1999).

Standard enzyme assay mixtures were prepared as given in Table 2.1 and contained 0.1 M Tris-HCl, pH 8.5, 2.5 mM $CaCl_2$, 2 M NaCl, 40 µL serum, and 1.2 mM paraoxon. The substrate solution (6mM paraoxon) was prepared daily from stock (120 mM) paraoxon, using 50 mM Tris-HCl pH 8.5 for dilution. 120 mM stock paraoxon was prepared in acetone and was stable for approximately 3 weeks when stored at 4°C.

The assay was initiated by the addition of the substrate solution (200 µL of 6 mM freshly prepared paraoxon substrate solution), and the formation of *p*-nitrophenol was continuously monitored by its absorbance at 412 nm and at 37°C with Shimadzu UV160-A double-beam spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). A molar extinction coefficient of 18.05 x 10^3 was used for calculation of enzyme activity, which was expressed in unit per liter (U/L). One unit of paraoxonase activity is defined as 1 µmol *p*-nitrophenol formed per min under the given conditions.

Table 2.1 Reaction medium constituents for determination of serum paraoxonase activity**.**

Constituent	**Stock concentration**	**Volume added (µL)**	**Final concentration in 1 mL reaction mixture**
Tris-HCl, pH 8.5	1 M	100	0.1 M
$CaCl_2$	50 mM	50	2.5 mM
NaCl	5 M	400	2 M
Serum	---	40	---
Paraoxon	6 mM	200	1.2 mM
dH_2O	---	210	---

2.2.1.2 Determination of Arylesterase Activity

The rate of phenylacetate hydrolysis (arylesterase activity; ARE) was measured by using the modified methods of Eckerson *et al.*, 1983b and Furlong *et al.*, 1988, by following the formation of phenol. This reaction is given in Figure 2.2.

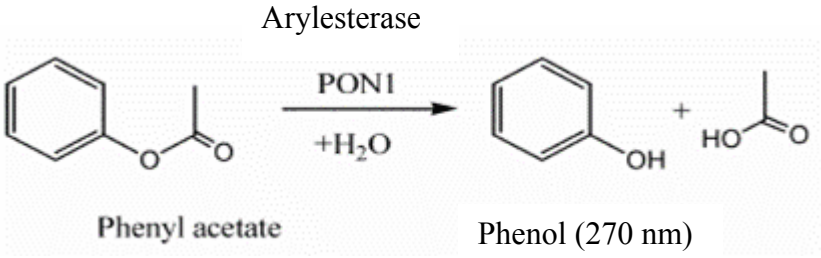


Figure 2.2 Hydrolysis of phenylacetate by arylesterase (PON1) into phenol and acetate.

The rate of formation of phenol at 37°C was monitored spectrophotometrically (Shimadzu UV160-A double-beam spectrophotometer) at 270 nm in a reaction mixture, which was prepared according to Table 2.2. The assays contained 3.6 mM phenylacetate, 20 µL of a 1:50 dilution of serum (in water) and 0.9 mM $CaCl_2$ in 3 mL 9 mM pH 8.0, Tris-HCl buffer and was initiated by addition of 100 µL of 108 mM substrate solution. Substrate solution (108 mM phenyl acetate) was prepared fresh each hour using a 1:1 mixture of dH_2O and ethanol. A molar extinction coefficient of 1.31 x 10^3 was used for calculation of enzyme activity, which was expressed in unit per mL (U/mL). One unit of arylesterase activity is defined as 1 µmol phenol formed per minute under the given conditions.

Table 2.2 Reaction medium constituents for determination of serum arylesterase activity.

Constituent	**Stock concentration**	**Volume added**	**Final concentration in 3 mL reaction mixture**
Tris-HCl, pH 8.0	10 mM	2.7 mL	9 mM
$CaCl_2$	50 mM	54 µL	0.9 mM
Serum (1:50 diluted)	---	20 µL	---
Phenyl acetate	108 mM	100 µL	3.6 mM
dH_2O	---	126 µL	---

2.2.1.3 Determination of Diazoxonase Activity

Diazoxonase activity (DIA) was determined by using the method described by Richter and Furlong, 1999, where the rate of hydrolysis of diazoxon is followed by the formation of pyrimidinol (IMHP; 2-isopropyl-4-methyl-6-hydroxy pyrimidine; Figure 2.3).

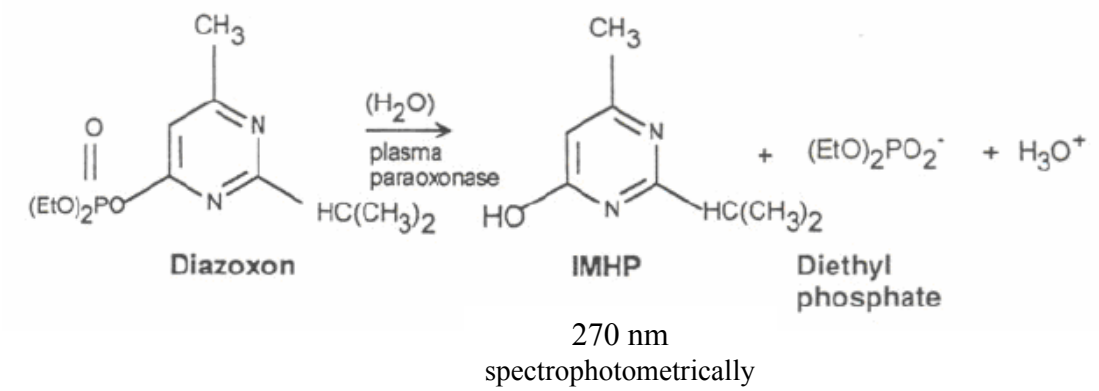


Figure 2.3 Hydrolysis of diazoxon by paraoxonase 1 into IMHP (2-isopropyl-4-methyl-6-hydroxy pyrimidine) and diethylphosphate (taken from Richter and Furlong, 1999).

Reaction medium constituents for determination of serum diazoxonase activity are given in Table 2.3. The assays contained 0.1 M Tris-HCl, pH 8.5; 2.0 M NaCl; 5.0 mM $CaCl_2$ and 500 µM diazoxon (diazinon O-analog) in a volume of 1 mL and were initiated by the addition of 5µL of a 1:1 dilution of serum (in water) at 37ºC. 5 mM diazoxon was prepared from a stock of 867 mM diazoxon, using dH_2O as diluent. 867 mM stock diazoxon was prepared using absolute ethanol and was stored at -80ºC. Rate of diazoxon hydrolysis was followed spectrophotometrically

(Shimadzu UV160-A double-beam spectrophotometer) by the appearance of IMHP at 270 nm. Rate of conversion of diazoxon to IMHP was calculated based on a change of 3.03 absorbance units mM^{-1} IMHP generated at 270 nm (1 cm path length). Diazoxonase activity was expressed in U/L and 1 unit is equivalent to 1 μmol substrate hydrolyzed per min per liter of serum under the given conditions.

Table 2.3 Reaction medium constituents for determination of serum diazoxonase activity.

Constituent	**Stock concentration**	**Volume added (μL)**	**Final concentration in 1 mL reaction mixture**
Tris-HCl, pH 8.5	1 M	100	0.1 M
$CaCl_2$	50 mM	100	5 mM
NaCl	5 M	400	2 M
Serum (1:1 diluted)	**---**	5	**---**
Diazoxon	5 mM	100	0.5 mM
dH_2O	---	295	---

2.2.2 Genotype Determination

2.2.2.1 Preparation of Genomic DNA for PCR

2.2.2.1.1 Isolation of Genomic DNA from Human Whole Blood Samples

DNA extractions were performed using a salting-out method according to the method described by Lahiri and Schnabel (1993), with some modifications. Whole blood collected to EDTA containing tube was used as DNA source. The procedure was as follows: 500 µL of whole blood was treated with an equal volume of low-salt buffer containing 10 mM Tris-HCl, pH 7.6, 10 mM KCl, 2 mM EDTA and 4 mM $MgCl_2$ (TKEM buffer). 12.5 µL of Triton X-100 was added and the cells were lysed by inverting the tube several times. The suspension was centrifuged at 1,000g for 10 min at room temperature. The pellet of mostly leukocytes was saved and washed two more times with TKEM buffer. The final pellet was resuspended in 0.1 mL of TKEM buffer. 10 µL of 10 % sodium dodecyl sulfate (SDS) was added, and the whole suspension was mixed thoroughly and incubated for 10 min at 58ºC. After adding 37.5 µL of saturated NaCl (~6 M), the tube was mixed well and centrifuged at 12,000g for 7 min. The supernatant contained DNA, which was precipitated using 2x volume ice-cold ethanol. The tubes were stored at -20ºC for at least 30 min and DNA was precipitated to pellet by centrifugation at 10,000g for 10 min at 4ºC. Supernatant was removed and the DNA containing pellet was solubilized with 0.1 mL 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer). For complete dissolvation, tubes were incubated at 37ºC for 1.5-2 hrs.

2.2.2.1.2 Quantification of DNA Concentration by Spectrophotometry

Genomic DNA isolated from each subject was quantified spectrophotometrically using Shimadzu UV160-A double-beam spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) by measuring its absorbance at 260 nm.

Concentration was calculated using the following formula:

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

2.2.2.1.3 Qualification of Genomic DNA by Spectrophotometry

In addition to 260 nm, absorbance of each DNA sample was also determined at 280 nm and the ratio between absorbance values at 260 nm and 280 nm (A_{260}/A_{280}) was used to estimate the purity of the nucleic acid. Pure DNA preparations give the ratio of 1.8, while the higher or lower values indicate RNA or protein contaminations, respectively.

2.2.2.1.4 Qualification of Genomic DNA by Agarose Gel Electrophoresis

Intactness of DNA samples was determined by 0.5% agarose gel electrophoresis, using a horizontal agarose gel electrophoresis unit, which had a gel tray in 8 cm x 9 cm dimensions. 0.5% agarose gel was prepared by adding 0.15 g agarose to 30 mL of 0.5x TBE (450 mM Tris, 450 mM Borate, 10 mM EDTA) buffer, pH 8.3 in an erlenmeyer flask, so that the buffer did not occupy more than half of the volume of the flask. The slurry in the erlenmeyer flask was heated in a microwave oven until all of the grains of agarose dissolved. Before adding ethidium bromide to the dissolved agarose, the flask was cooled to approximately 60°C on a magnetic stirrer, with continuous stirring. When cooled enough, ethidium bromide was added from a stock solution of 10 mg/mL in water to a final concentration of 0.5 µg/mL and the solution was mixed thoroughly. While the agarose gel is being heated, the mold, plastic tray and comb were cleaned with 70 % ethanol. The plastic tray was settled in the mold and the comb was placed 0.5-1.0 mm above the plate. The warm agarose solution was then poured into the mold and any air bubbles-if present-, especially under or between the teeth of the comb were removed with the help of a pipette tip. The gel was allowed to solidify completely for approximately

20-40 minutes at room temperature. The gel tank was filled with approximately 300 mL of 0.5x TBE buffer. The comb was carefully removed from the gel and the gel in the plastic tray was mounted in the electrophoresis tank so that the slots of the gel faced towards the negative pole-cathode. 0.5x TBE buffer was added to the tank until it covered the gel to a depth of about 1 mm. Any air bubbles in the wells, if present, were removed with the help of a pipette tip. 5 µL (0.25-0.5 ng) of DNA sample was mixed with 1 µL of gel loading buffer (0.25% bromophenol blue and 40% sucrose in dH_2O) by sucking in and out of a micropipette and the mixture was slowly loaded into the slots of the gel. The lid of the tank was closed and the electrical leads were attached to the power supply. The power supply was set to the constant voltage of 100 volts, so that a voltage of 5 V/cm (measured as the distance between the electrodes) was applied. The gel was run for 45 minutes and then examined under UV light and the photograph was taken by using Vilber Lourmat Gel Imaging System (Marre La Vallee, Cedex, France) and Bio-Capture (Version 99.03) computer software. Pure DNA preparations give a single band in agarose gel electrophoresis, while RNA contaminated preparations yield two bands. A smear indicates that the DNA is degraded.

2.2.2.2 Genotyping for 192Q/R, 55L/M and −107T/C Single Nucleotide Polymorphisms of PON1

Standard PCR protocols, followed by restriction enzyme digestions were used to genotype the PON1 192Q/R, 55L/M and −107T/C polymorphisms (see Table 2.4).

Table 2.4 Genotyping of PON1; table showing the regions of amplification, SNP position, primer pairs used for amplification, size of the PCR products, restriction endonuclease (RE) used for digestion of PCR products, and size of digestion products and their interpretation.

Region of amplification	**SNP position**	**Primers**	**PCR product size**	**RE**	**Size of digestion products and interpretation**
Coding region	192Q/R (192 A/G)	5′ TAT TGT TGC TGT GGG ACC TGA G 3′ 5′ CCT GAG AAT CTG AGT AAA TCC ACT 3′	238 bp	*AlwI* (*BspPI*)	**192QQ**= 238 bp **192QR**= 238, 172, 66 bp **192RR**= 172, 66 bp
Coding region	55L/M (55 T/A)	5′ CCT GCA ATA ATA TGA AAC AAC CTG 3′ 5′ TGA AAG ACT TAA ACT GCC AGT C 3′	172 bp	*NlaIII* (*Hin1II*)	**55LL**=172 bp **55LM**=172, 106, 66 bp **55MM**=106, 66 bp
Promoter region	-107T/C	5’ AGC TAG CTG CGG ACC CGG CGG GGA GGA G 3’ 5’ GGC TGC AGC CCT CAC CAC AAC CC 3’	240 bp	*BsrBI* (*MbiI*)	**-107TT**= 240 bp **-107TC**= 240, 212, 28 bp **-107CC**= 212, 28 bp

2.2.2.2.1 192Q/R Single Nucleotide Polymorphisms

2.2.2.2.1.1 Polymerase Chain Reaction for 192Q/R SNP

PON1 192Q/R SNP region was amplified using primer sequences given by Campo *et al.*, 2004 (Table 2.4). Sequence of the amplified fragment in coding region of PON1 gene that includes 192Q/R single nucleotide polymorphism is given in Figure 2.4.

Several modifications regarding the $MgCl_2$ concentration, primer and template DNA amount, and amplification program were carried out in order to obtain a single band amplified certainly from the 192Q/R SNP region of PON1. Components of the optimized PCR mixture for the amplification of 192Q/R SNP are given in Table 2.5. PCR reaction (total volume 50 µL) contained approximately 400 ng genomic DNA, 200 µM dNTPs, 400 nM of each primer, 2.0 mM $MgCl_2$ and 1.25 Unit of Taq polymerase (Table 2.5).

Table 2.5 Components of PCR mixture for PON1 192Q/R SNP.

Constituent	**Stock concentration**	**Volume added**	**Final Concentration in 50 µL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 µL	
Amplification Buffer	10X	5 µL	1X
$MgCl_2$	25 mM	4 µL	2.0 mM
dNTP mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmol/µL	2 µL	20 pmol (400 nM)
Reverse Primer	10 pmol/µL	2 µL	20 pmol (400 nM)
Template DNA	changes	changes	400 ng
Taq DNA Polymerase	5 U/µL	0.25 µL	1.25 U

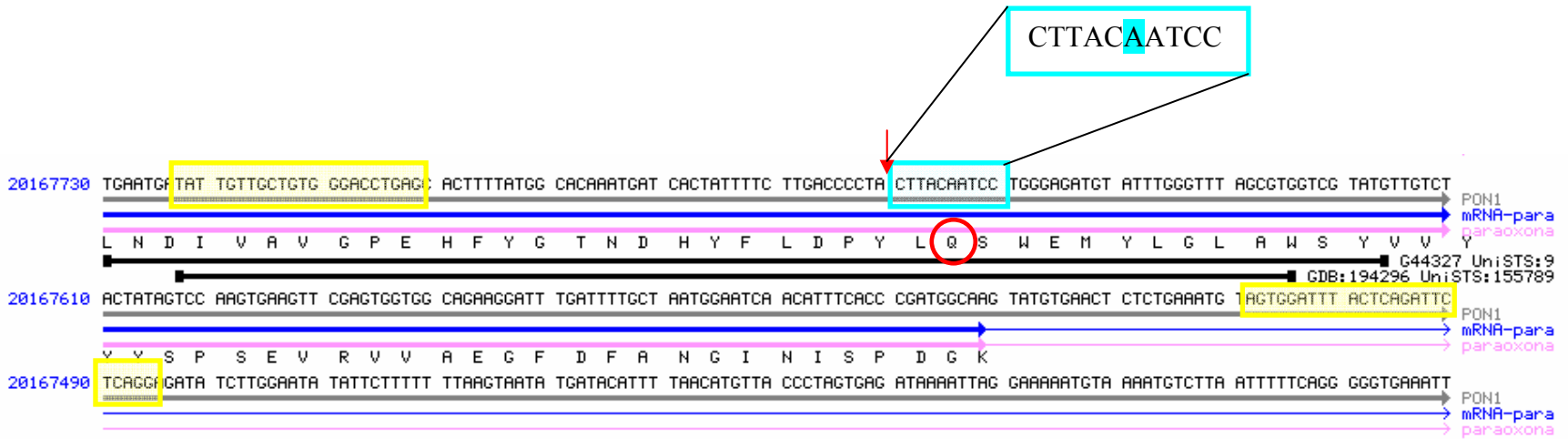


Figure 2.4 Sequence of the amplified fragment in coding region of PON1 gene that includes 192Q/R single nucleotide polymorphisms. The forward and reverse primers are marked with yellow boxes. Location of recognition sequence for *AlwI* restriction endonuclease is marked with blue box and the red arrow shows the cutting point for *AlwI*. The polymorphic amino acid (Q) is marked with a red circle. The recognition site is zoomed and the polymorphic nucleotide (A) is highlighted blue (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

The program of the thermalcycler used for the amplification of 192Q/R SNP region of PON1 were modified from those published (Campo *et al.*, 2004) and the optimized PCR program used in the present study are given in Table 2.6.

Table 2.6 PCR program used for the amplification of the 192Q/R SNP region of PON1.

Initial denaturation	94°C	3 min.	
Denaturation	95°C	30 sec.	35 cycles
Annealing	60°C	20 sec.	
Extension	72°C	50 sec.	
Final extension	72°C	10 min	

PCR products were analyzed on 2.0 % agarose gel which was prepared as described in section 2.2.2.1.4. Eight µL of PCR product was mixed with 1 µL of gel loading buffer and applied to the wells of gel. Six µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V.

2.2.2.2.1.2 Restriction Endonuclease Digestion of PCR Products for Determination of 192Q/R SNP

Schematic representation of the protocol for the determination of 192Q/R genotypes is given in Figure 2.5. Molecular basis of the 192Q/R polymorphism is a single nucleotide change in DNA from adenine (A) to guanine (G). The codon formed in the wild type allele, CAA, codes for glutamine. Thus, this allele is called 192*Q*. The codon formed in the polymorphic allele, CGA, codes for arginine, and the

allele is called 192*R*. The sequence around guanine (3' CCTAG$(N)_5$ 5') in the 192*R* allele is a recognition site for *AlwI*, which is also given below:

AlwI cuts the 238-bp PCR product into two fragments of 66-bp and 172-bp. In the wild type allele (192*Q*), this sequence is 3' CCTAA$(N)_5$ 5', which is not recognized by *AlwI*. In the agarose gel, a single 238-bp band indicates 192*QQ* genotype, while two bands of 66-bp and 172-bp indicate 192*RR* genotype. Heterozygotes contain two alleles together and are represented by three bands in agarose gel (Figure 2.5).

First step of the procedure to determine glutamine (Q) and arginine (R) alleles for the position 192 of PON1 consisted of incubation of 10 µL of 238-bp PCR product with 2U *AlwI* (*BspPI*), at 55°C for 20 hrs, in a reaction mixture as described in Table 2.7. At the end of the incubation period, digestion products were analyzed on 2.5 % agarose gel. 238-bp PCR product resulted in 66- and 172-bp fragments for the 192*R* allele and a no-digested 238-bp fragment for the 192*Q* allele.

Table 2.7 Constituents of reaction mixture for restriction endonuclease (*AlwI*) digestion of PCR products for the determination of PON1 192Q/R SNP.

Constituent	**Concentration**	**Volume added**	**Final concentration in 30 µL reaction mixture**
Buffer Tango	10 X	3 µL	1 X
Sterile Apyrogen dH_2O	---	Up to 30 µL	---
AlwI (*Bsp PI*)	2 U/ µL	1 µL	2 U
PCR product	---	10 µL	---

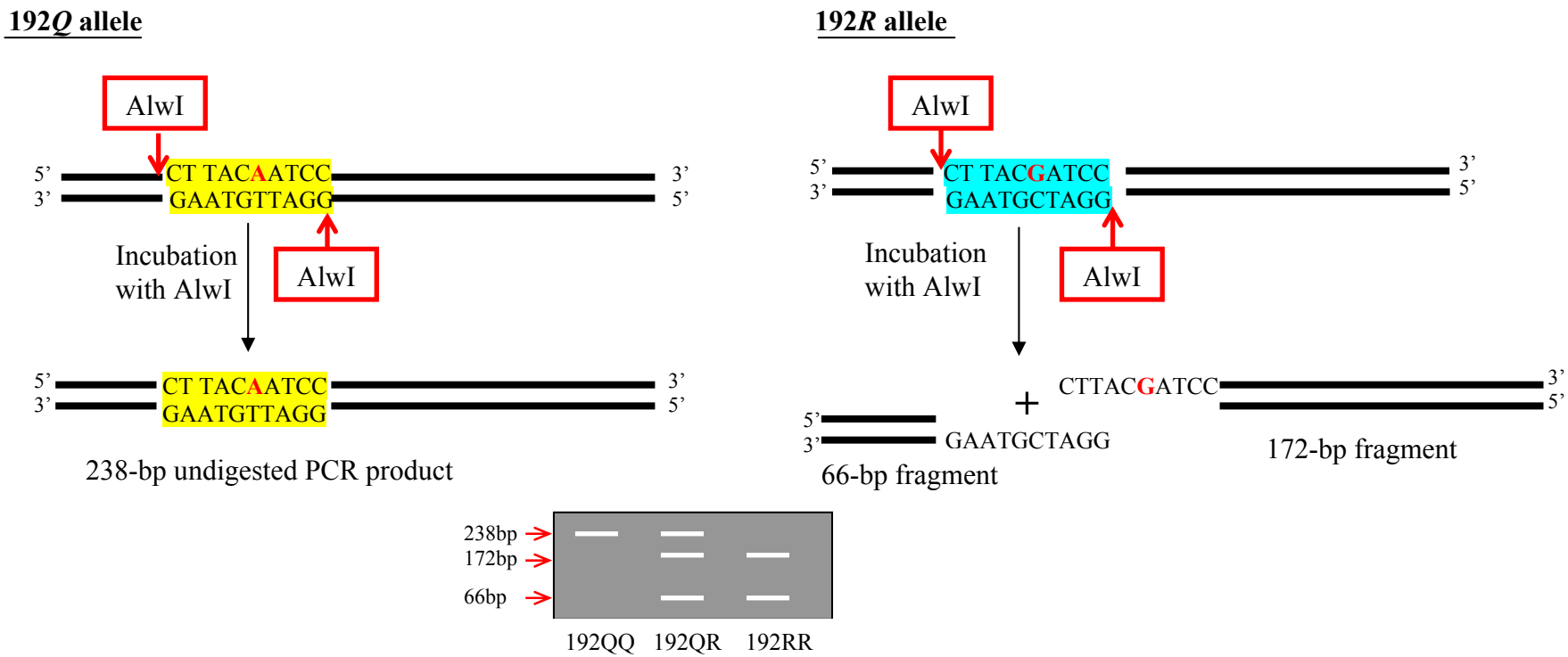


Figure 2.5 Schematic representation of 192Q/R genotype determination. In the left panel, the codon for the 192th amino acid glutamine (CAA) and the surrounding nucleotides in wild type allele (192*Q*) can be seen. In the polymorphic allele (192*R*; right panel), A is replaced with G, thus the codon becomes CGA. Since CGA codes for arginine, this allele is called 192*R*. A recognition site for *AlwI* (3’ CCTAG $(N)_5$ 5’) is also created within the polymorphic allele, which can be seen as the blue highlighted region, where G (typed in red) is the polymorphic nucleotide. *AlwI* cuts the 238-bp PCR product containing the recognition site after $(N)_5$. Digestion with *AlwI* results in two fragments of 66-bp and 172-bp. On the other hand, *AlwI* does not recognize the nucleotide sequence 3’ CCTAA $(N)_5$ 5’ (left panel; highlighted yellow) in the wild type allele, thus an undigested 238-bp fragment implies 192*Q* allele. In heterozygotes (192*QR*), two alleles exist together. At the bottom a representative agarose gel photograph is given.

2.2.2.2.2 55L/M Single Nucleotide Polymorphism

2.2.2.2.2.1 Polymerase Chain Reaction for 55L/M SNP

PON1 55L/M SNP region was amplified using primer sequences given by Campo *et al.*, 2004 (Table 2.4). Sequence of the amplified fragment in coding region of PON1 gene that includes 55L/M single nucleotide polymorphism is given in Figure 2.6. Several modifications regarding the $MgCl_2$ concentration, primer and template DNA amount, and amplification program were carried out in order to obtain a single band amplified from the 55L/M SNP region of PON1. Components of the optimized PCR mixture for the amplification of 55L/M SNP are given in Table 2.5. PCR reaction (total volume 50 µL) contained approximately 400 ng genomic DNA, 200 µM dNTPs, 350 nM of each primer, 2.0 mM $MgCl_2$ and 1.25 Unit of Taq polymerase (Table 2.8).

Table 2.8 Components of PCR mixture for PON1 55L/M SNP.

Constituent	**Stock concentration**	**Volume added**	**Final Concentration in 50 µL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 µL	
Amplification Buffer	10X	5 µL	1X
$MgCl_2$	25 mM	4 µL	2.0 mM
dNTP mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmol/µL	1.75 µL	17.5 pmol (350 nM)
Reverse Primer	10 pmol/µL	1.75 µL	17.5 pmol (350 nM)
Template DNA	changes	changes	400-500 ng
Taq DNA Polymerase	5 U/µL	0.25 µL	1.25 U

The program of the thermalcycler for the amplification of 55L/M SNP region of PON1 were modified from those published (Campo *et al.*, 2004) and the optimized PCR program used in the present study are given in Table 2.9.

Table 2.9 PCR program used for the amplification of the 55L/M SNP region of PON1.

Initial denaturation	94°C	3 min.	
Denaturation	95°C	30 sec.	35 cycles
Annealing	60°C	20 sec.	
Extension	72°C	50 sec.	
Final extension	72°C	10 min	

PCR products were analyzed on 2.0 % agarose gel which was prepared as described in section 2.2.2.1.4. Eight µL of PCR product was mixed with 1 µL of gel loading buffer and applied to the wells of gel. The gel was run for 1 hour at 100V.

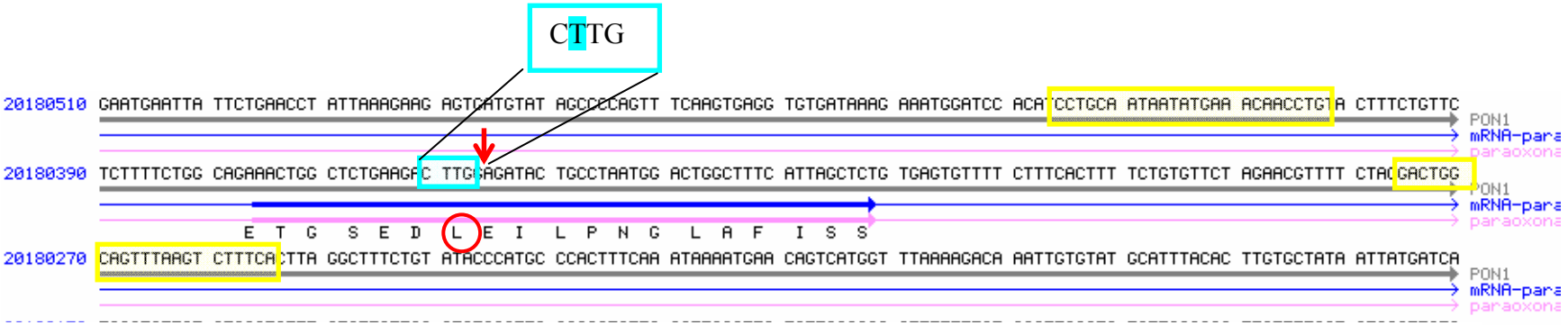


Figure 2.6 Sequence of amplified fragment in coding region of PON1 gene that includes 55L/M single nucleotide polymorphisms. The forward and reverse primers are marked with yellow boxes. Location of recognition sequence for *NlaIII* restriction endonuclease is marked with blue box and the red arrow shows the cutting point for *NlaIII*. The polymorphic amino acid (L) is marked with a red circle. The recognition site is zoomed and the polymorphic nucleotide (T) is highlighted blue (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

2.2.2.2.2.2 Restriction Endonuclease Digestion of PCR Products for 55L/M SNP

Schematic representation of the protocol for the determination of 55L/M genotypes is given in Figure 2.7. Molecular basis of the 55L/M polymorphism is a single nucleotide change in DNA from thymine (T) to adenine (A). The codon formed in the wild type allele, UUG, codes for leucine. Thus this allele is called 55*L*. The codon formed in the polymorphic allele, AUG, codes for methionine, and the allele is called 55*M*. The sequence around adenine (5' CATG 3') is a recognition site for *NlaIII*, which is also given below:

NlaIII cuts the 172-bp PCR product into two fragments of 66-bp and 106-bp. In the wild type allele, this sequence becomes 5' CTTG 3', which is not recognized by *NlaIII*. In the agarose gel, a single 172-bp band indicates 55*LL* genotype, while two bands of 66-bp and 106-bp indicate 55*MM* genotype. Heterozygotes (55*LM*) contain two alleles together and are represented by three bands in agarose gel (Figure 2.7).

The procedure to determine leucine (*L*) and methionine (*M*) alleles at position 55 of PON1 started with incubation of 10 µL of 172-bp PCR product with 2.5 U *NlaIII* (*Hin1II*) at 37°C for 20 hrs, in a reaction mixture as given in Table 2.10. At the end of the incubation period, digestion products were analyzed on 2.5 % agarose gel. The digestion resulted in 66-and 106-bp fragments for the 55*M* allele and in a non-digested 172-bp fragment for the 55*L* allele.

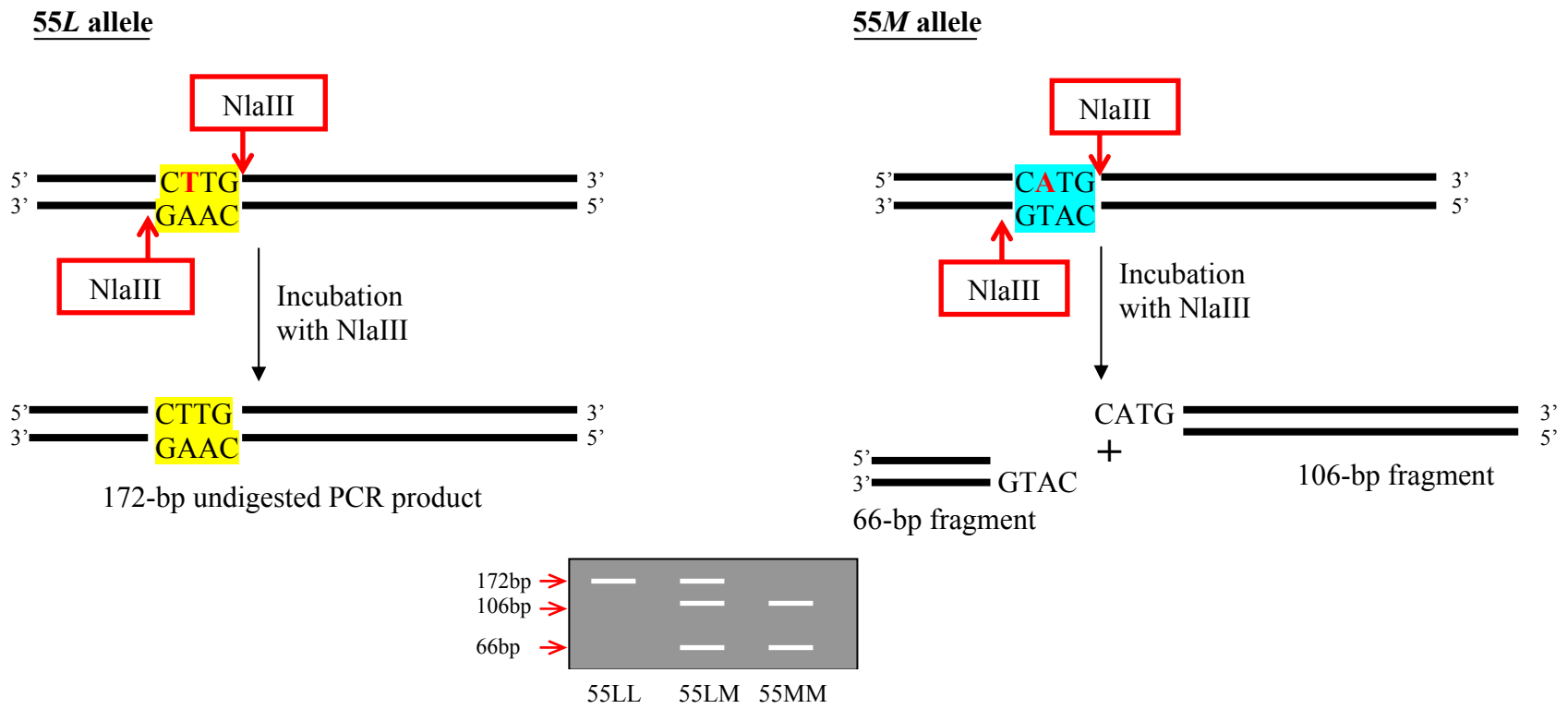


Figure 2.7 Schematic representation of 55L/M genotype determination. In the wild type allele 55*L* (left panel), the codon for the 55th amino acid leucine is TTG (UUG). In the polymorphic allele (55*M*; right panel), T is replaced with A, thus the codon becomes ATG (AUG). Since AUG codes for methionine, this allele is called 55*M*. A recognition site for *NlaIII* (3' CATG 5') is also created within the polymorphic allele, which can be seen as the blue highlighted region, where A (typed in red) is the polymorphic nucleotide. *NlaIII* cuts the 172-bp PCR product into two fragments of 66-bp and 106-bp. On the other hand, the sequence 3' GTTC 5' (highlighted yellow; left panel) in the wild type allele is not recognized by *NlaIII*, thus an undigested 172-bp fragment implies 55*L* allele. In heterozygotes (55*LM*), two alleles exist together. At the bottom a representative agarose gel photograph is given.

Table 2.10 Constituents of reaction mixture for restriction endonuclease (*NlaIII*) digestion of PCR products for the determination of PON1 55L/M SNP.

Constituent	Concentration	Volume added	Final concentration in 30 µL reaction mixture
Buffer Tango	10 X	3 µL	1 X
Sterile Apyrogen dH_2O		Up to 30 µL	
NlaIII (*Hin1 II*)	2 U/ µL	1.25 µL	2.5 U
PCR product		10 µL	

2.2.2.2.3 −107T/C Single Nucleotide Polymorphism

2.2.2.2.3.1 Polymerase Chain Reaction for −107T/C SNP

PON1 −107T/C SNP region was amplified using primer sequences and amplification program given by Campo *et al.*, 2004 (Table 2.1). Sequence of the DNA fragment in promoter region of PON1 gene to be amplified that includes −107T/C single nucleotide polymorphism is given in Figure 2.8. Note that the nucleotide sequence of the forward primer is different from that of DNA.

Primer's sequence: AGCTAGCTGCGGACCCGGCGGGGAGG**A**G

DNA's sequence: AGCTAGCTGCGGACCCGGCGGGGAGG**G**G

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

Several modifications regarding the $MgCl_2$ concentration, primer and template DNA amount, and amplification program were carried out in order to obtain a single band amplified for sure from the −107T/C SNP region of PON1. Final optimized reaction medium components for the amplification of −107T/C SNP are given in Table 2.11.

Table 2.11 Components of PCR mixture for PON1 −107T/C SNP.

Constituent	**Stock concentration**	**Volume added**	**Final Concentration in 50 µL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 µL	
Amplification Buffer	10X	5 µL	1X
$MgCl_2$	25 mM	5 µL	2.5 mM
dNTP mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmol/µL	1.75 µL	17.5 pmol (350 nM)
Reverse Primer	10 pmol/µL	1.75 µL	17.5 pmol (350 nM)
Template DNA	changes	changes	400-500 ng
Taq DNA Polymerase	5 U/µL	0.25 µL	1.25 U

The program of the thermalcycler for the amplification of −107T/C SNP region of PON1 were modified from those published (Campo *et al.*, 2004) and the optimized PCR program used in the present study are given in Table 2.12.

Table 2.12 PCR program used for the amplification of the −107T/C SNP region of PON1.

Initial denaturation	94°C	3 min.	
Denaturation	95°C	30 sec.	35 cycles
Annealing	67°C	20 sec.	
Extension	72°C	50 sec.	
Final extension	72°C	10 min	

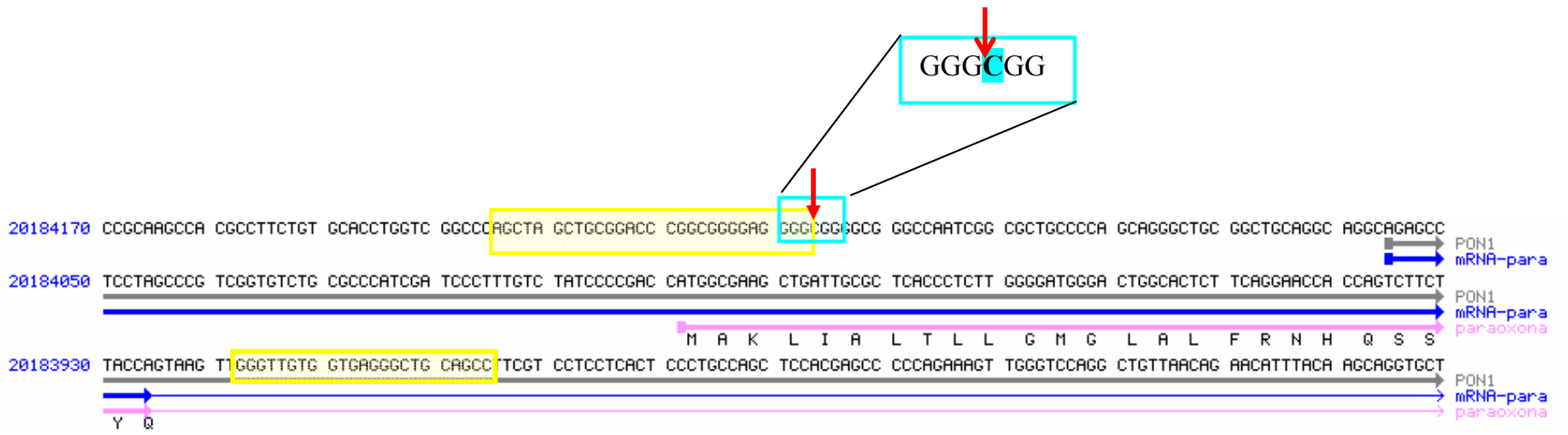


Figure 2.8 Sequence of the DNA fragment in promoter region of PON1 gene to be amplified that includes −107T/C single nucleotide polymorphism. The forward and reverse primers are marked with yellow boxes. Location of recognition sequence for *BsrBI* restriction endonuclease is marked with blue box and the red arrow shows the cutting point for *BsrBI*. The recognition site is zoomed and the polymorphic nucleotide (C) is highlighted blue (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

PCR products were analyzed on 2.0 % agarose gel which was prepared as described in section 2.2.2.1.4. Eight µL of PCR product was mixed with 1 µL of gel loading buffer and applied to the wells of gel. Six µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V.

2.2.2.2.3.2 Restriction Endonuclease Digestion of PCR Products for −107T/C SNP

Schematic representation of the protocol for the determination of −107T/C genotypes is given in Figure 2.9. Molecular basis of the −107T/C polymorphism is a single nucleotide change in DNA from thymine (T) to cytosine (C). The sequence around cytosine (5' GAGCGG 3') is a recognition site for *BsrBI*, which is also given below:

BsrBI cuts the 240-bp PCR product into two fragments of 28-bp and 212-bp. In the wild type allele, this sequence becomes 5' GAGTGG 3', which is not recognized by *BsrBI*. In the agarose gel, a single 240-bp band indicates −107*TT* genotype, while two bands of 28-bp and 212-bp indicate −107*CC* genotype. Heterozygotes (−107*TC*) contain two alleles together and are represented by three bands in agarose gel (Figure 2.9).

The procedure to determine *T* and *C* alleles at position −107 of PON1 started with incubation of 10 µL of 240-bp PCR product with 5 unit *MbiI* (*BsrBI*) at 37°C for 20 hrs, in a reaction mixture as given in Table 2.13. At the end of the incubation period, digestion products were analyzed on 2.5 % agarose gel. The digestion resulted in 212- and 28-bp fragments for the −107*C* allele and in a non-digested 240-bp fragment for the −107*T* allele.

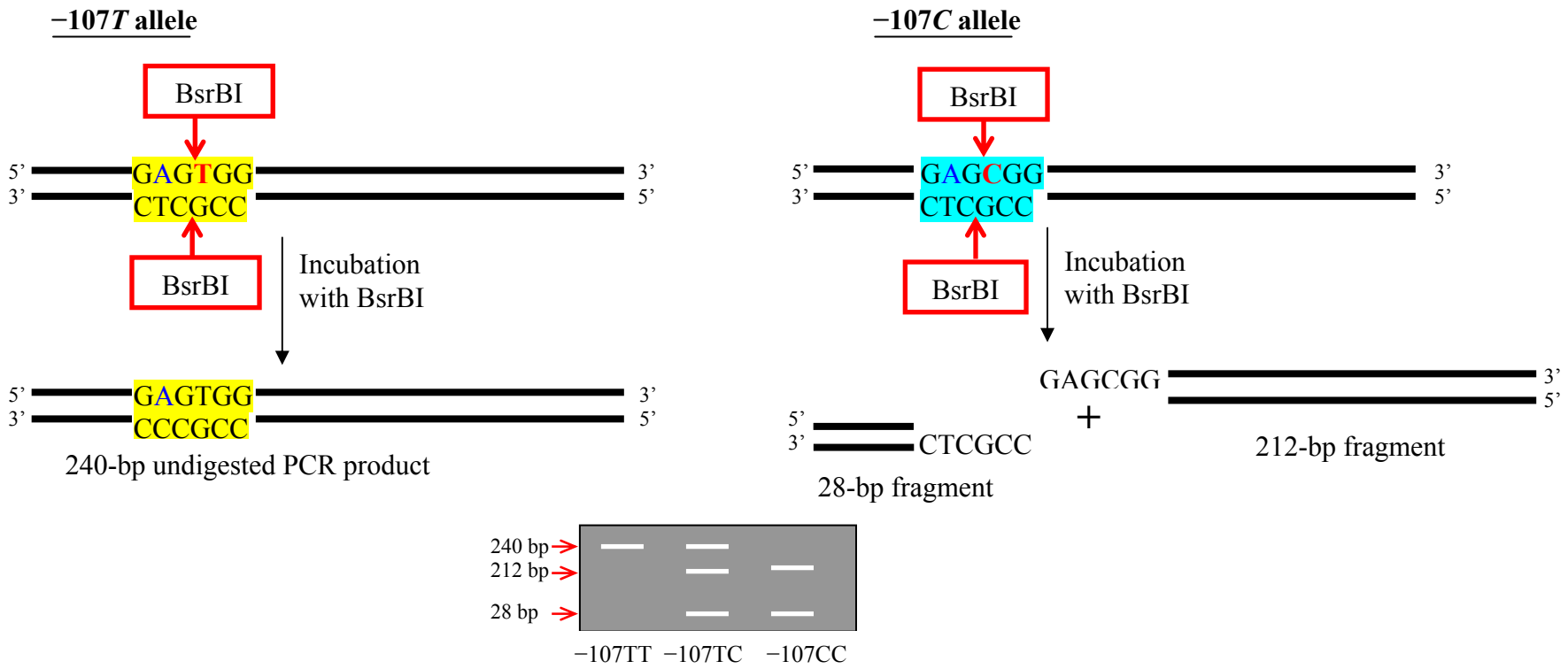


Figure 2.9 Schematic representation of −107T/C genotype determination. In the wild type allele (−107*T*; left panel), position 107 upstream from the translation start site is occupied by T. In the polymorphic allele (−107*C*; right panel), T is replaced with C. A recognition site for *BsrBI* (5’ GAGCGC 3’) is created within the polymorphic allele, which can be seen as the blue highlighted region, where **C** (typed in red) is the polymorphic nucleotide. *BsrBI* cuts the 240-bp PCR product containing the recognition site into two fragments of 28-bp and 212-bp, which imply −107*C* allele. On the other hand, the sequence 5’ GAGTGC 3’ (highlighted yellow; left panel) in the wild type allele is not recognized by *NlaIII*, thus an undigested 240-bp fragment implies −107*T* allele. In heterozygotes (−107*TC*), two alleles exist together. At the bottom a representative agarose gel photograph is given.

Table 2.13 Constituents of reaction mixture for restriction endonuclease (*BsrBI*) digestion of PCR products for the determination of PON1 −107T/C SNP.

Constituent	**Concentration**	**Volume added**	**Final concentration in 30 µL reaction mixture**
Buffer Tango	10 X	3 µL	1 X
Sterile Apyrogen dH_2O	---	Up to 30 µL	---
BsrBI (*MbiI*)	10 U/ µL	0.5 µL	5 U
PCR product	---	10 µL	---

2.2.3 Statistical Methods

Statistical analyses were conducted using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± SD. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov-Smirnov test. Differences of continuous variables were evaluated by the Student's t or Mann-Whitney U test, depending on the shape of the distribution curves. Values of paraoxonase activity, PON1 status, triglycerides and total cholesterol were log transformed before applying t-test, due to skewness. Categorical variables were expressed as proportions and compared using χ^2 test. Allele frequencies were determined by the gene counting method and departure from the Hardy-Weinberg equilibrium was evaluated by the χ^2 test. Comparisons of genotype distribution and allele frequencies were assessed by χ^2 statistics with 2 and 1 df, respectively. Correlation of PON1 genotypes and PON1 activities were determined by Pearson coefficient of correlation. The relations between PON1 haplotypes and PON1 activities were evaluated by analysis of variance (ANOVA) test.

Logistic regression analysis with backward selection method was used to asses the effects of vascular risk factors, PON1 activities and genotypes in the prediction of ischemic stroke cases (coded as 1) versus controls (coded as 0). Age and sex were

also included in the model as covariates. For each odds ratio, we estimated 2-tailed probability values with 95% confidence intervals and calibration was assessed using the Hosmer-Lemeshow goodness-of-fit test. A *P* value of less than 0.05 was evaluated as statistically significant.

CHAPTER III

RESULTS

3.1 Study Participants

Study population was comprised of 172 ischemic stroke patients and 105 controls. Demographic features, prevalence of vascular risk factors and serum lipids in the acute ischemic stroke and control populations are given in Table 3.1. Raw data including these parameters in addition to PON1 activities and PON1 genotypes of study participants are listed in Appendix D.

The study population was fairly old; age varied between 20 to 89 years in stroke patients and 36 to 88 years in controls. There was no statistically significant difference between the mean ages of the ischemic stroke patients (66.6 ± 14.8 years) and the control group (64.5 ± 12.8 years; P=0.093). Among stroke patients, 97 were male and 75 were female. Patient group contained slightly more males (56.4 %) than did controls (49.5%; P=0.266). The prevalences of conventional vascular risk factors, hypertension, diabetes and smoking were found to be higher in the patient group as compared to control subjects. Hypertension was observed in 61% of stroke patients, while 42.9% of control subjects were hypertensive (P=0.003). Out of 150 hypertensives, 105 (70 %) were stroke patients. Prevalence of diabetes mellitus was also significantly higher in the stroke patient group (29.1 %) when compared to controls (17.1 %; P=0.025). Among 68 diabetic subjects, 50 (73.5 %) had ischemic stroke. Among stroke patients, 22.7 % were smokers; while 13.3 % of controls declined to be smokers (P=0.055). Out of 53 smokers 39 (73.6%) were stroke patients.

Table 3.1 Clinical characteristics and laboratory data of ischemic stroke patients and controls.

Parameter	Patients (n=172)	Controls (n=105)	*P*
Age (years)[a]	66.6 ± 14.8	64.5 ± 12.8	0.093
Male, n (%)[b]	97 (56.4)	52 (49.5)	0.266
Hypertension, n (%)[b]	105 (61.0)	45 (42.9)	0.003
Diabetes mellitus, n (%)[b]	50 (29.1)	18 (17.1)	0.025
Smokers, n (%)[b]	39 (22.7)	14 (13.3)	0.055
Obesity, n (%)[b]	13 (7.6)	8 (7.6)	0.985
Stenosis, %[a]	38 ± 0.4	16 ± 0.2	0.000
Statin, n (%)	14 (8.1%)	4 (3.8%)	0.156
Total cholesterol (mmol/L)[c]	4.8 ± 1.3	4.9 ± 1.2	0.449
Triglycerides (mmol/L)[c]	1.5 ± 0.7	1.4 ± 0.7	0.376
HDL-cholesterol (mmol/L)[c]	1.1 ± 0.3	1.2 ± 0.3	0.007
LDL-cholesterol (mmol/L)[c]	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
[c] Independent Samples T-test is applied

The frequency of obese individuals among stroke patients was exactly the same as in controls (7.6%). Stroke patients had an average of 38 % stenosis of their carotid artery, while controls had 16 % blockage (*P*=0.000). The prevalence of statin drug users came out to be 8.1% in stroke patients and 3.8 % in controls (*P*=0.156).

Risk estimates for some of the common vascular risk factors are given in Table 3.2. Being hypertensive and diabetic both exhibited around 2-fold relative risk for ischemic stroke. Gender, smoking and obesity did not have significant effects on ischemic stroke risk. The risks associated with these situations, however, were not free from the effects of other risk factors, as regression analysis was not performed at this step.

Table 3.2 Risk of ischemic stroke associated with conventional vascular risk factors.

Parameter	**OR (95% CI)**	***P***
Gender (male)	1.318 (0.810-2.145)	0.266
Hypertension	2.090 (1.276-3.422)	0.003
Diabetes mellitus	1.981 (1.082-3.627)	0.025
Smoking	1.906 (0.979-3.711)	0.055
Obesity	0.991 (0.397-2.478)	0.985

As given in Table 3.1, the level of HDL-cholesterol was significantly (*P*=0.007) lower in patient group, while LDL-cholesterol and triglycerides were found to be slightly higher. Total cholesterol levels were almost the same in patients and controls. As stated above, slightly more of the stroke patients were statin users compared to controls. To see the effects of statins on lipids, we stratified the

population in terms of statin use. As given in Table 3.3, total cholesterol levels of statin users (4.27 ± 1.02 mmol/L) were slightly lower than those of individuals who did not use stain type of drugs (4.85 ± 1.31 mmol/L; *P*=0.068). LDL-cholesterol levels were found to be significantly lower in statin users (2.22 ± 0.69 mmol/L) when compared to those of non-statin users (2.74 ± 1.08 mmol/L; *P*=0.047).

Table 3.3 Effect of statin use on the levels of total cholesterol and LDL-cholesterol (LDL-C) levels in the combined population.

	Statin + (n=18)	Statin – (n=259)	*P*
Total cholesterol (mmol/L)	4.27 ± 1.02	4.85 ± 1.31	0.068
LDL-C (mmol/L)	2.22 ± 0.69	2.74 ± 1.08	0.047

3.2 PON1 Enzyme Activities

PON1 enzyme activities of stroke patients and controls were determined towards three substrates in the present study: paraoxon (paraoxonase activity; PON), phenyl acetate (arylesterase activity; ARE), and diazoxon (diazoxonase activity; DIA). Raw data including PON1 activities of patients and controls is given in Appendix D.

3.2.1 Range of PON1 Enzyme Activities

Serum PON1 activities towards all three substrates used in the present study exhibited interindividual variation (Table 3.4). The greatest variation was observed in PON1 activities towards paraoxon (paraoxonase activity; PON) in both stroke patient and control groups (Table 3.4). In patients group, maximum and minimum paraoxonase activities were 632.3 U/L and 33.9 U/L, respectively; thus there was an

18.6-fold variation. The variation in paraoxonase activities among controls was also 18.6-fold, with 568.8 U/L and 30.5 U/L being the highest and lowest values.

PON1 activities towards diazoxon (diazoxonase activity; DIA) also exhibited a wide variation (16.8-fold) in patients (Table 3.4), whereas a 10.5-fold variation was observed among controls (range: 3630.4-38231 U/L).

Table 3.4 Range of PON1 activities towards paraoxon (paraoxonase activity; PON), phenylacetate (arylesterase activity; ARE) and diazoxon (diazoxonase activity; DIA).

PON1 activity	Patients (n=172)			Controls (n=105)		
	Lowest	Highest	Variation fold	Lowest	Highest	Variation fold
PON (U/L)	33.9	632.3	18.6	30.5	568.8	18.6
ARE (U/mL)	49	200.0	4	43.0	218.0	5
DIA (U/L)	3102.3	52145	16.8	3630.4	38231.0	10.5

The variation in PON1 activities towards phenylacetate (arylesterase activity; ARE), which reflects enzyme level, was not as wide as the variation in paraoxonase and diazoxonase activities (Table 3.4). Arylesterase activities showed a 4x (range: 200-49 U/mL) and 5x (range: 218-43 U/mL) variation among patients and controls, respectively.

3.2.2 PON1 Enzyme Activities in Stroke Patients and Controls

As shown in Table 3.5, paraoxonase, arylesterase and diazoxonase activities of the patients were slightly lower when compared to those of control group, although none of the differences reached statistical significance. Mean paraoxonase activity of

172 stroke patients was 217.2 ± 136.9 U/L, while that of 105 controls was 230.3 ± 137.3 U/L (*P*=0.442). Arylesterase activity was found to be 109.1 ± 32.5 U/mL in patients and 113.5 ± 33.1 U/mL in controls. Mean diazoxonase activity of stroke patients (13543.0 ± 6221.6 U/L) was also slightly lower than that of controls (14329 ± 6057 U/L; *P*=0.305). PON1 activity ratio 1 (PON/ARE) did not differ much between the two groups. PON1 activity ratio 2 (PON/DIA), on the other hand, was slightly higher in patients than in controls (Table 3.5).

Table 3.5 Paraoxonase (PON), arylesterase (ARE) and diazoxonase (DIA) activities and PON1 activity ratios of stroke patients and controls.

PON1 Activity	**Patients (n=172)**	**Controls (n=105)**	***P***
PON (U/L)	217.2 ± 136.9	230.3 ± 137.3	0.442
ARE (U/mL)	109.1 ± 32.5	113.5 ± 33.1	0.284
DIA (U/L)	13543.0 ± 6221.6	14329 ± 6057	0.305
PON1 activity ratio 1 (PON/ARE)	1.974 ± 1.070	1.972 ± 0.980	0.705
PON1 activity ratio 2 (PON/DIA)	0.019 ± 0.014	0.017 ± 0.011	0.372

Data are mean ± SD. Independent Samples T-test is applied for calculation of *P* values, except for PON1 activity ratio 1 (PON/ARE), for which Mann-Whitney test was applied.

3.2.3 PON1 Enzyme Activities in Different Subgroups of Stroke Patients and Controls

Effects of vascular risk factors; age, gender, hypertension, diabetes and smoking and statin drug use on PON1 enzyme activities were analyzed in the combined (stroke patient + control) population and in patients and controls separately.

3.2.3.1 Effect of Age

We stratified the study population by age, so that any possible effect of age on enzyme activities could be minimized, and difference in PON1 enzyme activities between patients and controls could be better determined. As can be seen in Table 3.6, paraoxonase, arylesterase and diazoxonase activities of older subjects (>59 years) were lower than those of the younger group (<60 years). Arylesterase activity of the elderly group was 108.3 U/mL, which was significantly lower than arylesterase activity of the younger group (117.6 U/mL; *P*=0.035). The difference in diazoxonase activities of elderly and younger subjects was also statistically significant (*P*=0.001; Table 3.6).

Table 3.6 PON1 activities in elderly and younger subjects.

PON1 Activity	Elderly group (>59) (n=203)	Younger group (<60) (n=74)	P
PON (U/L)	216.9 ± 136.6	236.6 ± 137.6	0.289
ARE (U/mL)	108.3 ± 33.9	117.6 ± 28.4	0.035
DIA (U/L)	13123.6 ± 5638.7	15828.5 ± 7078.7	0.001

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

Since we observed that PON1 enzyme activities are affected by age, comparison of PON1 enzyme activities of patients and controls was also conducted separately in the elderly and younger age groups (Table 3.7). In both age groups, paraoxonase and arylesterase activities were lower in the patients than the respective control group. Although the difference in paraoxonase activities between patients and controls was almost lost in the elderly group (216.1 ± 141.4 U/L vs. 218.4 ± 128.8 U/L, P=0.908), this difference was more pronounced in the younger group (220.7 ± 123.7 U/L vs. 258.7 ± 154.2 U/L), although still not significant (P=0.261). A similar trend was also observed in arylesterase activities (Table 3.7).

Mean diazoxonase activity of the elderly patients (12549.1 U/L) was lower than that of the elderly controls (14109.3 U/L), while in the younger group this observation was reversed; i.e. diazoxonase activity of the younger patients was higher than that of younger controls (Table 3.7). The difference in diazoxonase activities of elderly patients (12549.1 ± 5016.3 U/L) and younger patients (16548.3 ± 8304.8 U/L) was significant (P=0.005).

PON1 activities (paraoxonase, arylesterase and diazoxonase) of the older (>59) group (patients or controls) were reduced with respect to those of the respective younger (<60) group (Table 3.7). Paraoxonase and arylesterase activities decreased in the order of young control>young patient>old control>old patient. There were no statistically significant differences between PON1 activity ratios of patients and controls, in neither the elderly group, nor the younger group.

Table 3.7 PON1 activities and activity ratios in stroke patients and controls stratified by age.

PON1 Activity	Elderly group (>59)			Younger group (<60)		
	Stroke patient (n=129)	Control (n=74)	*P*	Stroke patient (n=43)	Control (n=31)	*P*
PON (U/L)	216.1 ± 141.4	218.4 ± 128.8	0.908	220.7 ± 123.7	258.7 ± 154.2	0.261
ARE (U/mL)	107.3 ± 33.2	110.0 ± 35.1	0.579	114.7 ± 29.8	121.7 ± 26.3	0.296
DIA (U/L)	12549.1 ± 5016.3*	14109.3 ± 6489.8	0.058	16548.3 ± 8304.8	14853.3 ± 4928	0.315
PON1 activity ratio 1 (PON/ARE)	1.974 ± 1.070	1.946 ± 0.965	0.810	1.973 ± 1.083	2.034 ± 1.032	0.809
PON1 activity ratio 2 (PON/DIA)	0.020 ± 0.014	0.017 ± 0.011	0.174	0.016 ± 0.013	0.018 ± 0.011	0.491

Data are mean ± SD Independent Samples T-test is applied for calculation of *P* values, except for PON1 activity ratio 1 of elderly group, for which Mann-Whitney test was applied. * Significantly different to diazoxonase activity of younger patients (*P*=0.005).

3.2.3.2 Effect of Gender

Paraoxonase, arylesterase and diazoxonase activities of male subjects (stroke patients and controls combined) were lower than those of the female subjects (Table 3.8). The difference in arylesterase activities of males (106.6 ± 29.2 U/mL) and females (115.6 ± 35.9) was statistically significant (*P*=0.026).

Table 3.8 PON1 activities in male and female subjects.

PON1 Activity	Male (n=149)	Female (n=128)	*P*
PON (U/L)	216.4 ± 137.7	228.8 ± 136.3	0.453
ARE (U/mL)	106.6 ± 29.2	115.6 ± 35.9	0.026
DIA (U/L)	13559.4 ± 6146.8	14183.7 ± 6182.8	0.404

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

Thus, PON1 enzyme activity comparisons between stroke patients and controls were handled in males and females separately. As shown in Table 3.9, in females group, paraoxonase, arylesterase and diazoxonase activities of stroke patients were lower than those of controls. In males group however, patients had almost the same paraoxonase, arylesterase and diazoxonase activities as controls. While PON1 activity ratio 1 was slightly lower in both male and female patients compared to the respective controls, PON1 activity ratio 2 was slightly higher in patients than controls. None of the differences were, however, statistically significant.

Table 3.9 PON1 activities in stroke patients and controls stratified by gender.

PON1 Activity	Male (n=149)			Female (n=128)		
	Stroke patients (n=97)	Controls (n=52)	*P*	Stroke patients (n=75)	Controls (n=53)	*P*
PON (U/L)	217.6 ± 133.9	214.3 ± 145.8	0.892	216.8 ± 141.5	245.9 ± 127.9	0.234
ARE (U/mL)	107.1 ± 29.5	105.8 ± 28.9*	0.808	111.8 ± 36.1	120.9 ± 35.4	0.155
DIA (U/L)	13658.5 ± 6646	13374.5 ± 5142.8	0.789	13387.5 ± 5641.5	15265.5 ± 6754	0.093
PON1 activity ratio 1 (PON/ARE)	1.914 ± 1.037	2.014 ± 1.097	0.714	1.921 ± 1.040	2.029 ± 0.928	0.546
PON1 activity ratio 2 (PON/DIA)	0.019 ± 0.014	0.017 ± 0.012	0.464	0.019 ± 0.014	0.018 ± 0.010	0.585

Data are mean ± SD, comparisons are by T-test
* significantly different from female controls (*P*=0.019)

This table can also be used for the comparison of PON1 enzyme activities of males and females separately in stroke patients and controls. No significant differences were found between the PON1 enzyme activities of male patients and female patients. In controls group, however, males had significantly lower arylesterase activities (105.8 ± 28.9 U/mL) when compared to female controls (120.9 ± 35.4; *P*=0.019). Paraoxonase activities of the male controls (214.3 ± 145.8 U/L) were also lower than those of the female controls (245.9 ± 127.9 U/L), although the difference did not reach statistical significance (*P*=0.240; Table 3.9).

3.2.3.3 Effect of Hypertension

PON1 activities of hypertensive and normotensive subjects (stroke patients and controls combined) are compared in Table 3.10. Diazoxonase activities of hypertensives (13147.7 ± 5187.8 U/L) were significantly lower than those of the normotensive subjects (14686.8 ± 7092.6 U/L). Although hypertensives had higher

paraoxonase activities than normotensives, the difference was not significant (Table 3.10). Arylesterase activity was almost the same in both groups (110 ± 32 U/mL versus 111.6 ± 33.6 U/mL).

Table 3.10 PON1 activities in hypertensive and normotensive subjects.

PON1 Activity	Hypertensive (n=150)	Normotensive (n=127)	*P*
PON (U/L)	235.2 ± 140.3	206.8 ± 131.7	0.086
ARE (U/mL)	110 ± 32	111.6 ± 33.6	0.694
DIA (U/L)	13147.7 ± 5187.8	14686.8 ± 7092.6	0.039

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

PON1 activity comparisons between stroke patients and controls were also carried out in hypertensives and normotensives separately. As given in Table 3.11, PON1 enzyme activities of stroke patients and controls having hypertension were very close. PON1 activity ratio 2 was significantly higher in hypertensive stroke patients (0.021 ± 0.014) than hypertensive controls (0.017 ± 0.009; P=0.048). In normotensives, patients had slightly lower paraoxonase and arylesterase activities than controls. Diazoxonase activities were, however, almost the same in both patient and control groups in normotensives.

In addition, comparison of PON1 enzyme activities between hypertensives and normotensives separately in patients or controls yielded significant results for paraoxonase activity. Hypertensive stroke patients had significantly higher paraoxonase activities (237.2 ± 142.7 U/L) than normotensive stroke patients (185.9 ± 121.6 U/L, P=0.013).

Table 3.11 PON1 activities in stroke patients and controls stratified by being hypertensive or normotensive.

PON1 Activity	Hypertensive (n=150)			Normotensive (n=127)		
	Stroke patients (n=105)	Controls (n=45)	*P*	Stroke patients (n=67)	Controls (n=60)	*P*
PON (U/L)	237.2 ± 142.7*	230.4 ± 135.8	0.787	185.9 ± 121.6	230.2 ± 139.5	0.058
ARE (U/mL)	110.3 ± 32.7	109.5 ± 30.8	0.881	107.2 ± 32.4	116.5 ± 34.6	0.123
DIA (U/L)	12892.4 ± 5198.6	13743.5 ± 5171.3	0.359	14610.6 ± 7533.0	14768.1 ± 6653.4	0.902
PON1 activity ratio 1 (PON/ARE)	2.134 ± 1.089	2.030 ± 0.966	0.582	1.723 ± 0.996	1.929 ± 0.998	0.137
PON1 activity ratio 2 (PON/DIA)	0.021 ± 0.014	0.017 ± 0.009	0.048	0.015 ± 0.014	0.018 ± 0.012	0.187

Data are mean ± SD, comparisons are by T-test except for PON1 activity ratio 1 and 2 of normotensives.
* significantly different from normotensive patients (*P*=0.013).

3.2.3.4 Effect of Diabetes

Diabetic and non-diabetic subjects' PON1 activities were compared in Table 3.12. PON1 activities of diabetic subjects (stroke patients and controls combined) were lower than those of the non-diabetic subjects, although the differences were not significant.

Table 3.12 PON1 activities in diabetic and non-diabetic subjects.

PON1 Activity	Diabetic (n=68)	Non-diabetic (n=209)	*P*
PON (U/L)	215 ± 124. 2	224.5 ± 141.0	0.618
ARE (U/mL)	107.5 ± 35.8	111.8 ± 31.7	0.343
DIA (U/L)	13752.6 ± 6473.0	13874.5 ± 6068.9	0.888

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

PON1 activities of stroke patients and controls were thus compared in subgroups defined by being diabetic or non-diabetic (Table 3.13). As given in Table 3.13, in diabetics, paraoxonase activity was insignificantly higher in patients (216.6 ± 127.3 U/L) than controls (210.5 ± 118 U/L; *P*=0.860). In non-diabetics, however, paraoxonase activity of stroke patients (217.5 ± 141.1 U/L) was slightly lower than that of controls (234.4 ± 141.1 U/L; *P*=0.394). Arylesterase and diazoxonase activities followed similar patterns in diabetics and non-diabetics. Stroke patients had lower arylesterase and diazoxonase activities than controls, both in diabetics and non-diabetics (Table 3.13).

In addition, PON1 enzyme activity comparisons between diabetics and non-diabetics can be performed in stroke patients and controls separately (Table 3.13). Diabetic stroke patients had almost the same paraoxonase activities (216.6 ± 127.3 U/L) as the non-diabetic stroke patients (217.5 ± 141.1 U/L). Arylesterase (105.2 ± 33.4 U/mL) and diazoxonase (13221.0 ± 5286.7 U/L) activities of diabetic stroke patients were slightly lower than the arylesterase (110.7 ± 32.1 U/mL) and diazoxonase activities (13678.3 ± 6591.1 U/L) of non-diabetic stroke patients. In addition, paraoxonase activities of diabetic controls (210.5 ± 118 U/L) were lower than those of non-diabetic controls (234.4 ± 141.1 U/L), although the differences were not significant.

Table 3.13 PON1 activities in stroke patients and controls stratified by being diabetic or nondiabetic.

	Diabetic (n=68)			Non-diabetic (n=209)		
PON1 Activity	Stroke patients (n=50)	Controls (n=18)	*P*	Stroke patients (n=122)	Controls (n=87)	*P*
PON (U/L)	216.6 ± 127.3	210.5 ± 118	0.860	217.5 ± 141.1	234.4 ± 141.1	0.394
ARE (U/mL)	105.2 ± 33.4	114 ± 41.9	0.374	110.7 ± 32.1	113.4 ± 31.2	0.556
DIA (U/L)	13221.0 ± 5286.7	15229.0 ± 9024.3	0.262	13678.3 ± 6591.1	14142.8 ± 5297.5	0.589
PON1 activity ratio 1 (PON/ARE)	2.016 ± 0.999	1.881 ± 0.917	0.617	1.957 ± 1.102	1.991 ± 0.997	0.606
PON1 activity ratio 2 (PON/DIA)	0.018 ± 0.012	0.016 ± 0.008	0.475	0.019 ± 0.015	0.018 ± 0.011	0.457

Data are mean ± SD, comparisons are by Independent Samples T-test, except for PON1 activity ratio 1 of non-diabetics.

3.2.3.5 Effect of Smoking

As given in Table 3.14, there was no statistically significant difference between the smoker and nonsmoker subjects (stroke patients and controls combined) in terms of PON1 activities. Paraoxonase activity was almost the same, while arylesterase and diazoxonase activities were lower in smokers than in nonsmokers.

Nevertheless, PON1 activities of stroke patients and controls were compared separately in smoker and non-smoker subgroups. As given in Table 3.15, smoker stroke patients had lower paraoxonase, arylesterase and diazoxonase activities than smoker controls. Nonsmoker stroke patients also had lower PON1 activities than nonsmoker controls. PON1 activity ratios did not differ much between patients and controls in either smoker or nonsmoker groups.

Table 3.14 PON1 activities in smokers and nonsmokers.

PON1 Activities	Smoker (n=53)	Non-smoker (n=224)	*P*
PON (U/L)	226.1 ± 134.3	221.2 ± 137.8	0.816
ARE (U/mL)	105.2 ± 27.5	112.1 ± 33.8	0.123
DIA (U/L)	12491.1 ± 5027.1	14153.7 ± 6360.1	0.082

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

Table 3.15 PON1 activities in stroke patients and controls stratified by being smoker or nonsmoker.

	Smoker (n=53)			Non-smoker (n=224)		
PON1 Activity	Stroke patients (n=39)	Controls (n=14)	*P*	Stroke patients (n=133)	Controls (n=91)	*P*
PON (U/L)	213.7 ± 130.7	260.6 ± 142.9	0.266	218.2 ± 139.1	225.6 ± 136.6	0.695
ARE (U/mL)	101.7 ± 27.7	115.1 ± 25.1	0.118	111.3 ± 33.6	113.2 ± 34.2	0.676
DIA (U/L)	12056.0 ± 5099	13641 ± 4820	0.320	13959.9 ± 6457.3	14434.8 ± 6241.2	0.585
PON1 activity ratio 1 (PON/ARE)	2.085 ± 1.162	2.176 ± 1.084	0.799	1.941 ± 1.044	1.941 ± 0.967	0.998
PON1 activity ratio 2 (PON/DIA)	0.020 ± 0.012	0.020 ± 0.011	0.897	0.018 ± 0.014	0.017 ± 0.011	0.369

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

Comparison of PON1 enzyme activities of smoker patients to nonsmoker patients revealed that smoker patients have got lower enzyme activities (Table 3.15). This trend was not observed for controls; paraoxonase and arylesterase activities of smoker controls were insignificantly higher than those of nonsmoker controls. The difference in PON1 activity ratios of stroke patients who smoke and who do not smoke; and smoker and non-smoker controls were not statistically significant.

3.2.3.6 Effect of Statin Drug Use

PON1 activities were lower in statin drug users than in subjects who did not use statins (Table 3.16). While the differences in paraoxonase and diazoxonase activities between statin users and statin non-users were insignificant, arylesterase activity of statin users (91.6 ± 19.9 U/mL) was significantly lower than that of statin non-users (112.2 ± 33.0 U/mL; *P*=0.000).

Table 3.16 PON1 activities in subjects who used statin (statin +) and who did not use statin (statin-).

PON1 Activity	Statin + (n=14)	Statin − (n=259)	*P*
PON (U/L)	198.7 ± 118.9	223.9 ± 138.4	0.452
ARE (U/mL)	91.6 ± 19.9	112.2 ± 33.0	0.000
DIA (U/L)	11797.5 ± 4356.0	13999 ± 6258.1	0.144

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

PON1 activities of stroke patients were lower when compared to those of controls, both in statin users and non-users (Table 3.17). In addition, stroke patients who used statins had lower PON1 activities and PON1 activity ratio 2 than those of

stroke patients who did not use statins. The difference between arylesterase activities of stroke patients who used (89.4 ± 18.2 U/mL) and didn't use statins (110.9 ± 32.9 U/mL) was significant (*P*=0.001). Within controls, while arylesterase and diazoxonase activities were lower in statin users, paraoxonase activity and PON1 activity ratios were higher when compared to statin non-users.

Table 3.17 PON1 activities in stroke patients and controls stratified by being statin user or not.

	Statin + (n=18)			Statin − (n=259)		
PON1 Activity	Stroke patients (n=14)	Controls (n=4)	*P*	Stroke patients (n=158)	Controls (n=101)	*P*
PON (U/L)	177.1 ± 96.6	274.1 ± 172.9	0.156	220.8 ± 139.5	228.8 ± 137.1	0.651
ARE (U/mL)	89.4 ± 18.2*	99.0 ± 26.8	0.414	110.9 ± 32.9	114.4 ± 33.2	0.401
DIA (U/L)	11768.9 ± 4794.6	11897.7 ± 2808.9	0.960	13703.3 ± 6322.6	14457.3 ± 6160.2	0.349
PON1 activity ratio 1 (PON/ARE)	2.003 ± 1.015	2.682 ± 1.275	0.279	1.971 ± 1.078	1.937 ± 0.967	0.796
PON1 activity ratio 2 (PON/DIA)	0.017 ± 0.010	0.023 ± 0.011	0.397	0.019 ± 0.014	0.017 ± 0.011	0.265

Data are mean ± SD, comparisons are by Independent Samples T-test.
* *P*=0.001 when compared to stroke patients who do not use statins.

3.3 PON1 Genotypes and Allele Frequencies in Stroke Patients and Controls

Before going into genotype information in stroke patients and controls, representative agarose gel photographs showing isolated genomic DNA, PCR and digestion products for the three genetic polymorphisms (two coding regions and one promoter region) of PON1 gene are given.

3.3.1 Isolation of Genomic DNA from Whole Blood Samples

Genomic DNA isolated from whole blood was tested for its quality by agarose gel electrophoresis. Figure 3.1 shows a representative agarose gel photograph. Quality of DNA was also tested spectrophotometrically. DNA preparations which gave A_{260}/A_{280} below 1.6 or above 2.0 were discarded and DNA isolation was repeated for these samples. Quantity of DNA was determined by spectrophotometry and this information was used for the calculation of amount of DNA preparation to be added to PCR mixture.

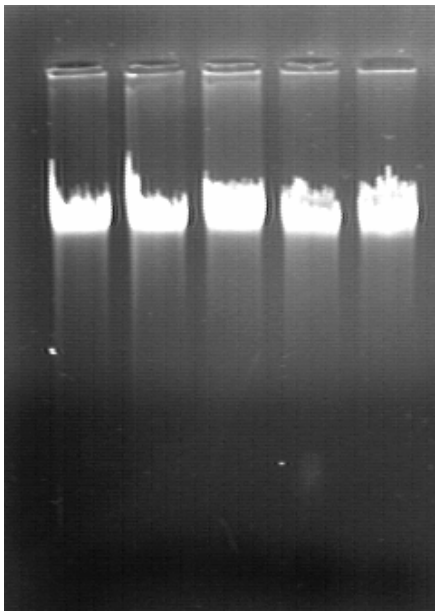


Figure 3.1 Determination of intactness of isolated genomic DNA on 0.7 % agarose gel electrophoresis. The gel was run for 1 hr at 100 V.

3.3.2 Determination of PON1 192Q/R, 55L/M and −107T/C Genotypes by Polymerase Chain Reaction Followed by Restriction Fragment Digestion

3.3.2.1 PON1 192Q/R SNP

3.3.2.1.1 PCR Result for the PON1 192Q/R SNP

Amplification of the region in PON1 gene including the 192Q/R SNP was carried out as described in section 2.2.2.2.1.1. PCR products were analyzed on 2.0 % agarose gel which was prepared as described in section 2.2.2.1.4. Eight µL of PCR product was mixed with 1 µL of gel loading buffer and applied to the wells of gel. Six µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V. Figure 3.2 shows a representative agarose gel electrophoresis pattern for the 192Q/R polymorphism of PON1 gene.

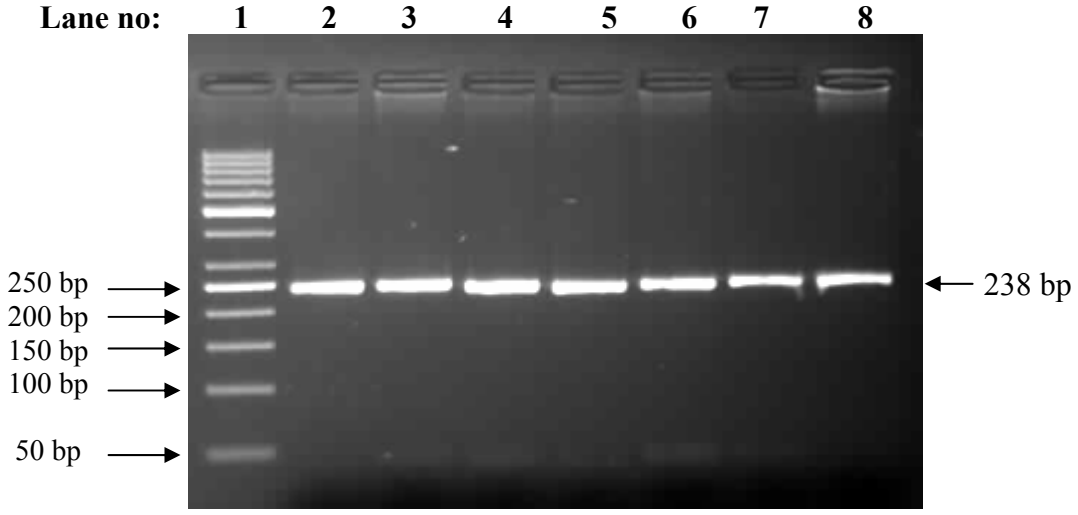


Figure 3.2 2.0 % Agarose gel electrophoresis of PCR products for the 192Q/R SNP region of PON1. Gel was run at 100V for 1 hour. Expected size of PCR product is 238 bp. Lane 1 contained DNA ladder (1031-50bp), and lanes 2-8 contained PCR products of subjects 156, 163, 174, 179, 180, 181, and 182, respectively.

3.3.2.1.2 Restriction Endonuclease Digestion Result for the PON1 192Q/R SNP

PCR products belonging to the 192Q/R SNP region of PON1 were subjected to digestion with *AlwI* (*BspPI*) restriction endonuclease as described in section 2.2.2.2.1.2. Digestion products were analyzed on 2.5% agarose gel. Figure 3.3 shows a representative agarose gel photograph. 238-bp PCR products resulted in 66- and 172-bp fragments for the 192*R* allele and a non-digested 238-bp fragment for the 192*Q* allele.

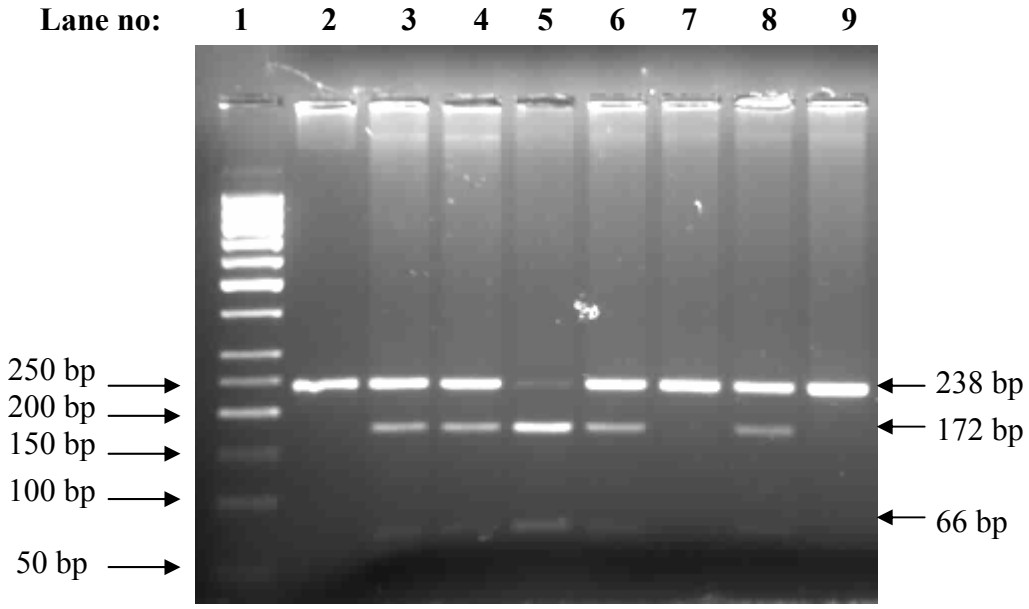


Figure 3.3 2.5 % Agarose gel electrophoresis of restriction endonuclease (*BspPI*) digestion products for the 192Q/R SNP of PON1. Gel was run at 100V for 1.5 hours. Lane 1 contained DNA ladder (1031-50 bp), lane 2 contained PCR product which was not subjected to digestion, and lanes 3-9 contained digestion products of subjects 156, 163, 174, 179, 180, 181, and 182, respectively.

Thus, the genotypes were decided to be;

Subject 156 (lane 3):	*QR*	Subject 180 (lane 7):	*QQ*
Subject 163 (lane 4):	*QR*	Subject 181 (lane 8):	*QR*
Subject 174 (lane 5):	*RR*	Subject 182 (lane 9):	*QQ*
Subject 179 (lane 6):	*QR*		

3.3.2.2 PON1 55L/M SNP

3.3.2.2.1 PCR Result for the PON1 55L/M SNP

Amplification of the region in PON1 gene including the 55L/M SNP was carried out as described in section 2.2.2.2.2.1 PCR products were analyzed on 2.0 % agarose gel, which was prepared as described in section 2.2.2.1.4. Eight µL of PCR product was mixed with 1 µL of gel loading buffer and applied to the wells of gel. Six µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V. Figure 3.4 shows a representative agarose gel electrophoresis pattern for the 55L/M polymorphism of PON1 gene.

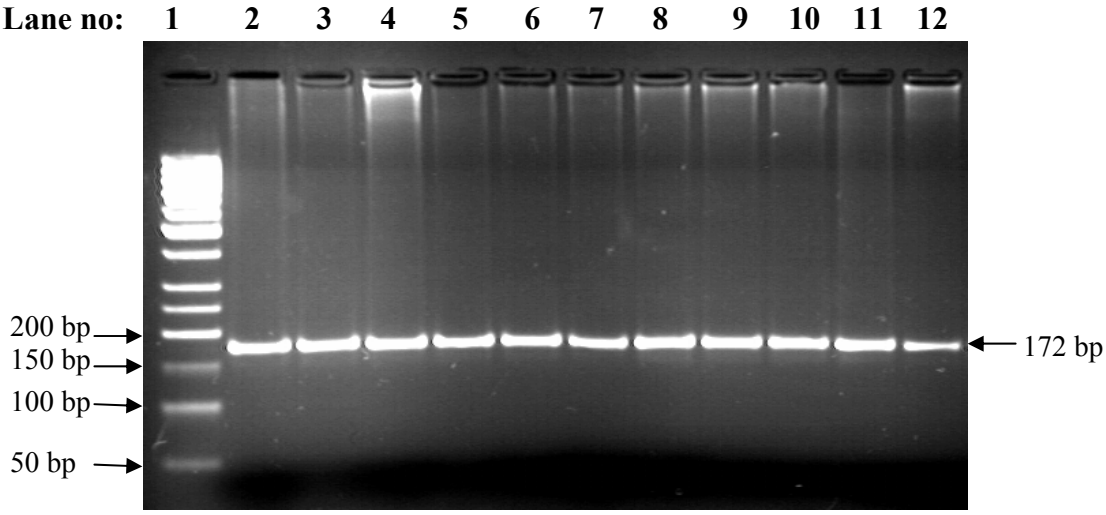


Figure 3.4 2.0 % Agarose gel electrophoresis of PCR products for the 55L/M SNP region of PON1. Gel was run at 100V for 1 hour. Expected size of PCR product is 172 bp. Lane 1 contained DNA ladder (1031-50bp) and lanes 2-12 contained PCR products of subjects 141, 142, 143, 144, 145 147, 148, 149, 150, 151, and 152, respectively.

3.3.2.2.2 Restriction Endonuclease Digestion Result for the PON1 55L/M SNP

PCR products belonging to the 55L/M SNP region of PON1 were subjected to digestion with *NlaIII* (*Hin1II*) restriction endonuclease as described in section 2.2.2.2.2.2. Digestion products were analyzed on 2.5 % agarose gel electrophoresis. Figure 3.5 shows a representative agarose gel photo. The digestion resulted in 66-and 106-bp fragments for the 55*M* allele and in a non-digested 172-bp fragment for the 55*L* allele.

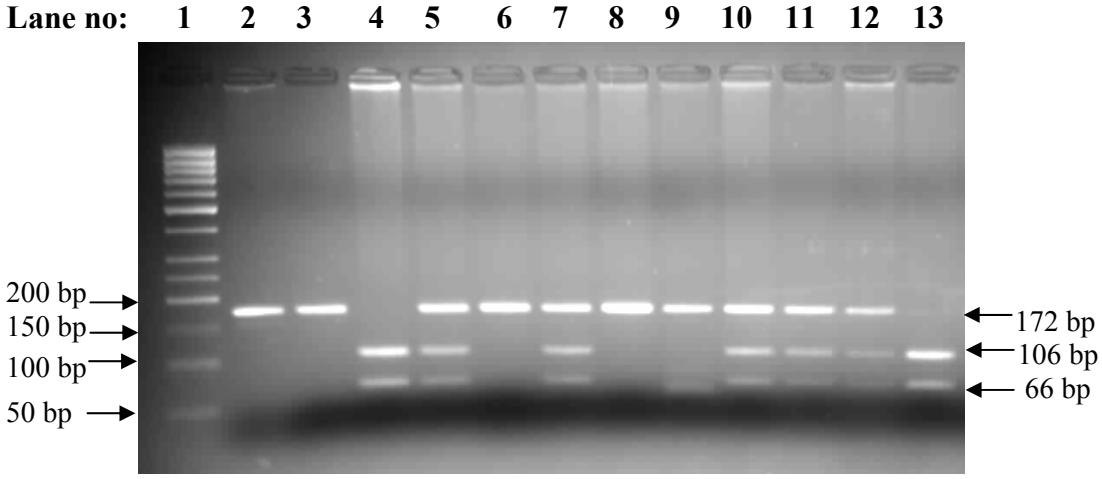


Figure 3.5 2.5 % Agarose gel electrophoresis of restriction endonuclease (*Hin1II*) digestion products for the 55L/M SNP of PON1. Gel was run at 100V for 1.5 hours. Lane 1 contained DNA ladder (1031-50bp), lane 2 contained PCR product which was not subjected to digestion, lanes 3-13 contained digestion products of subjects 82, 85, 96, 97, 98, 99, 104, 108, 110, 113, 114, respectively.

Thus, genotypes were decided as:

Subject 82 (lane 3): *LL*
Subject 85 (lane 4): *MM*
Subject 96 (lane 5): *LM*
Subject 97 (lane 6): *LL*
Subject 98 (lane 7): *LM*
Subject 99 (lane 8): *LL*
Subject 104 (lane 9): *LL*
Subject 108 (lane 10): *LM*
Subject 110 (lane 11): *LM*
Subject 113 (lane 12): *LM*
Subject 114 (lane 13): *MM*

3.3.2.3 PON1 −107T/C SNP

3.3.2.3.1 PCR Result for the PON1 −107T/C SNP

Amplification of the region in PON1 gene including the −107T/C SNP was carried out as described in section 2.2.2.2.3.1. PCR products were analyzed on 2.0 % agarose gel, which was prepared as described in section 2.2.2.1.4. Eight µL of PCR product was mixed with 1 µL of gel loading buffer and applied to the wells of gel. Six µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V. Figure 3.6 shows a representative agarose gel photograph displaying PCR products belonging to the −107T/C SNP region of PON1 gene.

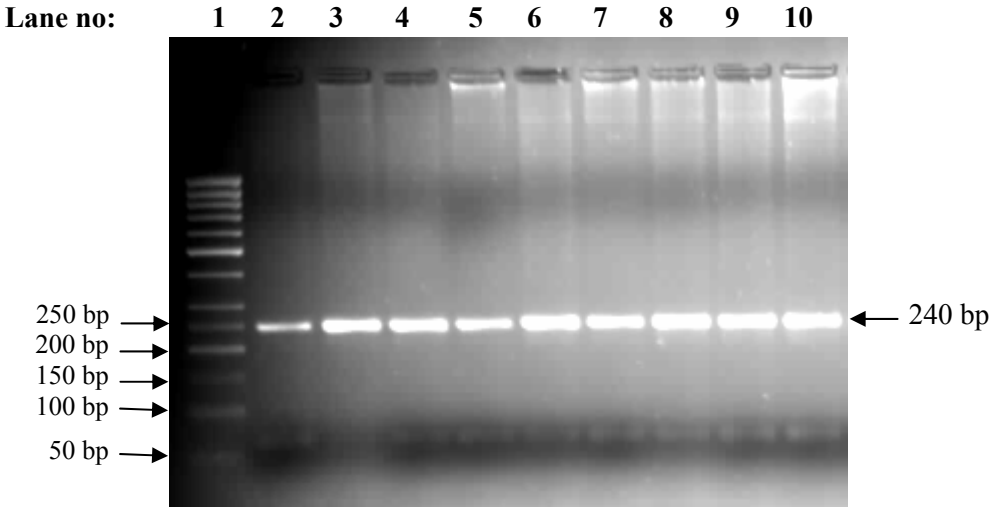


Figure 3.6 2.0 % Agarose gel electrophoresis of PCR products for the −107T/C SNP region of PON1. Gel was run at 100V for 1 hour. Expected size of PCR product is 240 bp. Lane 1 contained DNA ladder (1031-50bp), and lanes 2-10 contained PCR products of subjects 137, 138, 139, 140, 141 142, 143, 144, and 145, respectively.

3.3.2.3.2 Restriction Endonuclease Digestion Result for the PON1 −107T/C SNP

PCR products belonging to the −107T/C SNP region of PON1 were subjected to digestion with *BsrBI* (*MbiI*) restriction endonuclease as described in section 2.2.2.2.3.2. Digestion products were analyzed on 2.5 % agarose gel electrophoresis. Figure 3.7 shows a representative agarose gel photo. The digestion resulted in 212- and 28-bp fragments for the −107*C* allele and in a non-digested 240-bp fragment for the −107*T* allele.

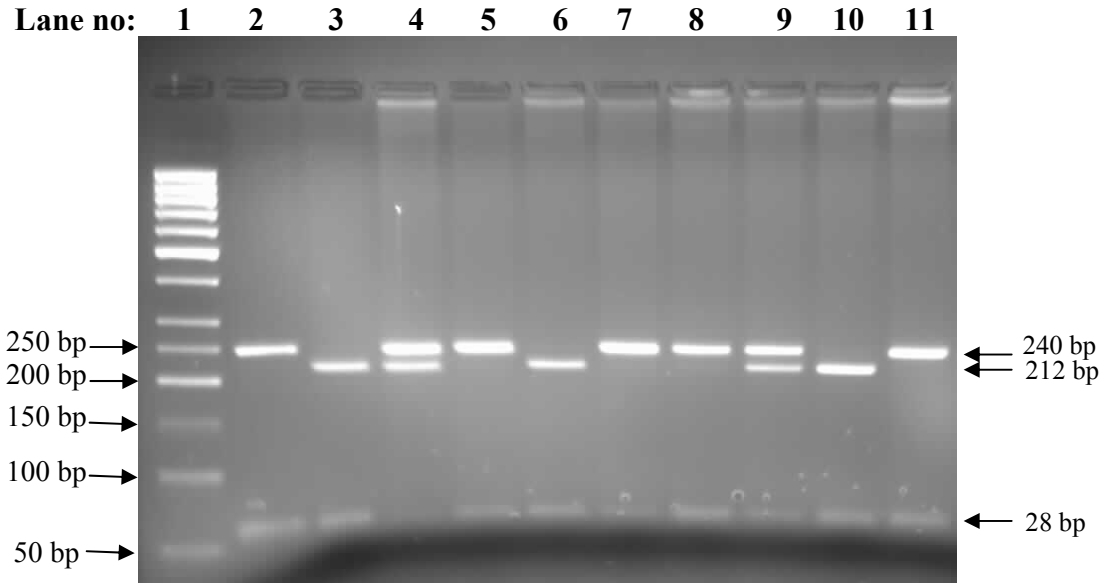


Figure 3.7 2.5 % Agarose gel electrophoresis of restriction endonuclease (*MbiI*) digestion products for the −107T/C SNP of PON1. Lane 1 contained DNA ladder (1031-50bp), lane 2 contained PCR product which was not subjected to digestion, and lanes 3-11 contained digestion products of subjects 137, 138, 139, 140, 141 142, 143, 144, 145, respectively.

Thus, genotypes were decided as;

Subject 137 (lane 3): *CC*
Subject 138 (lane 4): *TC*
Subject 139 (lane 5): *TT*
Subject 140 (lane 6): *CC*
Subject 141 (lane 7): *TT*
Subject 142 (lane 8): *TT*
Subject 143 (lane 9): *TC*
Subject 144 (lane 10): *CC*
Subject 145 (lane 11): *TT*

3.4 Relationship between PON1 Activities and PON1 Genotypes

3.4.1 PON1 Activities in Different PON1 Genotypes

Each of the three possible genotypes resulting from the 192Q/R, 55L/M and −107T/C SNPs differed in terms of PON1 activities. As expected, among 192Q/R genotypes, 192*RR* individuals, either control or patient, had the highest mean paraoxonase and arylesterase activities and individuals with *QQ* genotype had the lowest paraoxonase and arylesterase activities. Order of diazoxonase activities within 192Q/R genotypes was complete reverse of the order of paraoxonase and arylesterase activities. Diazoxonase activities decreased in the order of *QQ* > *QR* > *RR,* both in patients and controls (see Figures 3.11, 3.12 and 3.13). Among 55L/M genotypes, *LL* had the highest paraoxonase, arylesterase and diazoxonase activities. Individuals with *MM* genotype had the lowest paraoxonase, arylesterase and diazoxonase activities. The order of −107T/C genotypes in terms of paraoxonase, arylesterase and diazoxonase activities was *CC* > *TC* > *TT* both in patients and controls. The order of paraoxonase, arylesterase and diazoxonase activities of three different PON1 genotypes, in patients and controls, from highest to lowest is given in Figures 3.8, 3.9 and 3.10, can also be visualized in Figures 3.11 3.12 and 3.13.

Patients' paraoxonase activity
RR > CC > LL > QR > TC > LM > TT > QQ > MM

Controls' paraoxonase activity
RR > LL > QR > CC > TC > LM > TT > QQ > MM

Figure 3.8 Order of paraoxonase activities of 192Q/R, 55L/M and −107T/C genotypes in patients and controls, from highest to lowest.

Mean paraoxonase activity was highest in 192*RR* and lowest in 55*MM* individuals (Figure 3.8). Similarly, arylesterase activity was lowest in 55*MM*. In contrast to paraoxonase activity, arylesterase activity was highest in −107*CC* individuals (Figure 3.9).

Patients' arylesterase activity
CC > RR > LL > TC > QR > QQ > LM > TT > MM

Controls' arylesterase activity
CC > LL > RR > QR > TC > LM > QQ > TT > MM

Figure 3.9 Order of arylesterase activities of 192Q/R, 55L/M and −107T/C genotypes in patients and controls, from highest to lowest.

Diazoxonase activity, similar to arylesterase activity, was highest in −107*CC* genotype group and lowest in −107*TT* in patients and 192*RR* in controls (Figure 3.10).

Patients' diazoxonase activity													
CC	>	LL	>	QQ	>	TC	>	QR	>	LM	>	RR	>	MM	>	TT

Controls' diazoxonase activity													
CC	>	LL	>	QQ	>	QR	>	TC	>	LM	>	TT	>	MM	>	RR

Figure 3.10 Order of diazoxonase activities of 192Q/R, 55L/M and −107T/C genotypes in patients and controls, from highest to lowest.

3.4.2 Comparison of PON1 Activities and Activity Ratios of Patients and Controls in the Same Genotype Group

Figures 3.11-3.15 were prepared in order to compare PON1 enzyme activities and activity ratios of patients and controls in the same genotype class, so that the effects of genotype could be eliminated. As can be seen in Figure 3.11, paraoxonase activities of patients were, in general, lower than that of controls in each genotype group, except for −107*CC*. However, none of the differences were statistically significant.

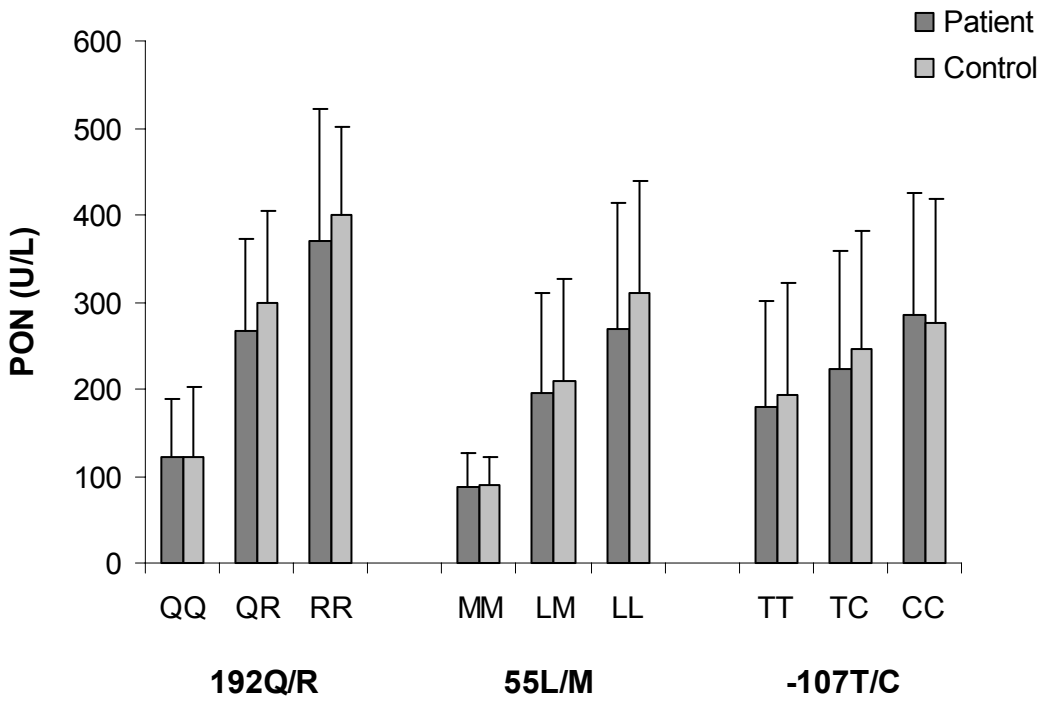


Figure 3.11 Paraoxonase activities (PON) of patients and controls in different PON1 192Q/R, 55L/M and −107T/C genotypes.

A similar trend was also observed in arylesterase activities; controls had higher arylesterase activities than patients (Figure 3.12). However, differences were not statistically significant.

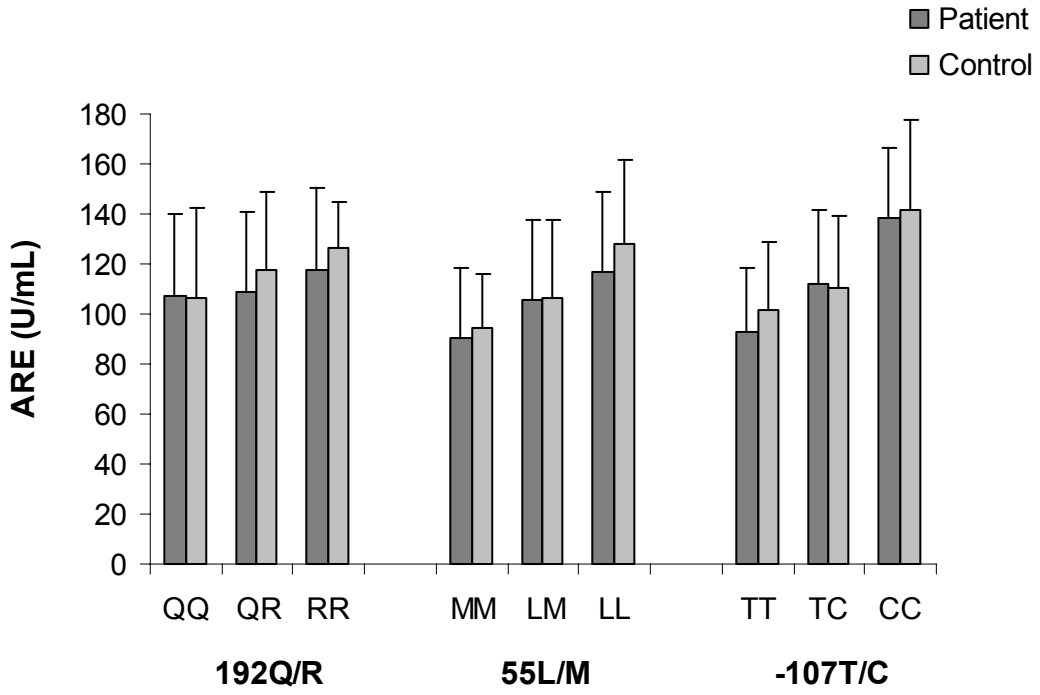


Figure 3.12 Arylesterase activities (ARE) of patients and controls in PON1 192Q/R, 55L/M and −107T/C genotypes.

Diazoxonase activities did not follow a general trend (Figure 3.13). Stroke patients with *QQ*, *QR, LL*, *LM MM*, *TT* and *CC* genotypes had lower diazoxonase activities than the respective controls. Patients with the remaining genotypes, namely *RR* and *TC*, had higher diazoxonase activities than controls in the respective genotype classes. However, none of the differences were statistically significant.

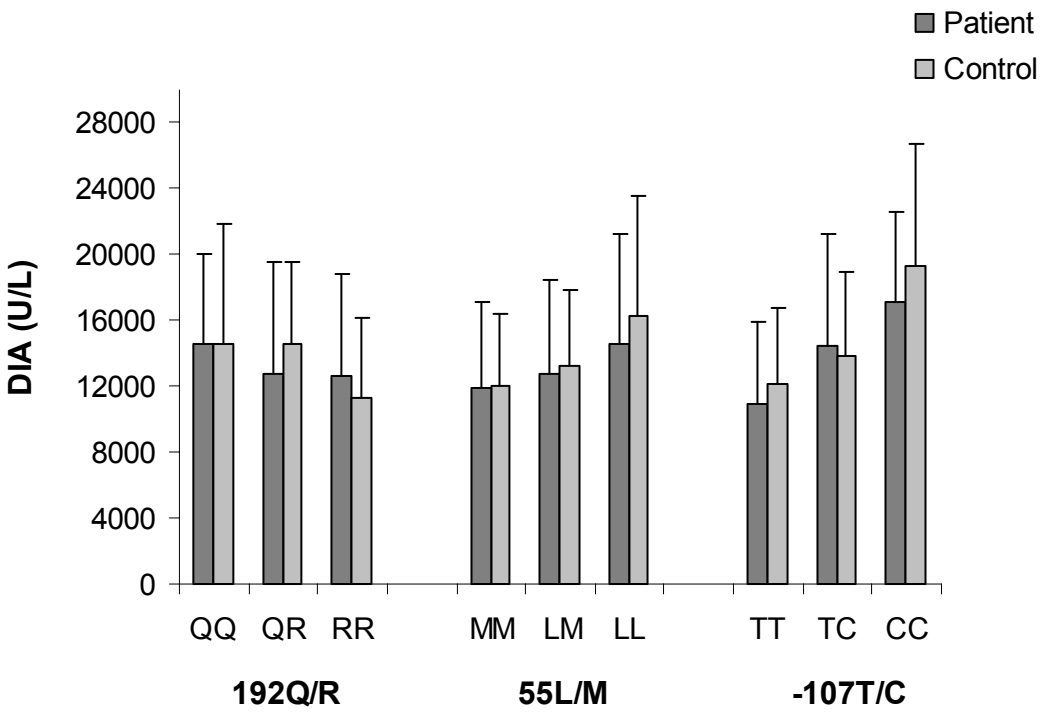


Figure 3.13 Diazoxonase activities (DIA) of patients and controls in PON1 192Q/R, 55L/M and −107T/C genotypes.

We could not find any significant differences between the PON1 activity ratio 1 (PON/ARE) of patients and controls in any of the genotype groups (Figure 3.14). Similarly, there was no significant difference between PON1 activity ratio 2 (PON/DIA) of patients and controls in any of the genotype groups (Figure 3.15).

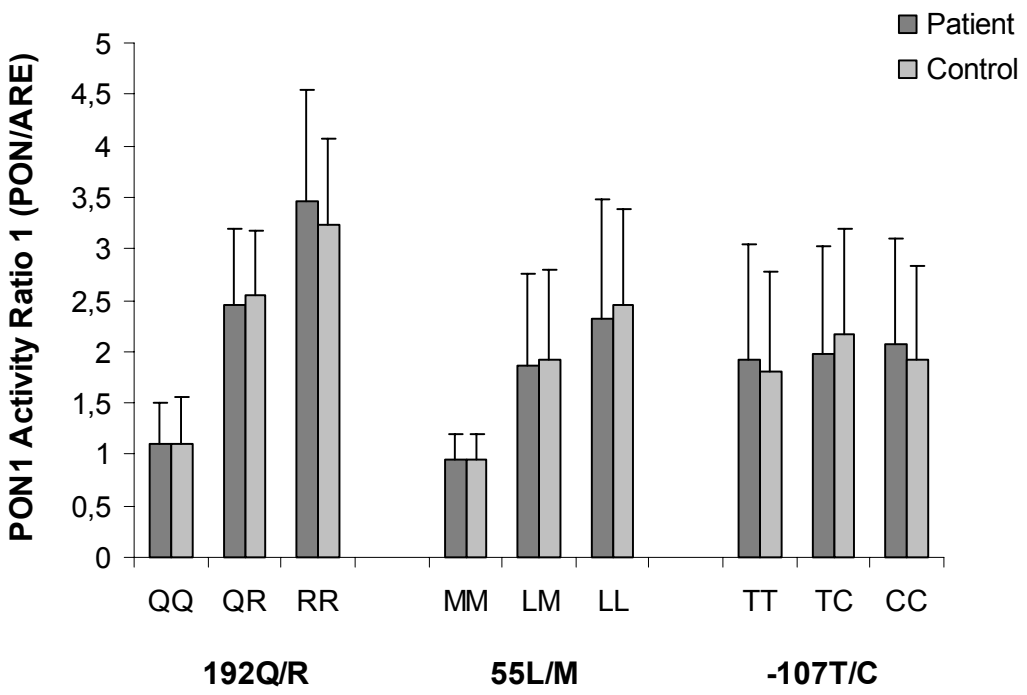


Figure 3.14 PON1 activity ratio 1 (PON/ARE) of patients and controls in PON1 192Q/R, 55L/M and −107T/C genotypes.

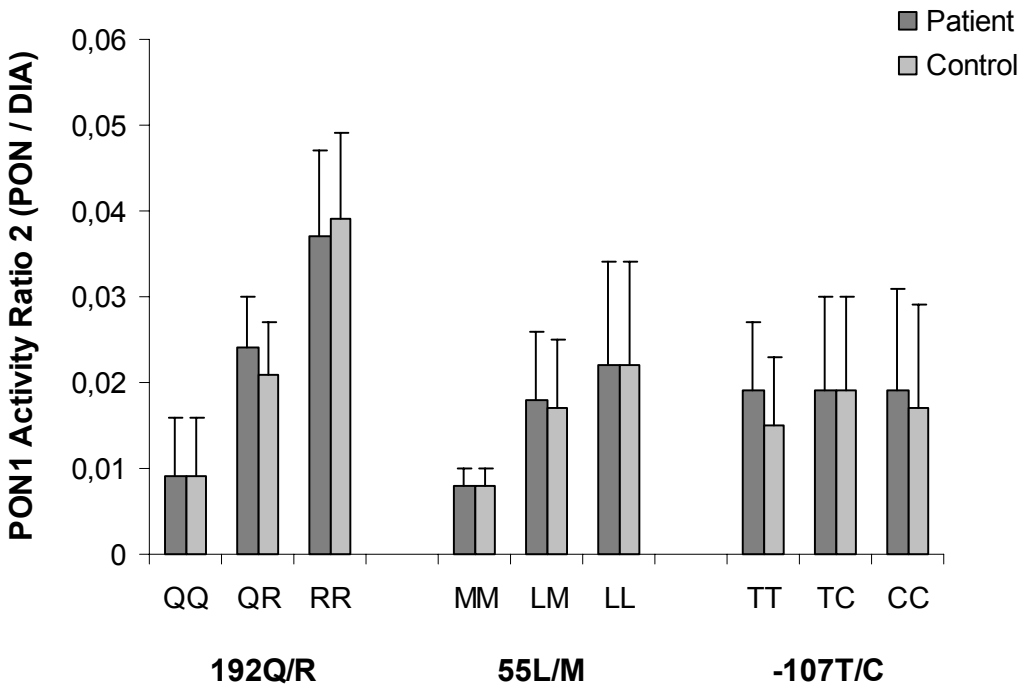


Figure 3.15 PON1 activity ratio 2 (PON/DIA) of patients and controls in PON1 192Q/R, 55L/M and −107T/C genotypes.

3.4.3 Association of PON1 Enzyme Activities with PON1 Genotypes

Dependence of paraoxonase, arylesterase and diazoxonase activities and PON1 activity ratios 1 (PON/ARE) and 2 (PON/DIA) on PON1 192Q/R (Table 3.18), 55L/M (Table 3.19) and −107T/C (Table 3.20) genotypes was analyzed by analysis of variance (ANOVA) test separately in controls and patients.

Paraoxonase activity was significantly associated with 192Q/R genotypes (ANOVA *P*=0.000). Distribution of paraoxonase activity within the 192Q/R genotypes was trimodal in patients and bimodal in controls (Table 3.18). In other words, in patients, the mean paraoxonase activity of *QQ* genotype (121.1 ± 67.2 U/L) was significantly different from that of *QR* (266.2 ± 106.5 U/L; *P*=0.000), which was in turn, significantly different from that of *RR* (396.7 ± 152.7 U/L; *P*=0.003). In control group, however, two groups were created by 192Q/R genotypes, namely *QQ* and *QR*+*RR*. This was because while the difference in paraoxonase activities between *QQ* and *QR* was significant (*P*=0.000), the difference between *QR* and *RR* was not significant (*P*=0.053), thus, creating two groups (Table 3.18). On the other hand, 192Q/R genotypes were not discriminative of arylesterase activities in neither controls (ANOVA *P*=0.123) nor patients (ANOVA *P*=0.438; Table 3.18). Similarly, diazoxonase activities were not associated with 192Q/R genotype groups (ANOVA *P*=0.148 in patients, ANOVA *P*=0.302 in controls). PON1 activity ratios 1 and 2 were both associated with 192Q/R genotypes (ANOVA *P*=0.000) both in patients and controls.

Table 3.18 PON1 activities and PON1 activity ratios in 192Q/R genotype groups in stroke patients and controls.

PON1 activities	192Q/R					
	QQ	*P*	QR	*P*	RR	ANOVA *P*
Patients						
PON (U/L)	121.1 ± 67.2	0.000	266.2 ± 106.5	0.003	396.7 ± 152.7	0.000
ARE (U/mL)	107.2 ± 32.7	0.988	108.7 ± 32.2	0.647	117.5 ± 33.3	0.438
DIA (U/L)	14589.7 ± 5480.4	0.199	12735.4 ± 6834.9	1.000	12612.3 ± 6200.7	0.148
PON1 Activity Ratio 1 (PON/ARE)	1.110 ± 0.384	0.000	2.452 ± 0.746	0.002	3.455 ± 1.084	0.000
PON1 Activity Ratio 2 (PON/DIA)	0.009 ± 0.004	0.000	0.024 ± 0.012	0.004	0.037 ± 0.016	0.000
Controls						
PON (U/L)	121.4 ± 81.4	0.000	299.7 ± 104.3	0.053	401.1 ± 100.7	0.000
ARE (U/mL)	106.5 ± 36.2	0.294	117.6 ± 31.1	0.632	126.2 ± 19	0.123
DIA (U/L)	14617.2 ± 7231.9	1.000	14603.3 ± 4927.6	0.238	11331.8 ± 4792.0	0.302
PON1 Activity Ratio 1 (PON/ARE)	1.094 ± 0.458	0.000	2.554 ± 0.612	0.129	3.228 ± 0.838	0.000
PON1 Activity Ratio 2 (PON/DIA)	0.009 ± 0.007	0.000	0.021 ± 0.006	0.002	0.039 ± 0.010	0.000

Data are mean ± SD, comparisons are by ANOVA.

As given in Table 3.19, paraoxonase activity was associated with 55L/M genotypes (ANOVA *P*=0.000). In both patients and controls, paraoxonase activity was trimodally distributed within the 55L/M genotypes (Table 3.19). Arylesterase activities, as in the case of 192Q/R genotypes, were not discriminative of 55L/M genotype groups, except for *LM* vs. *LL* in controls, where the difference was significant (Table 3.19; *P*=0.007). Diazoxonase activity was not associated with 55L/M genotypes in patients (ANOVA *P*=0.078). Although this activity of PON1 was associated with 55L/M genotypes in controls (ANOVA *P*=0.011), no significant separation in diazoxonase activity was observed between *LL* vs. *LM* and *LM* vs. *MM* genotype groups. In addition, PON1 activity ratios (PON/ARE and PON/DIA) were also associated with 55L/M genotypes (ANOVA *P*=0.000).

Among −107T/C genotypes, PON1 hydrolysis rates towards all the three substrates studied were highest in −107*CC* and lowest in −107*TT* genotypes. The differences in paraoxonase activities between genotype groups were, however, not significant. As can be inferred from the *P* values in Table 3.20, paraoxonase activity was not discriminative of −107T/C genotypes, in neither controls, nor patients. Arylesterase activity was, on the other hand, associated well with the −107T/C genotypes (ANOVA *P*=0.000). This enzyme activity of PON1 was trimodally distributed within −107T/C genotypes in patients. In control group, arylesterase activity was segregated into two groups; *CC* and *TC*+*TT* (Table 3.20). Diazoxonase activity was associated with −107T/C genotypes (ANOVA *P*=0.000), which created two groups in both patients (*CC*+*TC* and *TT*) and controls (*CC* and *TC*+*TT*). PON1 activity ratios were, however, not associated with −107T/C genotypes.

Table 3.19 PON1 activities and PON1 activity ratios in 55L/M genotype groups in stroke patients and controls.

PON1 activities	55L/M					
	MM	*P*	LM	*P*	LL	ANOVA *P*
Patients						
PON (U/L)	87.3 ± 38.4	0.000	195.7 ± 114.2	0.002	267.7 ± 144.0	0.000
ARE (U/mL)	90.7 ± 27.6	0.134	105.4 ± 32.2	0.092	116.8 ± 31.8	0.002
DIA (U/L)	11925.8 ± 5184.5	0.919	12695.5 ± 5730.0	0.175	14616 ± 6687.3	0.078
PON1 Activity Ratio 1 (PON/ARE)	0.951 ± 0.249	0.000	1.864 ± 0.896	0.019	2.323 ± 1.148	0.000
PON1 Activity Ratio 2 (PON/DIA)	0.008 ± 0.003	0.000	0.018 ± 0.012	0.255	0.022 ± 0.016	0.000
Controls						
PON (U/L)	90.6 ± 31.0	0.000	209.3 ± 118.1	0.001	310.6 ± 127.5	0.000
ARE (U/mL)	94.1 ± 22.3	0.213	106.7 ± 30.7	0.007	128.3 ± 33.3	0.000
DIA (U/L)	12045.2 ± 4380.8	0.692	13273.3 ± 4585.8	0.066	16322.9 ± 7292.8	0.011
PON1 Activity Ratio 1 (PON/ARE)	0.978 ± 0.346	0.000	1.923 ± 0.872	0.026	2.448 ± 0.981	0.000
PON1 Activity Ratio 2 (PON/DIA)	0.008 ± 0.002	0.000	0.017 ± 0.008	0.046	0.022 ± 0.012	0.000

Data are mean ± SD, comparisons are by ANOVA.

Table 3.20 PON1 activities and PON1 activity ratios in −107T/C genotype groups in stroke patients and controls.

PON1 activities	−107T/C					
	CC	*P*	TC	*P*	TT	ANOVA *P*
Patients						
PON (U/L)	284.1 ± 142.2	0.112	222.4 ± 137.1	0.166	179.7 ± 121.5	0.001
ARE (U/mL)	138.2 ± 28.4	0.000	111.7 ± 29.7	0.000	92.5 ± 25.5	0.000
DIA (U/L)	17100.6 ± 5489.7	0.103	14417.3 ± 6781.0	0.004	10975.8 ± 4887.9	0.000
PON1 Activity Ratio 1 (PON/ARE)	2.07 ± 1.035	0.970	1.983 ± 1.043	0.979	1.918 ± 1.119	0.782
PON1 Activity Ratio 2 (PON/DIA)	0.019 ± 0.012	1.000	0.019 ± 0.014	0.997	0.019 ± 0.015	0.980
Controls						
PON (U/L)	275.6 ± 143.3	0.799	244.9 ± 135.8	0.206	192.3 ± 128.5	0.047
ARE (U/mL)	141.2 ± 36.2	0.005	110.6 ± 28.6	0.400	101.8 ± 27.3	0.000
DIA (U/L)	19329.9 ± 7442.5	0.013	13879.9 ± 5095.9	0.288	12147.9 ± 4567.3	0.000
PON1 Activity Ratio 1 (PON/ARE)	1.929 ± 0.905	0.721	2.167 ± 1.023	0.273	1.805 ± 0.965	0.238
PON1 Activity Ratio 2 (PON/DIA)	0.015 ± 0.008	0.394	0.019 ± 0.011	0.838	0.017 ± 0.012	0.451

Data are mean ± SD, comparisons are by ANOVA.

Paraoxonase activity of PON1 was associated well with the three PON1 genotypes in double combinations (Table 3.21). Paraoxonase activity of *QQMM* patients (82.8 ± 33.4 U/L) was significantly lower than that of the *QRMM* patient (176.8 U/L; *P*=0.013). However, there was no significant difference in paraoxonase activities of *QQMM* and *QRMM* controls. Paraoxonase activities of *QQLM* patients were significantly lower than that of *QRLM* patients. A similar trend was also observed for these genotype classes in controls. There was no individual carrying the *RRMM* and *RRLM* genotype. Within 55*LL* genotype group, paraoxonase activity was associated with 192Q/R genotypes both in stroke patients and controls. Paraoxonase activity was also associated with 55L/M genotypes within the 192*QQ* genotype group, both in patients and controls. In both patients and controls, paraoxonase activity was associated with 192Q/R genotypes within *TT*, *TC* and *CC* genotype groups (*P*=0.000). Association of paraoxonase activity with 55L/M genotypes was significant only within *TT* and *TC* genotypes. In addition, paraoxonase activities of patients with *QQMM*, *QRLM*, *QQLL*, *QRLL*, *QQTT*, *QRTC*, *RRTC*, *RRCC*, *MMTT*, *LMTT*, *LLTT*, *LMTC*, *LLTC*, *LLCC* genotypes were lower than those of the respective controls. None of the differences were statistically significant (Table 3.21).

Table 3.21 Association of paraoxonase activities with double combined haplotypes of PON1 192Q/R, 55L/M and −107T/C polymorphisms in the patient and control groups.

	Patients				Controls			
	QQ	**QR**	**RR**	***P***	**QQ**	**QR**	**RR**	***P***
MM	82.8 ± 33.4	176.8	---	0.013	89.1 ± 31.2	118.3	---	0.375
LM	126.3 ± 87.5	261.1 ± 97.1	---	0.000	108.8 ± 40.9	284.7 ± 98.9	---	0.000
LL	146.1 ± 37.5	273.3 ± 115.9	396.7 ± 152.7	0.000	202.3 ± 135.8	321.3 ± 103.6	401.1 ± 100.7	0.001
P	0.005	0.628	---		0.001	0.099	---	
TT	87.3 ± 36.5	205.7 ± 75.2	402.4 ± 116.9	0.000	109.3 ± 101.3	244.1 ± 62.0	375.5 ± 121.5	0.000
TC	135.9 ± 84.1	264.2 ± 86.6	373.0 ± 185.7	0.000	115.4 ± 55.8	308.9 ± 111.4	400.9 ± 33.2	0.000
CC	157.6 ± 50.8	386.4 ± 82.4	489.9 ± 153.0	0.000	155.9 ± 61.2	361.3 ± 104.3	529.5	0.000
P	0.000	0.000	0.631		0.318	0.011	0.432	
	MM	**LM**	**LL**	**P**	**MM**	**LM**	**LL**	**P**
TT	67.1 ± 16.5	164.9 ± 78.9	276.3 ± 145.5	0.000	91.3 ± 33.7	173.7 ± 85.2	322.7 ± 126.6	0.000
TC	117.6 ± 30.5	194.5 ± 126.2	256.2 ± 143.4	0.035	85.7 ± 28.1	221.1 ± 128.0	315.9 ± 119.4	0.003
CC	187.9	312.1 ± 133.7	276.8 ± 148.4	0.647	100.4	257.4 ± 146.3	294.5	0.416
P	0.000	0.001	0.820		0.913	0.283	0.832	

Data are mean ± SD, comparisons are by ANOVA.

3.4.4 Correlation of PON1 Activities with PON1 Genotypes in Stroke Patients and Controls

Correlations between PON1 activities and PON1 genotypes were calculated separately in controls (Table 3.22) and patients (Table 3.23). The strongest correlation was found between paraoxonase activity and 192Q/R genotype, with a correlation coefficient of r=0.722 (*P*=0.000) in controls group and r=0.699 (*P*=0.000) in stroke patients. Paraoxonase activity was also positively correlated with 55L/M genotype in controls (r=0.585, *P*=0.000) and patients (r=0.428, *P*=0.000). There was a negative correlation between paraoxonase activity and −107T/C genotype (r = −0.238, *P*=0.014 in controls and r = −0.286, *P*=0.000 in patients). Paraoxonase activity was also found to be highly correlated with arylesterase activity both in controls (r=0.580, *P*=0.000) and patients (r=0.513, *P*=0.000). Although correlation between paraoxonase activity and diazoxonase activity was also positive in controls (r=0.297, *P*=0.002; Table 3.22), this correlation was lost in patients (Table 3.23).

Arylesterase activity was found to be strongly correlated with the PON1 55L/M genotype both in controls (r=0.401, *P*=0.000) and in patients (r=0.266, *P*=0.000). On the other hand, arylesterase activity was weakly correlated within the PON1 192Q/R genotype (r=0.200, *P*=0.041) in controls, and not correlated at all, in patients (r=0.085, *P*=0.268). The correlation between arylesterase activity and −107T/C polymorphism was negative with a correlation coefficient of r = −0.421 (*P*=0.000) in controls and r = −0.528 (*P*=0.000) in patients. Arylesterase activity and diazoxonase activity were strongly correlated both in controls (r=0.689, *P*=0.000) and patients (r=0.517, *P*=0.000).

No correlation was found between diazoxonase activity and 192Q/R genotype in either controls (Table 3.22) or patients (Table 3.23). On the other hand, diazoxonase activity was positively correlated with 55L/M genotype both in controls (r=0.281, *P*=0.000) and in patients (r=0.169, *P*=0.028). In both controls and patients, diazoxonase activity was found to be negatively correlated with −107T/C genotypes (r = −0.421 and r = −0.385, respectively).

PON1 192Q/R and 55L/M genotypes were highly correlated in controls (r=0.528, *P*=0.000) and patients (r=0.461, *P*=0.000). 192Q/R genotype was, however, not correlated with −107T/C genotype in either controls or patients. 55L/M and −107T/C genotypes were highly correlated in both controls (r=−0.377, *P*=0.000) and patients (r=−0.323, *P*=0.000).

Table 3.22 Correlation of PON1 activities and genotypes in controls.

		PON	ARE	DIA	192Q/R	55L/M	−107T/C
PON	Pearson Correlation *P*	1	0.580** 0.000	0.297** 0.002	0.722** 0.000	0.585** 0.000	−0.238* 0.014
ARE	Pearson Correlation *P*	0.580** 0.000	1	0.689** 0.000	0.200* 0.041	0.401** 0.000	−0.421** 0.000
DIA	Pearson Correlation *P*	0.297** 0.002	0.689** 0.000	1	−0.101 0.307	0.281* 0.004	−0.421** 0.000
192Q/R	Pearson Correlation *P*	0.722** 0.000	0.200* 0.041	−0.101 0.307	1	0.528** 0.000	0.001 0.992
55L/M	Pearson Correlation *P*	0.585** 0.000	0.401** 0.000	0.281* 0.004	0.528** 0.000	1	−0.337** 0.000
−107T/C	Pearson Correlation *P*	−0.238* 0.014	−0.421** 0.000	−0.421** 0.000	0.001 0.992	−0.337** 0.000	1

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table 3.23 Correlation of PON1 activities and genotypes in patients.

		PON	ARE	DIA	192Q/R	55L/M	−107T/C
PON	Pearson Correlation *P*	1	0.513** 0.000	0.126 0.102	0.699** 0.000	0.428** 0.000	−0.286** 0.000
ARE	Pearson Correlation *P*	0.513** 0.000	1	0.517** 0.000	0.085 0.268	0.267** 0.000	−0.528** 0.000
DIA	Pearson Correlation *P*	0.126 0.102	0.517** 0.000	1	−0.137 0.076	0.169* 0.028	−0.385** 0.000
192Q/R	Pearson Correlation *P*	0.699** 0.000	0.085 0.268	−0.137 0.076	1	0.461** 0.000	0.056 0.464
55L/M	Pearson Correlation *P*	0.428** 0.000	0.267** 0.000	0.169* 0.028	0.461** 0.000	1	−0.323** 0.000
−107T/C	Pearson Correlation *P*	−0.268** 0.000	−0.528** 0.000	−0.385** 0.000	0.056 0.464	−0.323** 0.000	1

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

3.5 PON1 Genotype and Allele Frequencies

Table 3.24 shows the distribution of PON1 genotypes arising from the 192Q/R, 55L/M and −107T/C polymorphisms. There was no deviation of genotype frequencies from Hardy-Weinberg equilibrium. The frequencies of the 192*R*, 55*L* and −107*T* alleles and prevalence of the so-called risky genotypes 192*RR*, 55*LL* and −107*TT* were increased in the patient group (Table 3.24).

Table 3.24 Distribution of PON1 192Q/R, 55L/M and −107T/C genotypes and allele frequencies in stroke patient and controls.

	Patients (n=172)	Controls (n=105)	OR (95% CI)	*P*
PON1 192 Genotypes, n (%)				
QQ	77 (44.8)	46 (43.8)		
QR	74 (43)	50 (47.6)		
RR	21 (12.2)	9 (8.6)	1.483[a] (0.652-3.374)	0.345
Alleles				
Q	0.663	0.676		
R	0.337	0.324	1.065[b] (0.880-1.278)	0.536
PON1 55 Genotypes, n (%)				
LL	83 (47.7)	44 (41.9)	1.293[c] (0.792-2.110)	0.303
LM	68 (39.5)	42 (40.0)		
MM	21 (12.2)	19 (18.1)		
Alleles				
L	0.690	0.628	1.318[d] (1.095-1.587)	0.003
M	0.310	0.372		
PON1 −107 Genotypes, n (%)				
TT	73 (42.4)	42 (40)	1.106[e] (0.675-1.812)	0.689
TC	63 (36.6)	41 (39)		
CC	36 (20.9)	22 (21)		
Alleles				
T	0.608	0.595	1.056[f] (0.883-1.263)	0.553
C	0.392	0.405		

[a]RR vs. QQ+QR, [b] R vs. Q, [c]LL vs. LM+MM , [d] L vs. M, [e]TT vs. TC+CC, [f]T vs. C

The *L* allele frequency was significantly higher among patients (0.690) than controls (0.628; *P*=0.003), while the increase in the frequency of *R* allele (0.337 vs. 0.324, *P*=0.536) and *T* allele (0.608 vs. 0.595, *P*=0.553) in patients compared to controls were insignificant. As can be seen in Table 3.25, none of the differences in the prevalence of the so called "risky genotypes" were significant (192*RR*; 12.2% vs. 8.6%, *P*=0.345, 55*LL*; 47.7% vs. 41.9%, *P*=0.303, −107*TT*; 42.4% vs. 40%, *P*=0.689).

Distribution of haplotypes resulting from 192Q/R, 55L/M and −107T/C SNPs in double and triple combinations are given in Tables 3.25 and 3.26, respectively. Frequency of the presence of two risky genotypes together in patients was increased when compared to controls (Table 3.25). For example, *RRLL* (12.2 % vs. 8.6 %), *RRTT* (5.2 % vs. 4.8 %) and *LLTT* (12.8 % vs. 12.4 %) genotypes were seen more common in patients than controls, although the differences were not significant. We did not find any subject with *RRLM* and *RRMM* genotype, confirming the linkage disequilibrium between *R* and *L* alleles (Blatter-Garin *et al.*, 1997).

Prevalence of presence of three heterozygote genotypes together (*QRLMTC*) was significantly lower in stroke patients (4.1 %) when compared to controls (11.4 %; *P*=0.019). Three risky genotypes (*RRLLTT*) were seen more common among patients (5.2 %) than controls (4.8 %), although the difference was not significant (Table 3.26).

Table 3.25 Distribution of double combined haplotypes in stroke patients and controls.

Double combined haplotypes, n (%)	Patient (n=172)	Control (n=105)	OR	*P*
192Q/R and 55L/M				
QQMM	20 (11.6%)	18 (17.14%)	0.636	0.196
QQLM	33 (19.2%)	18 (17.14%)	1.147	0.670
QQLL	24 (14%)	10 (9.52%)	1.545	0.276
QRMM	1 (0.6%)	1 (0.95%)	0.608	0.723
QRLM	35 (20.3%)	24 (22.86%)	0.862	0.621
QRLL	38 (22.1%)	25 (23.80%)	0.908	0.741
RRMM	0	0	–	–
RRLM	0	0	–	–
RRLL	21 (12.2%)	9 (8.60%)	1.483	0.344
192Q/R and −107T/C				
QQCC	17 (9.9%)	10 (9.5%)	1.042	0.922
QQTC	29 (16.9%)	15 (14.3%)	1.217	0.570
QQTT	31 (18.0%)	21 (20%)	0.879	0.683
QRCC	17 (9.9%)	11 (10.5%)	0.937	0.874
QRTC	24 (14%)	23 (21.9%)	0.578	0.087
QRTT	33 (19.2%)	16 (15.2%)	1.321	0.403
RRCC	2 (1.2%)	1 (0.95%)	1.223	0.869
RRTC	10 (5.8%)	3 (2.9%)	2.037	0.280
RRTT	9 (5.2%)	5 (4.8%)	1.104	0.862
55L/M and −107T/C				
MMCC	1 (0.6%)	1 (0.95%)	0.608	0.723
MMTC	6 (3.5%)	4 (3.8%)	0.913	0.889
MMTT	14 (8.1%)	14 (13.3%)	0.576	0.164
LMCC	10 (5.8%)	6 (5.7%)	0.988	0.981
LMTC	21 (12.2%)	21 (20%)	0.556	0.079
LMTT	37 (21.5%)	15 (14.3%)	1.644	0.135
LLCC	25 (14.5%)	15 (14.3%)	1.020	0.954
LLTC	36 (20.9%)	16 (15.2%)	1.472	0.239
LLTT	22 (12.8%)	13 (12.4%)	1.038	0.921

Table 3.26 Distribution of triple combined haplotypes in stroke patients and controls.

Triple combined haplotypes, n (%)	Patient (n=172)	Control (n=105)	OR	P
QQMMCC	1 (0.6%)	1 (0.95%)	0.608	0.723
QQMMTC	5 (2.9%)	4 (3.8%)	0.756	0.681
QQMMTT	14 (8.1%)	13 (12.4%)	0.627	0.248
QQLMCC	4 (2.3%)	3 (2.86%)	0.809	0.784
QQLMTC	14 (8.1%)	9 (8.6%)	0.945	0.899
QQLMTT	15 (8.7%)	6 (5.7%)	1.576	0.359
QQLLCC	12 (7.0 %)	6 (5.7%)	1.237	0.679
QQLLTC	10 (5.8%)	2 (1.9%)	3.179	0.121
QQLLTT	2 (1.2%)	2 (1.9%)	0.606	0.615
QRMMCC	0	0	–	–
QRMMTC	1 (0.6%)	0	–	–
QRMMTT	0	1 (0.95%)	–	–
QRLMCC	6 (3.5%)	3 (2.86%)	1.229	0.774
QRLMTC	7 (4.1%)	12 (11.4%)	0.329	**0.019**
QRLMTT	22 (12.8%)	9 (8.6%)	1.564	0.280
QRLLCC	11 (6.4%)	8 (7.6%)	0.828	0.696
QRLLTC	16 (9.3%)	11 (10.5%)	0.877	0.749
QRLLTT	11 (6.4 %)	6 (5.7 %)	1.093	0.865
RRMMCC	0	0	–	–
RRMMTC	0	0	–	–
RRMMTT	0	0	–	–
RRLMCC	0	0	–	–
RRLMTC	0	0	–	–
RRLMTT	0	0	–	–
RRLLCC	2 (1.2%)	0	–	–
RRLLTC	10 (5.8%)	3 (2.9 %)	2.099	0.259
RRLLTT	9 (5.2%)	5 (4.8 %)	1.104	0.862

3.6 Further Analysis of Effects of Vascular Risk Factors in Different PON1 Genotype Groups

Three vascular risk factors, hypertension, smoking and diabetes were analyzed in terms of the proportion of stroke patients to controls within the risky group and the non-risky group. The results were also stratified by the genotypes resulting from the three genetic polymorphisms of PON1; 192Q/R (Table 3.27), 55L/M (Table 3.28) and −107T/C (Table 3.29).

In Table 3.27 one could see that in each genotype group, the proportion of stroke patients to controls was increased in hypertensive, smoker or diabetic group when compared to normotensive, nonsmoker or nondiabetic group. For example, out of 16 hypertensive individuals with a 192*RR* genotype, 14 were stroke patients and 2 were not. This proportion came out to be 7 stroke patients and 7 controls for normotensive 192*RR* individuals. Thus, odds ratio (OR) was 7 (95% CI= 1.140-42.970, *P*=0.025). Among hypertensives, the greatest approximate relative risk for stroke was found for 192*RR* individuals, followed by 192*QR* and 192*QQ*. Among smokers, none of the relative odds was statistically significant (Table 3.27). For diabetes, the OR calculated for 192*QR* individuals was 2.949 (95% CI= 1.157-7.514, *P*=0.02).

Among 55L/M genotypes, risk of ischemic stroke in hypertensives compared to normotensives was highest within 55*LL* genotype (Table 3.28; OR against normotensive 55*LL*s=2.66; 95% CI= 1.25-5.65; *P*=0.010). In diabetics and smokers, risk of stroke within none of the 55L/M genotypes was significant. Similarly, among −107T/C genotypes, a significant risk for ischemic stroke was found in hypertensives versus normotensives wihin the −107*TT* genotype (Table 3.29; OR against normotensive −107*TT*s=2.89; 95% CI= 1.32-6.36; *P*=0.007). Risk associated with ischemic stroke in diabetics and smokers was not significant in any of the −107T/C genotypes.

Table 3.27 Stratification of hypertensive-normotensive, smoker-nonsmoker, diabetic-nondiabetic individuals according to 192Q/R genotypes and stroke-control status.

Status	192*RR* (n=30)	Stroke / control	192*QR* (n=124)	Stroke / control	192*QQ* (n=123)	Stroke / control
Hypertensive (n=150)	16	14 / 2[a]	78	52 / 26[b]	56	39 / 17[c]
Normotensive (n=127)	14	7 / 7	46	22 / 24	67	38 / 29
Smoker (n=53)	8	6 / 2[d]	22	15 / 7[e]	23	18 / 5[f]
Nonsmoker (n=224)	22	15 / 7	102	59 / 43	100	59 / 41
Diabetic (n=68)	6	5 / 1[g]	31	24 / 7[h]	31	21 / 10[i]
Nondiabetic (n=209)	24	16 / 8	93	50 / 43	92	56 / 36

[a] OR calculated against normotensive 192*RR*s= 7.000 (95% CI= 1.140-42.970), *P*=0.025
[b] OR calculated against normotensive 192*QR*s= 2.182 (95% CI= 1.035-4.600), *P*=0.039
[c] OR calculated against normotensive 192*QQ*s= 1.751 (95% CI= 0.829-3.695), *P*=0.140
[d] OR calculated against nonsmoker 192*RR*s= 1.400 (95% CI= 0.223-8.768), *P*= 0.718
[e] OR calculated against nonsmoker 192*QR*s= 1.562 (95% CI= 0.586-4.159), *P*=0.370
[f] OR calculated against nonsmoker 192*QQ*s= 2.502 (95% CI= 0.860-7.279), *P*=0.085
[g] OR calculated against nondiabetic 192*RR*s= 2.500 (95% CI= 0.248-25.154), *P*= 0.425
[h] OR calculated against nondiabetic 192*QR*s= 2.949 (95% CI= 1.157-7.514), *P*=0.020
[i] OR calculated against nondiabetic 192*QQ*s= 1.350 (95% CI= 0.570-3.195), *P*=0.494

Table 3.28 Stratification of hypertensive-normotensive, smoker-nonsmoker, diabetic-nondiabetic individuals according to 55L/M genotypes and stroke-control status.

Status	55*LL* (n=127)	Stroke / control	55*LM* (n=110)	Stroke / control	55*MM* (n=40)	Stroke / control
Hypertensive (n=150)	69	52 / 17[a]	60	28 / 22[b]	21	13 / 8 [c]
Normotensive (n=127)	58	31 / 27	50	40 / 20	19	8 / 11
Smoker (n=53)	22	16 / 6[d]	24	18 / 6[e]	7	5 / 2[f]
Nonsmoker (n=224)	105	67 / 38	86	50 / 36	33	16 / 17
Diabetic (n=68)	26	21 / 5[g]	35	24 / 11[h]	7	5 / 2[i]
Nondiabetic (n=209)	101	62 / 39	75	44 / 31	33	16 / 17

[a] OR calculated against normotensive 55*LL*s=2.66 (95% CI= 1.25-5.65), *P*=0.010
[b] OR calculated against normotensive 55*LM*s=0.63 (95% CI=0.29-1.38), *P*=0.251
[c] OR calculated against normotensive 55*MM*s=2.23 (95% CI=0.629-7.932), *P*=0.210
[d] OR calculated against nonsmoker 55*LL*s=1.512 (95% CI=0.545-4.191), *P*=0.424
[e] OR calculated against nonsmoker 55*LM*s= 2.160 (95% CI=0.780-5.980), *P*=0.132
[f] OR calculated against nonsmoker 55*MM*s= 2.565 (95% CI=0.450-15.693), *P*=0.270
[g] OR calculated against nondiabetic 55*LL*s=2.642 (95% CI=0.920-7.583), *P*=0.064
[h] OR calculated against nondiabetic 55*LM*s= 1.537 (95% CI=0.658-3.592), *P*=0.319
[i] OR calculated against nondiabetic 55*MM*s=2.656 (95% CI=0.450-15.693), *P*=0.270

Table 3.29 Stratification of hypertensive-normotensive, smoker-nonsmoker, diabetic-nondiabetic individuals according to −107T/C genotypes and stroke-control status.

Status	−107*TT* (n=115)	Stroke / control	−107*TC* (n=104)	Stroke / control	−107*CC* (n=58)	Stroke / control
Hypertensive (n=150)	60	45 / 15[a]	52	35 / 17[b]	38	25 / 13[c]
Normotensive (n=127)	55	28 / 27	52	28 / 24	20	11 / 9
Smoker (n=53)	22	17 / 5[d]	26	18 / 8[e]	5	4 / 1[f]
Nonsmoker (n=224)	93	56 / 37	78	45 / 33	53	32 / 21
Diabetic (n=68)	28	21 / 7[g]	25	18 / 7[h]	15	11 / 4[i]
Nondiabetic (n=209)	87	52 / 35	79	45 / 34	43	25 / 18

[a] OR calculated against normotensive -107*TT*s=2.89 (95% CI= 1.32-6.36), *P*=0.007
[b] OR calculated against normotensive -107*TC*s=1.76 (95% CI=0.80-3.91), *P*=0.160
[c] OR calculated against normotensive -107*CC*s=1.57 (95% CI=0.52-4.76), *P*=0.421
[d] OR calculated against nonsmoker -107*TT*s=2.25 (95% CI=0.76-6.62), *P*=0.135
[e] OR calculated against nonsmoker -107*TC*s=1.65 (95% CI=0.64-4.25), *P*=0.297
[f] OR calculated against nonsmoker -107*CC*s=2.63 (95% CI=0.0.27-25.14), *P*=0.387
[g] OR calculated against nondiabetic -107*TT*s=2.02 (95% CI=0.0.78-5.26), *P*=0.145
[h] OR calculated against nondiabetic -107*TC*s=1.94 (95% CI=0.73-5.18), *P*=0.180
[i] OR calculated against nondiabetic -107*CC*s=1.98 (95% CI=0.0.54-7.23), *P*=0.296

Effects of individual PON1 alleles on stroke risk in risk groups were also evaluated. Table 3.30 compares PON1 192*R* allele frequency of stroke patients and controls in hypertensives and diabetics to that found in the respective non-risk groups. 192*R* allele was associated with a significant 1.233 fold increased risk of stroke in hypertensives. In normotensives, however, *R* allele was associated with a negative risk. Thus, 192*R* allele of PON1 represented 1.554 times increased risk (1.233/0.793) for ischemic stroke in hypertensives relative to normotensives. This allele of PON1 constituted a 1.546 times increased risk in diabetics.

Table 3.30 PON1 192*R* allele frequency in risk groups.

Group	192*R* allele frequency		OR	*P*
	Stroke	Control		
Hypertensive	0.381	0.333	1.233	0.020
Normotensive	0.269	0.317	0.793	0.018
Diabetic	0.340	0.250	1.546	0.000
Non-diabetic	0.336	0.339	0.987	0.887

A similar analysis was carried out with 55*L* allele and the results obtained are given in Table 3.31. While 55*L* allele of PON1 was associated with a 1.66 times increased risk of stroke in hypertensives, this allele was not associated significantly to stroke risk in normotensives. In diabetics, this allele constituted 1.388 fold increased stroke risk, while the risk was reversed in non-diabetics. Thus, 55*L* allele was associated with a 2.6 (1.388/0.534) times increased risk for stroke in diabetics.

Table 3.31 PON1 55*L* allele frequency in risk groups.

Group	55*L* allele frequency		OR	*P*
	Stroke	Control		
Hypertensive	0.710	0.596	1.660	0.000
Normotensive	0.646	0.638	1.035	0.709
Diabetic	0.660	0.583	1.388	0.000
Non-diabetic	0.688	0.805	0.534	0.000

As given in Table 3.32, −107*T* allele also represented a significant risk for stroke in hypertensives (OR=1.345, 95% CI=1.006-1.388, *P*=0.001). However, this allele was not associated with stroke risk in normotensive individuals. This allele of PON1 was not significantly associated with stroke in neither diabetics nor non-diabetics.

Table 3.32 PON1 −107*T* allele frequency in risk groups.

Group	−107*T* allele frequency		OR	*P*
	Stroke	Control		
Hypertensive	0.595	0.522	1.345	0.001
Normotensive	0.627	0.650	0.905	0.284
Diabetic	0.600	0.583	1.073	0.439
Non-diabetic	0.611	0.598	1.056	0.552

3.7 Logistic Regression Analysis

Different binary logistic regression models, all with backward selection (backward likelihood ratio), were set up including different combinations of the following parameters in the overall population or in different subgroups of subjects defined by being non-user of statin drugs, having 55*LL* genotype or being elderly (age > 59): vascular risk factors (age, sex, hypertension, diabetes, smoking), either 192*RR*, 55*LL* and −107*TT* genotypes or PON1 *QRLMTC* haplotype, either PON1 activities separately (PON, ARE, DIA) or PON1 activity ratios 1 (PON/ARE) and 2 (PON/DIA).

MODEL 1

In MODEL 1 (Table 3.33), age, sex, hypertension, smoking status, diabetes, PON1 activities (paraoxonase, arylesterase and diazoxonase), PON1 192*RR*, 55*LL* and −107*TT* genotypes were added as covariates, and logistic regression revealed hypertension (OR=2.435 95% CI=1.468-4.038, *P*=0.001) and smoking (OR=2.687 95% CI=1.339-5.390, *P*=0.001) to be the strongest determinants of stroke. Paraoxonase activity (OR=0.998 95% CI=0.996-0.999, *P*=0.012) and PON1 55*LL* genotype (OR=1.783 95% CI=1.012-3.141, *P*=0.045) were also associated with stroke versus control status. 67.8% of cases were predicted correctly by the model and the calibration was satisfactory (chi-square=9.4; 7 degrees of freedom; *P*=0.227).

MODEL 2

When PON1 activities (paraoxonase, arylesterase and diazoxonase) were removed and instead PON1 activity ratio 1 (PON/ARE) and PON1 activity ratio 2 (PON/DIA) were included into the model (MODEL 2), hypertension and smoking status were still significant predictors of stroke. PON1 activity ratio 1 (OR=0.793, 95% CI=0.638-0.984, *P*=0.035) was found to be a significant predictor of stroke versus control status (Table 3.34). The model correctly predicted 67.8 % of the cases and the Hosmer-Lemeshow goodness-of-fit test demonstrated that the calibration of the model was satisfactory (chi-square= 6.3; 8 degrees of freedom; *P*= 0.614) for logistic regression.

Table 3.33 Logistic regression analysis of vascular risk factors (age, sex, hypertension, diabetes and smoking), 192*RR*, 55*LL* and −107*TT* genotypes and PON1 activities (PON, ARE, DIA) (MODEL 1).

Parameter	OR	95 % CI	*P*
Hypertension	2.435	1.468-4.038	0.001
Smoking	2.687	1.339-5.390	0.005
PON	0.998	0.996-0.999	0.012
55*LL*	1.783	1.012-3.141	0.045

Age, sex, diabetes, ARE, DIA, PON1 192*RR* and −107*TT* genotypes were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

Table 3.34 Logistic regression analysis of vascular risk factors (age, sex, hypertension, diabetes, smoking), 192*RR*, 55*LL* and −107*TT* genotypes and PON1 activity ratio 1 (PON/ARE) and ratio 2 (PON/DIA) (MODEL 2).

Parameter	OR	95 % CI	*P*
Hypertension	2.378	1.415-3.996	0.001
Smoking	2.688	1.320-5.476	0.006
PON1 activity ratio 1 (PON/ARE)	0.793	0.638-0.984	0.035

Age, sex, diabetes, PON1 activity ratio 2, PON1 192*RR*, 55*LL* and −107*TT* genotypes were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 3

In a third logistic regression model (MODEL 3), statin users were excluded from the population and only those who did not use statin drugs were included. Parameters of MODEL 1 and MODEL 2 were tested separately. In this analysis, hypertension and smoking remained to be risk factors for stroke, in addition to PON (Table 3.35). PON1 activity ratios were not found to be risk factors in this subgroup. 64.6% of cases were predicted correctly by the model and the calibration was satisfactory (chi-square=6.1; 8 degrees of freedom; *P*=0.634).

Table 3.35 Logistic regression analysis of age, sex, vascular risk factors (hypertension, diabetes and smoking), 192*RR*, 55*LL* and −107*TT* genotypes and PON1 activities (PON, ARE, DIA) or PON1 activity ratios 1 and 2 in non-statin users (MODEL 3).

Parameter	OR	95 % CI	*P*
Hypertension	2.925	1.687-5.073	0.000
Smoking	3.058	1.471-6.357	0.003
PON	0.998	0.997-1.000	0.025

Age, sex, diabetes, HDL, triglycerides, ARE, DIA, PON1 192*RR*, 55*LL* and −107*TT* genotypes were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 4

Lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C) were included as covariates together with vascular risk factors and PON1 activities or activity ratios in MODEL 4 (Table 3.36). No distinction was made as statin users and non-users. Hypertension and smoking were still associated with stroke, however,

PON1 activity ratio 1 or PON was not a predictor of stroke anymore. Instead, 55*LL* genotype (OR=1.909, 95% CI= 1.066-3.416, *P*=0.030), total cholesterol (OR=0.705, 95% CI=0.545-0.913, *P*=0.008) and LDL-C (OR=1.662, 95% CI=1.115-2.477, *P*=0.013) were significantly associated with case versus control status. This model correctly predicted more of the cases and controls (68.5%) than the first and second models. Calibration of the model was tested by Hosmer-Lemeshow goodness-of-fit test and turned out to be satisfactory (chi-square= 12.1; 8 degrees of freedom; *P*=0.147) for logistic regression.

Table 3.36 Logistic regression analysis of vascular risk factors (age, sex, hypertension, diabetes and smoking), lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C), 192*RR*, 55*LL* and −107*TT* genotypes and PON1 activities (PON, ARE, DIA) or PON1 activity ratios 1 and 2 (MODEL 4).

Parameter	OR	95 % CI	*P*
Hypertension	2.925	1.687-5.073	0.000
Smoking	3.058	1.471-6.357	0.003
Total cholesterol	0.705	0.545-0.913	0.008
LDL-C	1.662	1.115-2.477	0.013
55*LL*	1.909	1.066-3.416	0.030

Age, sex, diabetes, HDL, triglycerides, PON, ARE, DIA, PON1 192*RR* and −107*TT* genotypes were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 5

The same analysis was repeated in a group of individuals who did not use statin drugs (MODEL 5) and the results given in Table 3.37 were obtained. Hypertension and smoking remained to be significant predictors of stroke, in addition to LDL-cholesterol. In this analysis, low HDL-cholesterol was found to be a risk factor for stroke (Table 3.37). The model correctly predicted 67.3 % of the cases and the Hosmer-Lemeshow goodness-of-fit test demonstrated that the calibration of the model was satisfactory (chi-square= 4.1; 8 degrees of freedom; *P*= 0.849) for logistic regression.

Table 3.37 Logistic regression analysis of vascular risk factors (age, sex, hypertension, diabetes and smoking), lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C), 192*RR*, 55*LL* and −107*TT* genotypes and PON1 activities (PON, ARE, DIA) or PON1 activity ratios 1 and 2 in individuals who did not use statins (MODEL 5).

Parameter	OR	95 % CI	*P*
Hypertension	2.819	1.661-4.782	0.000
Smoking	2.336	1.125-4.849	0.023
LDL-C	1.343	1.052-1.715	0.018
HDL-C	0.400	0.215-0.746	0.004

Age, sex, diabetes, HDL, triglycerides, PON1 activities and PON1 genotypes were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 6

In a new logistic regression model (MODEL 6), triple combined heterozygote (*QRLMTC*) haplotype was added in the analysis instead of 192*RR*, 55*LL* and −107*TT* (Table 3.38). Presence of three heterozygote genotypes in the same person was significantly associated with control versus case status. In other words, having this haplotype has 4.587 (1/0.218) times increased protective effect against stroke. The model correctly predicted 64.2 % of the cases and the Hosmer-Lemeshow goodness-of-fit test demonstrated that the calibration of the model was satisfactory (chi-square=3.2; 4 degrees of freedom; *P*= 0.524) for logistic regression.

Table 3.38 Logistic regression analysis of vascular risk factors (age, sex, hypertension, diabetes, smoking), PON1 activities and PON1 *QRLMTC* combined haplotype (MODEL 6).

Parameter	OR	95 % CI	*P*
Hypertension	2.472	1.708-3.576	0.000
Smoking	2.703	1.395-5.236	0.003
PON1 *QRLMTC*	0.218	0.077-0.614	0.004

Age, sex, diabetes and PON1 activities, were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 7

When lipid parameters were added to the above model as covariates (MODEL 7), in addition to hypertension, smoking and PON1 *QRLMTC* combined haplotype, total cholesterol and LDL-C were found to be significantly associated with stroke (Table 3.39). The protective effect of PON1 *QRLMTC* combined haplotype increased from 4.587 to 5.650 (1/0.177). In addition, effects of hypertension and smoking were also increased (Table 3.39). Moreover, this model predicted more (70.8 %) of the

cases than the previous model. The calibration of the model was found to be satisfactory (chi-square=12.4; 8 degrees of freedom; *P*=0.136) by Hosmer-Lemeshow test.

Table 3.39 Logistic regression analysis of vascular risk factors (age, sex, hypertension, diabetes, smoking), lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C), PON1 activities and *QRLMTC* combined haplotype (MODEL 7).

Parameter	OR	95 % CI	*P*
Hypertension	3.147	1.810-5.474	0.000
Smoking	2.975	1.377-6.427	0.002
Total cholesterol	0.663	0.516-0.853	0.001
LDL-C	1.691	1.134-2.523	0.010
PON1 *QRLMTC*	0.177	0.060-0.522	0.002

Age, sex, diabetes, triglycerides, HDL-C and PON1 activities were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 8

When the above analysis was repeated in subjects who did not use statin drugs (MODEL 8; Table 3.40), PON1 *QRLMTC* combined haplotype remained to be significantly associated with stroke. Moreover, its protective effect was increased from 5.650 to 6.944 (1/0.144). The model correctly predicted 67.8 % of the cases and the Hosmer-Lemeshow goodness-of-fit test demonstrated that the calibration of the model was satisfactory (chi-square=5.2; 8 degrees of freedom; *P*=0.731) for logistic regression. When the risky genotypes 192*RR*, 55*LL* and −107*TT* were added to the

above model, the parameters given in Table 3.40 stayed still and OR values did not change much (data not given).

Table 3.40 Logistic regression analysis of age, sex, vascular risk factors (hypertension, diabetes, smoking), lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C), PON1 activities and *QRLMTC* combined haplotype in individuals who did not use statins (MODEL 8).

Parameter	OR	95 % CI	*P*
Hypertension	3.080	1.750-5.422	0.000
Smoking	2.829	1.331-6.015	0.007
Total cholesterol	0.663	0.516-0.853	0.001
LDL-C	1.691	1.134-2.523	0.010
PON1 *QRLMTC* genotype	0.144	0.044-0.468	0.001

Age, sex, diabetes, triglycerides, HDL-C and PON1 activities were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 9

Within a subgroup of individuals carrying 55*LL* genotype, when age, sex, hypertension, smoking status, diabetes, PON, ARE, DIA, PON1 192*RR*, and -107*TT* genotypes were selected as covariates (MODEL 9, Table 3.41), logistic regression analysis revealed that hypertension, sex and PON are significantly associated with stroke versus control status. Being male was not previously found to be a risk factor, but was associated with 2.2 times increased risk for stroke relative to females within individuals carrying PON1 55*LL* genotype. (OR=2.229, 95% CI=1.034-4.804, *P*=0.041). The effect of hypertension was increased substantially within 55*LL*

individuals; being hypertensive was associated with more than 4-fold increased risk of having an ischemic stroke compared to normotensives (Table 3.41). The model correctly predicted 69.3 % of the cases and the Hosmer-Lemeshow goodness-of-fit test demonstrated that the calibration of the model was satisfactory (chi-square= 5.2; 8 degrees of freedom; *P*= 0.741) for logistic regression.

Table 3.41 Logistic regression analysis of vascular risk factors (age, sex, hypertension, diabetes and smoking), 192*RR* and −107*TT* genotypes and PON1 activities (PON, ARE, DIA) in 55*LL* genotype group (MODEL 9).

Parameter	OR	95 % CI	*P*
Hypertension	4.280	1.839-9.961	0.001
Sex	2.229	1.034-4.804	0.041
PON	0.997	0.995-0.999	0.010

Age, diabetes, smoking, PON, ARE, DIA, PON1 192*RR* and -107*TT* genotypes were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

MODEL 10

In an attempt to see whether risk factors for stroke change with advancing age, MODEL 10 was constituted, in which only elderly subjects (aged > 59 years) who did not use statin drugs were considered. Covariates were chosen identical to those in MODEL 4, except that age is not included, i.e.; sex, hypertension, diabetes, smoking, lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C), PON, ARE, DIA, PON1 192*RR*, 55*LL* and −107*TT* genotypes. This model revealed hypertension, smoking, total cholesterol, LDL-cholesterol and PON1 −107*TT* genotype as significant predictors of stroke (Table 3.42). The low expressor genotype −107*TT* was associated with a 1.973 times increased risk for stroke (95% CI=1.014-3.839,

P=0.045). Total cholesterol came out to be a significant predictor of control versus stroke status. When the statin users were included into the model, PON1 −107*TT* genotype was no more a predictor of stroke (data not given).

Table 3.42 Logistic regression analysis of vascular risk factors (sex, hypertension, diabetes and smoking), lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C), 192*RR*, 55*LL* and −107*TT* genotypes and PON1 activities (PON, ARE, DIA) in elderly population (age > 59) who did not use statins (MODEL 10).

Parameter	OR	95 % CI	*P*
Hypertension	3.000	1.609-5.592	0.001
Smoking	3.235	1.093-9.578	0.034
Total cholesterol	0.655	0.486-0.882	0.005
LDL-C	1.579	1.000-2.494	0.050
PON1 −107*TT*	1.973	1.014-3.839	0.045

Sex, diabetes, triglycerides, HDL, PON1 activities, PON1 192*RR* and 55*LL* genotypes were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

It was noteworthy that the risk associated with smoking was increased (OR=3.235 95% CI=1.093-9.578, *P*=0.034) in the elderly group, compared to the risk of smoking in the overall population containing all age groups (OR=2.688 for MODEL 1, OR=3.058 for MODEL 4). The model correctly predicted 67 % of the cases and the calibration of the model was satisfactory (chi square=10.6; 8 degrees of freedom, *P*=0.225).

MODEL 11

In another logistic regression analysis, PON1 *QRLMTC* combined haplotype was included as a covariate instead of 192*RR*, 55*LL* and −107*TT* genotypes (MODEL 11; Table 3.43). The protective effect of this haplotype against stroke increased to 10.4-fold (1/0.096) in this subgroup of statin non-user elderly subjects. Hypertension, smoking, total cholesterol and LDL-cholesterol were also significantly associated with stroke versus control status (Table 3.43). The effect of smoking further increased to 6.472 in this model.

Table 3.43 Logistic regression analysis of vascular risk factors (sex, hypertension, diabetes and smoking), lipid parameters (total cholesterol, triglycerides, HDL-C and LDL-C), PON1 *QRLMTC* combined haplotype and PON1 activities (PON, ARE, DIA) in elderly population (age > 59) who did not use statins (MODEL 11).

Parameter	OR	95 % CI	*P*
Hypertension	3.078	1.582-5.992	0.001
Smoking	6.472	2.062-20.313	0.001
Total cholesterol	0.670	0.480-0.937	0.019
LDL-C	1.590	1.004-2.516	0.048
PON1 *QRLMTC*	0.096	0.022-0.409	0.002

Sex, diabetes, triglycerides, HDL and PON1 activities were also included in the analysis but were not significantly associated with the presence of ischemic stroke.

CHAPTER IV

DISCUSSION

Stroke is the third-leading cause of death and the leading cause of severe neurological disability worldwide. In addition, stroke is one of the major public health problems in developed countries. Major advances have been made in understanding the etiology, pathology and management of the disease. However considerable progress is still needed both in research and implementation of research findings to reduce the burden of stroke to society.

Nothing is accidental about stroke, even though it is implied by the misnomer of cerebral vascular accident. Instead, stroke is usually the result of predisposing conditions that originated years before the ictus. Atherosclerosis is a precursor for atherothrombotic infarction and carotid atherosclerosis is a risk factor for this stroke subtype. Although the consequences of atherosclerosis generally manifest in middle-aged or elderly persons, the process of atherogenesis generally begins during childhood, with a preclinical phase that can last for decades (Fuster *et al.*, 1992; Berliner *et al.*, 1995).

Recent evidence indicates that modified forms of low density lipoprotein (LDL) are associated with increased atherogenicity (Steinberg *et al.*, 1989). Of special interest is the oxidative modification of LDL, the mechanism of which involves cellular lipid peroxidation. Inhibition of such LDL modifications may arrest the development of the atherosclerotic lesion (Aviram, 1993).

High-density lipoprotein (HDL) is today regarded as one of the most important protective factors against arteriosclerosis. Numerous cohort studies have confirmed association between a low HDL-cholesterol concentration and an increased risk of coronary heart disease (Miller and Miller, 1975; Rhoads *et al.*, 1976; Gordon *et al.*, 1977). In experimental animals, an inverse correlation between HDL concentration and the development of arteriosclerosis has been demonstrated (Tomás *et al.*, 2004). In addition, it has been observed that arteriosclerotic lesions tend to regress *in vivo* as the concentration of HDL or its apolipoproteins increases (Badimon *et al.*, 1990; Miyazaki *et al.*, 1995; Nissen *et al.*, 2003; Tomás *et al.*, 2004). There does not appear to be any single explanation for the inverse relationship between serum HDL and risk of atherosclerosis. Traditionally, HDL's protective function has been attributed to its active participation in the reverse transport of cholesterol (Durrington *et al.*, 2001). However, in recent years the greatest interest is into the capacity of HDL to protect LDL against lipid peroxidation (Mackness *et al.*, 2000a). Two enzyme systems associated with normal HDL have been reported to inhibit LDL oxidation *in vitro*. Stafforini and colleagues (Stafforini *et al.*, 1993) reported that the platelet activating factor acetylhydrolase (PAFAH) was effective in preventing metal ion–dependent oxidation of LDL. Mackness and colleagues (Mackness *et al.*, 1991b) reported that paraoxonase, an HDL-associated enzyme, also inhibited LDL oxidation *in vitro*. Both enzymes have been found to protect against LDL modification (Watson *et al.*, 1994 and 1995b; Berliner *et al.*, 1995). Many lines of evidence indicate, however, that the PAFAH activity of HDL is due to paraoxonase (Rodrigo *et al.*, 2001a; Durrington *et al.*, 2001).

Human serum paraoxonase 1 (PON1) is an HDL associated ester hydrolase. PON1 endows HDL with its antioxidant properties and is probably responsible for the principal mechanism inhibiting the oxidation of both low-density lipoproteins (LDLs) and HDL itself, a process that is directly involved in the initial phases of arteriosclerosis (Mackness *et al.*, 1991b; Mackness *et al.*, 1993b; Berliner *et al.*, 1995; Aviram *et al.*, 1998b; Tomás *et al.*, 2004). It has also been shown to hydrolyze the platelet-activating factor (Rodrigo *et al.*, 2001a) and the L-homocysteine thiolactone (Jakubowski, 2000), thereby contributing to the prevention of

atherogenesis and inflammation in blood vessel walls (Josse *et al.*, 2002). The observation that mice with a genomic deletion of the PON1 gene develop atherosclerosis (Shih *et al.*, 1998) helped to strengthen the role of PON1 in atherosclerosis. Moreover, human paraoxonase-1 overexpression inhibited atherosclerosis in mice (Tward *et al.*, 2002; Oda *et al.*, 2002; Mackness *et al.*, 2006).

As stated above, atherosclerosis in the carotid arteries represents a risk for ischemic stroke (Fuster *et al.*, 1992; Berliner *et al.*, 1995). Thus, PON1 might have a protective role for the development of ischemic stroke. Activity and level of PON1 is, however, variable between individuals. PON1 activity is in part determined by single nucleotide polymorphisms (SNPs) in the coding (192Q/R and 55L/M; Brophy *et al.*, 2002) and the promoter region (–107T/C, –162A/G, and –909(907)C/G) of the gene (Brophy *et al.*, 2001b). Physiological and pathological conditions, diet, exogenous compounds such as drugs and environmental chemicals also have demonstrated effects on PON1 activity (see Tables 1.13 and 1.14).

In this study we aimed to test three PON1 activities (paraoxonase, arylesterase and diazoxonase) and three genetic polymorphisms (192Q/R, 55L/M and –107T/C) as risk factors for atherothrombotic ischemic stroke. In addition, roles of conventional risk factors and lipid parameters in this type of stroke were also evaluated.

Demographic characteristics and lipid parameters

The study population was composed of 172 atherothrombotic ischemic stroke patients and 105 control subjects. Since age is the strongest nonmodifiable determinant of stroke (Petitti *et al.*, 1997; Goldstein *et al.*, 2006), great effort was spent in the present study to recruit healthy elderly individuals who perfectly meet the exclusion criteria into the control group, so that there was no statistically significant difference in mean age of patient and control groups. Mean age was 66.6 years in stroke patients and 64.5 years in controls (P=0.093). In this way, results

regarding PON1 activities and PON1 genotype frequencies would not be influenced by age. It should be noted that the sample was clinically well defined and strictly selected. In the group of controls, there was no history of ischemic heart disease, myocardial infarction within 3 weeks and carotid stenosis >50%.

Another nonmodifiable risk factor for stroke is sex. Stroke incidence was found to be 1.25 times greater among men in previous studies (Sacco *et al.*, 1997; Sacco *et al.*, 1998; Kumral *et al.*, 1998). In this study, within 172 ischemic stroke patients, 97 were male and 75 were female. Thus, males proved to have a greater risk (1.3 times) for ischemic stroke in the present study, too. For this reason, in the study population attention was paid so as to balance the number of males included into the control group with the number of males in stroke patient group. There was no statistically significant difference in the number of males included into each group. Males constituted 56.4% of patients and 49.5% of the control group (P=0.266).

Prevalences of hypertension and diabetes were significantly higher in the stroke patient group compared to controls in this study (Table 3.1). Being hypertensive or diabetic both exhibited around 2-fold relative risk for ischemic stroke (Table 3.2). This was an expected result, since these two are regarded as conventional, but modifiable as well, risk factors for stroke (Lawes *et al.*, 2004; Sacco, 2005). The common factor linking these conditions and stroke is oxidative stress. It was suggested that oxidative stress may constitute a major pathogenic factor in the development of hypertension and type 2 diabetes (de Champlain *et al.*, 2004), as in the case of atherosclerosis and ischemic stroke (Steinberg *et al.*, 1989; Fuster *et al.*, 1992; Berliner *et al.*, 1995).

Another modifiable risk factor for stroke is smoking (Rudd *et al.*, 1997). Even the number of cigarettes smoked was found to make a difference. The relative risk of stroke in heavy smokers (greater than 40 cigarettes per day) was twice that of light smokers (fewer than ten cigarettes per day) in a 26-year follow-up study (Wolf *et al.*, 1988). Stroke risk decreased significantly by two years and was at the level of nonsmokers by five years after cessation of cigarette smoking (Wolf *et al.*, 1988). In

the present study, smoking was seen more common among patients (22.7%) than controls (13.3%; *P*=0.055). Among 53 smokers, 39 had ischemic stroke, while 14 did not have stroke. Smoking may effect the development of stroke in two ways: First; cigarette smoking triggers oxidative stress (Agarwal, 2005), which leads to atherosclerosis according to the oxidative theory of atherogenesis (Steinberg *et al.*, 1989). Second; increased oxidative stress (Shih *et al.*, 1996; Navab *et al.*, 1997) or more directly cigarette smoke (Nishio and Watanabe, 1997) inhibits paraoxonase activity, which is a potential antiatherogenic enzyme. Decrease in paraoxonase activity thereby leads to increased oxidative stress, which gives way to atherosclerosis. Effect of smoking on PON1 enzyme activities were also analyzed in the present work, and will be discussed under the heading of "PON1 activities".

Total cholesterol levels were found to be slightly lower in stroke patients than in controls in the present work (Table 3.1). In a large case-control study, higher total cholesterol levels were associated with increased risk of ischemic stroke (Tirschwell *et al.*, 2004). On the other hand, total cholesterol levels were not significantly associated with ischemic stroke risk in another case-control study (Bowman *et al.*, 2003). Thus, there is no consensus at the moment on whether or not high level of cholesterol is a risk factor for stroke. Furthermore, it was suggested that in the elderly, for whom statins are most commonly prescribed, high total cholesterol and LDL-C are not risk factors and quite the opposite is true (Schupf *et al.*, 2005; Kauffman, 2007). Thus, in our study group where the mean age was 66.6 years in stroke patients and 64.5 years in controls, it is possible that low levels of total cholesterol is a risk factor for stroke. This was really what we observed in logistic regression analysis. Total cholesterol was found to be associated with a 0.705 times increased risk for stroke (Table 3.36). In other words, low levels of total cholesterol is 1.42 (1/0.705) times risky in this population (*P*=0.008). However, when individuals who used cholesterol lowering statin drugs were excluded from the study group, total cholesterol was no more a predictor (Table 3.37). These findings corroborate with the observation that cholesterol is only a very weak cardiovascular risk factor for the elderly (Casiglia *et al.*, 2002). This could simply indicate that elderly persons are the survivors in a population where significant mortality has

already made its mark, eliminating those with the worst risk pattern (Casiglia *et al.*, 2002). However, it should be noted that there is considerable controversy over whether total cholesterol levels predict coronary heart disease risk in the elderly and whether cholesterol reduction is advisable for elderly patients. Some think that the war on cholesterol is misguided (Graveline, 2004).

Our finding of lower levels of total cholesterol in stroke patients might have been due to the insignificantly higher prevalence of the users of the statins, cholesterol biosynthesis inhibitor drugs, among patients. Although not our priority, effects of statin use on levels of total cholesterol and LDL-cholesterol (LDL-C) levels were also analyzed in this study. We observed significantly lower levels of LDL-C and substantially lower levels of total cholesterol in statin users compared to nonusers (Table 3.3). These results were in concordance with previous findings (Berger *et al.*, 1989; Bach *et al.*, 1990). In a pilot study with Turkish subjects, nine men were treated with simvastatin, 10 mg/day, for 8 weeks and at the end their LDL-C levels were dramatically reduced by 42% (Bersot and Mahley, 1998).

We also wondered whether statin use significantly affected these lipid parameters in the overall population which was composed of both statin users and those who did not use statin. When we calculated total cholesterol and LDL-C levels in stroke patients and controls who did not use statins, we reached almost the same values as obtained in the overall population (data not shown). Lack of difference was probably due to the small number of statin users (n=18) compared to nonusers (n=259).

High density lipoprotein-cholesterol (HDL-C) levels were significantly lower in stroke patients (1.1 mmol/L = 42.5 mg/dL), compared to controls (1.2 mmol/L = 46.3 mg/dL; P=0.007). A similar result was also obtained by other researchers (Ueno *et al.*, 2003; Pasdar *et al.*, 2006; Kim *et al.*, 2007), even though completely reverse result was found in other studies (Imai *et al.*, 2000; Aydin *et al.*, 2006; Baum *et al.*, 2006). Since the antioxidant role of HDL is well recognized, our finding of lower levels of HDL-C in ischemic stroke patients seems plausible.

In the present study, the level of HDL-C in the healthy population (controls; 1.2 mmol/L = 46.3 mg/dL) was low according to the recommended values. Recommended HDL-C levels are above 60-70 mg/dL, and considered low when < 55 mg/dL in men and < 60 mg/dL in women. Mahley and colleagues (1995) examined the plasma lipids and lipoproteins in approximately 9,000 men and women from six different regions of Turkey and reported low levels of HDL-C in men (34-38 mg/dL) and women (37-45 mg/dL). Turkish population appears to be unique in having unusually low levels of HDL-C, which was suggested to be genetic in origin (Mahley *et al.*, 1995) or due to increased hepatic lipase activity (Mahley *et al.*, 1999).

Since PON1 is associated with HDL-C in serum, and since in the present work HDL-C levels were significantly lower in patients, we expected to see significantly lower PON1 activities in patients compared to controls. However, as will be stated below, despite the significantly lower HDL levels in patients, none of the PON1 activities exhibited a significant reduction in stroke patients. This was also represented by the lack of correlation between HDL-C levels and PON1 activities (data not given). This is noteworthy, since today it is common knowledge that low HDL-C is a risk factor for cardiovascular disease. What is not very commonly recognized is that the beneficial effect of HDL comes mostly from paraoxonase that resides on it, and that this enzyme is polymorphic and might be at high levels in a person having low levels of HDL. Furthermore, PON1 is associated exclusively with a discrete subpopulation of HDL particles (Blatter *et al.*, 1993; Kelso *et al.*, 1994; Mackness *et al.*, 2002b). Thus, most of the time it is the quality of HDL, not the amount, which makes a change. Specific HDL fractions were not determined in this study, which may explain the poor correlation between PON1 and HDL found in this and other population studies (Mackness *et al.*, 2000a; Mackness *et al.*, 2002b).

Degree of stenosis in the carotid artery was significantly increased in the patient group. This was partly an artifact of the selection procedure; we have eliminated individuals having > 50% stenosis in their carotid artery from the control group. The rationale for such a selection was that increased carotid artery stenosis might increase stroke risk (Rothwell, 2000).

PON1 activities

The importance of determination of genotypes and serum PON1 activities and/or concentrations (PON1 status) simultaneously for correlation with disease susceptibility, has been strongly emphasized by several authors (Jarvik *et al.*, 2000; Richter and Furlong, 1999; Mackness *et al.*, 2001; Draganov and La Du, 2004; Brophy *et al.*, 2002). Although a few case-control studies on the role of PON1 genetic polymorphisms (Imai *et al.*, 2000; Voetsch *et al.*, 2002; Ueno *et al.*, 2003; Ranade *et al.*, 2005; Baum *et al.*, 2006; Pasdar *et al.*, 2006) and PON1 activity towards a single substrate (Aydin *et al.*, 2006; Kim *et al.*, 2007) in stroke were published, to our knowledge, no report appeared yet in the literature analyzing three PON1 activities and three PON1 genotypes simultaneously in ischemic stroke. It was therefore hard to compare the present findings. As a background, it was realized that atherosclerosis is a situation associated with increased oxidative stress, which is known to decrease paraoxonase activity (Shih *et al.*, 1996; Navab *et al.*, 1997). In addition, we considered studies investigating diseases of similar pathology for comparison. Since low paraoxonase activity was found in patients with cardiovascular disease (McElveen *et al.*, 1986; Ayub *et al.*, 1999; Ferré *et al.*, 2002a; Mackness *et al.*, 2003; Graner *et al.*, 2006) and cerebrovascular disease (Jarvik *et al.*, 2000 and 2003), we expected to measure lower PON1 activities in ischemic stroke patients, too. This was really the case (Table 3.5); serum paraoxonase, arylesterase and diazoxonase activities of patients were lower than those of the controls, although the differences were not significant. In two recent studies, which were published after we have started our investigation, PON1 activity was found to be significantly diminished in stroke patients compared to controls (Aydin *et al.*, 2006; Kim *et al.*, 2007).

In the present work, paraoxonase activity (PON) or PON1 activity ratio 1 (PON/ARE) were negatively associated with ischemic stroke by use of separate logistic regression models (Tables 3.33 and 3.34). Odds ratio calculated for PON1 activity ratio 1 was 0.793, which can be translated as, PON1 activity ratio 1 is associated with 0.793 times increased risk for stroke. To put it another way, this

parameter is associated with 1.261-fold (1/0.793) increase in control vs. stroke status (PON1 activity ratio 1 was 1.261 times protective against stroke). Similar discussion is valid for PON; although the predictive power of this parameter was less. Low paraoxonase activity was associated with 1.002 times increased risk of stroke, which can be translated as having no effect. Even though other researchers (Jarvik *et al.*, 2000 and 2003; Aydin *et al.*, 2006; Kim *et al.*, 2007) have found low PON1 activity as a risk factor for vascular disease, this is the first time PON1 activity ratio is demonstrated to be a protective factor for ischemic stroke.

Diazoxonase activity and PON1 activity ratio 2 (PON/DIA) were, however, not predictive of stroke in logistic regression. Similarly, in the Caerphilly prospective study there were no differences in PON1 activity toward diazoxon between CHD patients and controls (Mackness *et al.*, 2003).

Variability of PON1 activities between populations

There are marked differences in PON1 activities between populations, with populations of non-European origin having higher levels (Mackness *et al.*, 1998a). In part, this is because of the variability of the allele frequencies of 192Q/R polymorphism between populations; the higher activity 192 polymorphic alleles (192*R*) are prevalent in people of African and Asian origin (see Table 4.1). Comparison of allele frequencies of this and two other polymorphisms of PON1 in different populations is given in the next section. It is also likely that differences in nutrition and industrialization are critically important (Durrington *et al.*, 2002). However, one has to be cautious when comparing PON1 activities from different studies, as there are numerous PON1 enzyme activity determination methods, which make use of different buffer systems, include or exclude salt, utilize different reaction temperatures for product formation and measure the formation of product at different wavelengths. Thus, given the importance of actual enzyme activity determinations for disease association studies, PON1 enzyme activity measurements need to be uniform in laboratories all over the world. For the same reason,

comparison of the PON1 enzyme activities found in the present study with those found in other populations was not carried out.

Ethnic distribution of PON1 genetic polymorphisms

Ethnic distribution of human PON1 polymorphisms shows great variability around the world (Tables 1.10 and 1.11). Even within the same country, variances could be observed for the same allele (Table 4.1). Hence, while the frequency of the 192*R* allele in 105 Turkish controls was found to be 0.324 in the present study, the same allele frequency was reported to be 0.17 (Karaaslan-Biyikli *et al.*, 2006), 0.31 (Aynacioglu *et al.*, 1999) and 0.37 (Agachan *et al.*, 2005) within Turkish population. This frequency was previously found to be 0.346 in a smaller subgroup (n=78) of the control population of the present investigation (Can Demirdöğen *et al.*, 2008). The observed divergence of the frequency of the same allele within Turkish population might be explained by the ethnic diversity of our population. The frequency of 192*R* allele in control individuals in this study (0.324) was higher than found in most of the Caucasian European populations, including Swiss (Blatter-Garin *et al.*, 1994; James *et al.*, 2000a), British (Mackness *et al.*, 1997a; 2000c), Finnish (Antikainen *et al.*, 1996), French (Herrman *et al.*, 1996; Helbecque *et al.*, 1999), Italian (Ombres *et al.*, 1998), Austrian (Schmidt *et al.*, 1998), Russian (Akhmedova *et al.*, 1999), German (Cascorbi *et al.*, 1999) and Spanish (Senti *et al.*, 2000; Ferré *et al.*, 2002a). In addition, the frequency of the 192*R* allele in this population was also higher than Canadian Oji-cree (Fanella *et al.*, 2000), Inuit (Fanella *et al.*, 2000), Costa Rican (Sen-Banerjee *et al.*, 2000) and Thai populations (Phuntuwate *et al.*, 2005). This frequency was however lower than found in Chinese (Sanghera *et al.*, 1997 and 1998b; Ko *et al.*, 1998; Padungtod *et al.*, 1999) and Japanese (Suehiro *et al.*, 1996; Zama *et al.*, 1997; Ikeda *et al.*, 1998; Sakai *et al.*, 1998; Murata *et al.*, 1998; Sodeyama *et al.*, 1999; Kujiraoka *et al.*, 2000) populations (Table 4.1).

Table 4.1 Comparison of PON1 192R allele frequency found in the control group of the present study to that found in other populations.

Ethnicity	**Reference**	**192*R* allele frequency**
Turkish	present study	0.324
Turkish	Can Demirdöğen *et al.*, 2008	0.346
Turkish	Aynacioglu *et al.*, 1999	0.31
Turkish	Agachan *et al.*, 2005	0.37
Turkish	Karaaslan-Biyikli *et al.*, 2006	0.17
Swiss	Blatter-Garin *et al.*, 1994	0.25
	James *et al.*, 2000a	0.27
British	Mackness *et al.*, 1997a	0.26
	Mackness *et al.*, 2000c	0.25
Finnish	Antikainen *et al.*, 1996	0.26
French	Herrman *et al.*, 1996	0.30
	Helbecque *et al.*, 1999	0.28
Austrian	Schmidt *et al.*, 1998	0.25
Russian	Akhmedova *et al.*, 1999	0.26
German	Cascorbi *et al.*, 1999	0.27
Spanish	Senti *et al.*, 2000	0.30
	Ferré *et al.*, 2002a	0.25
Italian	Ombres *et al.*, 1998	0.30
Canadian Oji-Cree	Fanella *et al.*, 2000	0.24
Inuit	Fanella *et al.*, 2000	0.30
Costa Rican	Sen-Banerjee *et al.*, 2000	0.24
Thai	Phuntuwate *et al.*, 2005	0.29
Chinese	Sanghera *et al.*, 1997	0.58
	Sanghera *et al.*, 1998b	0.57
	Ko *et al.*, 1998	0.64
	Padungtod *et al.*, 1999	0.62

Table 4.1 (continued).

Ethnicity	Reference	192*R* allele frequency
Japanese	Kujiraoka *et al.*, 2000	0.34
	Murata *et al.*, 1998	0.47
	Sodeyama *et al.*, 1999	0.52
	Zama *et al.*, 1997	0.59
	Ikeda *et al.*, 1998	0.60
	Suehiro *et al.*, 1996	0.62
	Sakai *et al.*, 1998	0.69
African (African American)	Chen *et al.*, 2003	0.63

Frequency of PON1 55*L* allele in healthy controls in the present study (0.628) was the same with that found in a smaller subgroup (n=78) of the control population of the present study (Can Demirdöğen *et al.*, 2008), and more or less similar to that found in some of the European populations (Table 4.2; Malin *et al.*, 1998 and 1999; Mackness *et al.*, 2000c; Leus *et al.*, 2000). This allele frequency was however lower than found in other European populations (Jarvik *et al.*, 2000; Leviev and James, 2000a; Mackness *et al.*, 1997a; Cascorbi *et al.*, 1999) and that determined by other researchers for Turkish population (Aynacioglu *et al.*, 1999; Agachan *et al.*, 2005). PON1 55*L* allele frequency in the control population of the present study was also lower than that found in Chinese (Sanghera *et al.*, 1998b) and Japanese (Zama *et al.*, 1997; Ikeda *et al.*, 1998; Imai *et al.*, 2000; Suehiro *et al.*, 2000) populations (Table 4.2).

Table 4.2 Comparison of PON1 55*L* allele frequency found in the control group of the present study to that found in other populations.

Ethnicity	**Reference**	**55*L* allele frequency**
Turkish	present study	0.628
Turkish	Can Demirdöğen *et al.*, 2008	0.628
Turkish	Aynacioglu *et al.*, 1999	0.72
Turkish	Agachan *et al.*, 2005	0.70
Caucasian-Americans	Chen *et al.*, 2003	0.54
Finnish	Malin *et al.*, 1998	0.61
	Malin *et al.*, 1999	0.62
Dutch	Leus *et al.*, 2000	0.63
British	Mackness *et al.*, 2000c	0.63
British	Mackness *et al.*, 1997a	0.64
Caucasian	Jarvik *et al.*, 2000	0.64
Swiss	Leviev and James, 2000a	0.65
German	Cascorbi *et al.*, 1999	0.67
Caribbean	Chen *et al.*, 2003	0.71
Afro-Americans	Chen *et al.*, 2003	0.79
Chinese	Sanghera *et al.*, 1998b	0.96
Japanese	Zama *et al.*, 1997	0.91
	Ikeda *et al.*, 1998	0.94
	Imai *et al.*, 2000	0.92
	Suehiro *et al.*, 2000	0.94

The most influential of the promoter polymorphisms of PON1, –107T/C, was for the first time investigated in Turkish population in the present work. Frequency of the low expressor –107*T* allele was found to be 0.595 among the control group of the present study. As summarized in Table 4.3, this number was almost the same as the reported value for Italian (Campo *et al.*, 2004), and higher than found in Brazil (Voetsch *et al.*, 2004), North American (Brophy *et al.*, 2001b), Swiss (Leviev and James, 2000a; James *et al.*, 2000a), and Japanese populations (Suehiro *et al.*, 2000).

Table 4.3 Comparison of PON1 –107*T* allele frequency found in the control group of the present study to that found in other populations.

Ethnicity	**Reference**	**–107 *T* allele frequency**
Turkish	present study	0.595
Italian	Campo *et al.*, 2004	0.59
Swiss	James *et al.*, 2000a	0.56
	Leviev and James, 2000a	0.54
Japanese	Suehiro *et al.*, 2000	0.52
Caucasian (North America)	Brophy *et al.*, 2001b	0.50
	Chen *et al.*, 2003	0.38
Brazil	Voetsch *et al.*, 2004	0.41
Thai	Phuntuwate *et al.*, 2005	0.75
Caribbean	Chen *et al.*, 2003	0.65

Distribution of other promoter polymorphisms of PON1, such as –126, –162, –832 and –909 which are not investigated and reviewed here, might also be different in Turkish population compared to others and lead to different outcomes in gene-disease association studies.

Variability of PON1 activities in a particular population

Early work on paraoxonase 1 revealed a 10-40-fold interindividual difference in serum paraoxonase activity within a given population (Playfer *et al.*, 1976; Furlong *et al.*, 1988). In the present study, serum PON1 activities towards all three substrates used, namely paraoxon, phenyl acetate and diazoxon, exhibited wide variation. The greatest variation was observed in PON1 activities towards paraoxon (18.6 fold), followed by diazoxonase activities (16.8 fold in patients, 10.5 fold in controls). Arylesterase activity of PON1, which is accepted to be a measure of PON1 serum enzyme levels, exhibited 4X and 5X variation in patients and controls, respectively. What are the factors that add to such a wide variation between individuals? Dietary and lifestyle factors (such as smoking), physiological and pathological states (such as diabetes; see Tables 1.13 and 1.14) and functional polymorphisms in PON1 gene have documented effects on PON1 activities (Adkins *et al.*, 1993; Blatter-Garin *et al.*, 1997; Brophy *et al.*, 2001b). The allele and genotype frequencies of the most influential of these genetic polymorphisms (192Q/R, 55L/M and –107T/C) were determined for stroke patients and controls in this study. Before going into analyzing the effects of these genetic polymorphisms on PON1 activities, vascular risk factors were taken into account.

Effects of vascular risk factors on serum PON1 activities

We stratified the study population by vascular risk factors such as age, gender, hypertension, diabetes and smoking, to see whether these factors affect PON1 activities or not. By this way, any possible effects of these risk factors on enzyme activities could be minimized, and difference in PON1 enzyme activities between patients and controls could be better determined.

Oxidative stress, which is known to reduce PON1 synthesis both *in vitro* and in animal models (Shih *et al.*, 1996; Navab *et al.*, 1997), is increased with advancing age. Hence, paraoxonase and arylesterase activities were found to be significantly lower in a population aged 61 years and older (Leviev *et al.*, 2001b) and the differences in PON1 activities were dependent on differences in age (Mackness *et al.*, 2001). Therefore, the population of this study was divided into two subgroups: the elderly group (>59 years) and the younger group (<60 years). In the overall group (patients and controls combined), paraoxonase, arylesterase and diazoxonase activities of older subjects (>59 years) were lower than those of the younger group (<60 years). The differences in arylesterase and diazoxonase activities of elderly and younger subjects were statistically significant (Table 3.6). When the subjects were divided as stroke patient and control, two types of analysis could be performed (Table 3.7). First, enzyme activities of patients and controls could be compared within the two age groups created. In this analysis, paraoxonase and arylesterase activities of patients were lower than those of controls, in both age groups. Although not significant, the differences were higher in the younger group, while this difference seemed to vanish in elderly group. This effect would probably increase the susceptibility of elderly subjects who are still in the controls group to stroke. Second, enzyme activities in the two age groups could be compared within either patients or controls. As expected, all three enzyme activities of PON1 were lower in elderly patients compared to younger patients. The same was true for elderly controls' and younger controls' enzyme activities. Furthermore, the difference in diazoxonase activities between elderly patients and younger patients was statistically significant. PON1 activity ratios did not differ significantly between patients and controls, in any of the age groups (Table 3.7).

We observed that all three enzymatic activities of PON1 were lower in men when compared to women. Arylesterase activity, which is associated with enzyme levels, was significantly lower in males than in females. In a Thailander cohort, Phuntuwate and coworkers (2005) reported lower paraoxonase and arylesterase activities but higher diazoxonase activities for males. Sumegová *et al.*, 2006 also reported lower paraoxonase and arylesterase activities for males. In the present study,

when the analysis was continued with separation of the population into stroke patients and controls, arylesterase activity of male controls was found to be significantly lower than that of female controls. This may help explain the observed increased risk of stroke for males.

The effect of hypertension on serum PON1 activities was complex. Arylesterase and diazoxonase activities were lower, while paraoxonase activity was higher for hypertensives. Paraoxonase activity of hypertensive stroke patients was significantly higher than that of normotensive stroke patients. It was hypothesized that risk factors associated with atherosclerosis, such as hypertension and smoking, also promote the progression of chronic glomerulonephritides which may therefore be associated with perturbations in PON1 activity (Kovács *et al.*, 2006).

Diabetes mellitus has been extensively studied in recent years in terms of its effects on paraoxonase activity and vice versa. These studies consistently reported lower paraoxonase activities for both Type 1 (Patel *et al.*, 1990; Abbott *et al.*, 1995; Mackness *et al.*, 2002b) and Type 2 diabetes (Mackness *et al.*, 1998c; Agachan *et al.*, 2005). In the present study no distinction was made as Type 1 or Type 2 diabetes. Serum PON1 enzyme activities of diabetic individuals were found to be decreased with respect to nondiabetic subjects. Diabetic stroke patients had lower PON1 activities than the non-diabetic stroke patients. Similarly, PON1 activities of diabetic controls were lower than those of non-diabetic controls. The decrease in PON1 activities in diabetes is believed to be due to the heavy glycosylation of the PON1 protein in type 2 diabetes rather than a reduction in synthesis of the enzyme (Hedrick *et al.*, 2000b; Mackness *et al.*, 2002b). Type 1 diabetes is associated with an increase in oxidative stress (Hunt *et al.*, 1990; Hunt and Wolff, 1991), which has been shown to reduce PON1 synthesis in animal and cell culture models (Shih *et al.*, 1996; Navab *et al.*, 1997). This may account for or be a consequence of the low PON1 in type 1 diabetes.

Cigarette smoking is another mechanism that generates oxidative stress (Agarwal, 2005). Similar to the discussion on diabetes, PON1 enzyme activities were

expected to be lower in smokers. Hence, Boemi and colleagues (2004) determined decreased paraoxonase levels in smokers. In the present study, smoking was found to reduce arylesterase and diazoxonase activities of PON1 in the combined population (patients + controls). Paraoxonase activity was however higher in smokers than nonsmokers. When distinction was made as patient and control, we realized that smokers with high paraoxonase activities were controls. Smoker patients had lower paraoxonase, arylesterase and diazoxonase activities than smoker controls. Moreover, serum PON1 activities of smoker patients were lower than those of nonsmoker patients.

Statins are often the drug of choice for treating elevated cholesterol. Several small clinical trials have investigated the ability of statins to influence PON1 activity and concentration. In these studies (Tomás *et al.*, 2000; Leviev and James, 2000b; Jarvik *et al.*, 2002; Deakin *et al.*, 2003b), serum PON1 activity was found to be significantly increased in subjects treated with simvastatin. On the other hand, *in vitro* exposure of HuH7 human hepatoma cells to simvastatin caused a 25–50% decrease in PON1 activity in the culture medium and a similar decrease in PON1 mRNA (Gouedard *et al.*, 2003). In the present work, serum PON1 activities were lower in statin users than in subjects who did not use statin type of drugs (Table 3.16). Stroke patients who used statins had lower PON1 activities and PON1 activity ratio 2 than those of stroke patients who did not use statins (Table 3.17). It may be possible that statin treatment could not restore PON1 levels in stroke patients who already had an unfavourable environment (increased oxidative stress) for PON1 activity. On the other hand, paraoxonase activity of control subjects who used statins was higher than that non-statin user controls (Table 3.17).

Low PON1 activity, cause or consequence?

Lowered serum PON1 activity in stroke patients might well be a cause or consequence of the ischemic event. According to the oxidative theory of atherogenesis, oxidation of LDL has an important role in the pathogenesis of atherosclerosis (Steinberg *et al.*, 1989; Heinecke, 1998), which is, in turn, one of the

most important underlying causes of ischemic stroke (Virmani *et al.*, 2006). PON1 has antioxidative capacity and is protective against the damage due to oxidized LDL (Mackness *et al.*, 1991b). Thus, PON1 activity might have been low before the ischemic event and might have lead to plaque formation in the arterial wall due to insufficiently prevented lipid peroxidation, and in turn resulted in ischemic stroke. Another possibility is that, decreased paraoxonase activity in stroke patients was a consequence of the ischemic event; which is plausible when considering that increased oxidative stress reduces PON1 synthesis (Shih *et al.*, 1996; Navab *et al.*, 1997). Whether lowered PON1 activity is a cause or consequence of the ischemic event is a hot discussion, worth investigating in detail for the correct determination of risk factors for ischemic stroke. Low serum PON1 activity was suggested to have preceded myocardial infarction, because lower paraoxonase activity was found even within a few hours of the onset of cardiac ischemic chest pain in survivors of myocardial infarction (Ayub *et al.*, 1999). The only prospective investigation testing the relationship of PON1 status and CHD to be reported so far is the Caerphilly Prospective Study (Mackness *et al.*, 2003). In this study, PON1 activity toward paraoxon was 30 % lower in men who had a new coronary event (n=163) than in those who did not (n=1175) (Mackness *et al.*, 2003). Thus, low paraoxonase activity was found to be a predictive risk factor for subsequent coronary events (Mackness *et al.*, 2003). Future studies of prospective nature are also warranted to ascertain the role of PON1 as a risk factor for ischemic stroke.

Relationship of PON1 activities with PON1 genotypes

Dependence of paraoxonase activities on PON1 192Q/R genotypes has long been known (Ruiz *et al.*, 1995; Davies *et al.*, 1996). The mechanism of how this amino acid change could affect enzyme activity was, however, only recently explained (Harel *et al.*, 2004). The amino acid at the 192nd position of PON1 protein resides on the active site wall, and amino acid change at the number 192 probably reshapes the active site walls and perimeter, thereby improving the positioning of some substrates (and of their respective catalytic intermediates and transition states) and worsening that of others (Harel *et al.*, 2004). Another possibility is that increased

activity of 192*R* isoform is due to the linkage disequilibrium with the 55*L* allele, which is in turn in linkage disequilibrium with the high expressor allele (–107*C*) of the PON1 promoter polymorphism –107T/C. This means that, one will find *R* alleles to be associated exclusively with *L* allele, and almost no individuals would be found carrying the *R* and *M* alleles together. Such a linkage was really observed in the present study (Table 3.25) as in others (Blatter-Garin *et al.*, 1997; Brophy *et al.*, 2000).

In our sample, as expected (Davies *et al.*, 1996; Ferré e*t al.*, 2002a), 192*RR* genotype had the highest paraoxonase activities, and 192*QQ* had the lowest. The differences in paraoxonase activities between the genotype groups were significant (Table 3.18). In other words, both patients and controls were divided by their 192Q/R genotypes into three groups in terms of their paraoxon hydrolytic activities; low, intermediate and high metabolizers of paraoxon. From this observation, paraoxon appeared to be a discriminating substrate with respect to 192Q/R genotypes. The association of paraoxonase activities with the 192Q/R genotypes was also demonstrated by the strong correlation between these two parameters (Tables 3.22 and 3.23).

Arylesterase activities were also highest in *RR* and lowest in *QQ*; however, the differences between genotype groups were insignificant (Table 3.18). This finding also overlaps with previous reports stating that phenylacetate hydrolysis rates (arylesterase activity; ARE) are not polymorphic and both 192Q/R isoforms hydrolyze this substrate with equal rates (Eckerson *et al.*, 1983b; Davies *et al.*, 1996; Mackness *et al.*, 1997b; Brophy *et al.*, 2002). In other words, phenylacetate was not a discriminating substrate of 192Q/R genotypes. Thus, arylesterase activity was accepted to be a measure of the PON1 serum enzyme level, irrespective of the 192Q/R genetic polymorphism, which was also corroborated by the lack of correlation between arylesterase activity and 192Q/R genotypes (Tables 3.22 and 3.23). PON1 activity ratio 1 (PON/ARE) would then represent the real catalytic potential of PON1, since this calculation would correct for the differences in enzyme levels. PON1 activity ratio 1 was significantly different among the three genotype

classes. One could determine the 192Q/R genotype of an individual making use of his/her PON1 activity ratio 1 value, in this population.

Distribution of diazoxonase activity with respect to 192Q/R genotypic forms was the complete reverse of the paraoxonase activity; highest in *QQ* and lowest in *RR* (Table 3.18), again as expected (Davies *et al.*, 1996). There were no statistically significant differences between the genotype groups. This finding was also confirmed by the lack of correlation between diazoxonase activity and 192Q/R genotypes (Tables 3.22 and 3.23).

Paraoxonase activity of PON1 was strongly correlated with 55L/M polymorphism, within both the patient and the control populations (Tables 3.22 and 3.23). This was also evident from the significant association between paraoxonase activity and the 55L/M genotype classes, represented by the analysis of variance (ANOVA) test, with *MM* having the lowest and *LL* the highest paraoxonase activities (Table 3.19). Similar result was also obtained by Ferré and colleagues (Ferré e*t al.*, 2002a).

Serum arylesterase activity and PON1 55L/M polymorphism were strongly correlated (Tables 3.22 and 3.23). Although ANOVA test pointed to the significant association of these two parameters, arylesterase activity of PON1 could not discriminate between the 55L/M genotype classes (Table 3.19). Other studies have shown that the 55L/M substitution did not affect this activity (Adkins *et al.*, 1993; Humbert *et al.*, 1993). Diazoxonase activity and 55L/M polymorphism were weakly but significantly correlated (Tables 3.22 and 3.23). This activity of PON1 did not differ significantly between *LL*-*LM* and *LM*-*MM* genotypes (Table 3.19). ANOVA test revealed, however, that diazoxonase activity and 55L/M genotypes were significantly associated within controls.

The promoter polymorphism of PON1, –107T/C, was strongly and negatively correlated with arylesterase activity, diazoxonase activity and 55L/M polymorphism, both in controls (Table 3.22) and patients (Table 3.23). The correlation between PON

and –107T/C was strong in patients and weak in controls. The risky genotype for –107T/C polymorphism, –107*TT*, has been associated with the lowest serum enzyme levels (Brophy *et al.*, 2001b). In the present work, PON1 enzyme activities were highest in individuals with *CC* and lowest in *TT* (Table 3.20), as expected. This is completely the reverse case of 192Q/R and 55L/M polymorphisms, for which the high activity (towards paraoxon) alleles were the risky ones. For this reason, correlations of –107T/C polymorphism with the other parameters tested came out to be negative. As previously stated, both arylesterase activity and 55L/M are accepted to be measures of PON1 serum enzyme levels. Thus, the strong correlation of these parameters with the –107T/C promoter region polymorphism is logical.

Paraoxonase activity of PON1 was associated well with the three PON1 genotypes in double combinations (Table 3.21). In addition, paraoxonase activities of patients with *QQMM*, *QRLM*, *QQLL*, *QRLL*, *QQTT*, *QRTC*, *RRTC*, *RRCC*, *MMTT*, *LMTT*, *LLTT*, *LMTC*, *LLTC*, *LLCC* genotypes were lower than those of the respective controls. None of the differences between patients and controls were, however, statistically significant (Table 3.21). No individual was found carrying *RRMM* and *RRLM* genotypes (Tables 3.21 and 3.25), as in previous studies (Ferré *et al.*, 2002a).

Comparison of PON1 activities of stroke patients and controls according to their genotype group

As can be inferred from Figures 3.11, 3.12 and 3.13, paraoxonase, arylesterase and diazoxonase activities of stroke patients were in general lower than those of controls, irrespective of the genotype group they belong to. Exceptions were arylesterase activity of *QQ* and *TC*, and diazoxonase activity of *RR* and *TC*. In each genotype group, there were large variations in enzyme activities and those having lower PON1 activities were stroke patients. Similar results in stroke patients and controls were also obtained by Aydin *et al.*, 2006.

Although we tried to assign which of the PON1 genotypes might be a risk factor for ischemic stroke by stratifying the enzyme activities of the population with respect to their PON1 genotypes, these attempts might be rather insufficient. This was because there were large variations in PON1 enzyme activities within each genotype group, which could be appreciated from the large standard deviation values and visualized from Figures 3.11-3.13. This finding is in agreement with other studies which reported that there was at least a 13-fold variation in PON1 activity among individuals with the same genotype (Humbert *et al.*, 1993; Davies *et al.*, 1996; Richter and Furlong, 1999). What causes different enzyme activities in the same genotype group? Clearly, other modifications as to the enzyme activity must exist within each genotype group. These modifications may result from exogenous effectors such as smoking (James *et al.*, 2000b), diet (Debord *et al.*, 1998; Aviram *et al.*, 2000a) and drugs (Tomás *et al.*, 2000; Blatter-Garin *et al.*, 2003), as well as intrinsic properties such as physiological and pathological conditions including pregnancy (Geldmacher-Von Mallinckrodt and Diepgen, 1988) and diabetes (Abbott *et al.*, 1995; Mackness *et al.*, 1998c and 2002; Agachan *et al.*, 2005), and polymorphisms that affect expression levels of the PON1 protein (Brophy *et al.*, 2001b). Moreover, the possibility that these influences are likely to interact with each other to produce further variation should not be discounted. Thus, this analysis was far from unrevealing risk factors for stroke. A more sophisticated analysis, which brings vascular risk factors and parameters related to PON1 genotypes and/or activities together, such as logistic regression analysis, would probably help.

Association of PON1 genotypes with stroke risk

The capacity of PON1 allozymes to protect LDL from oxidation is the complete reverse of that of paraoxon hydrolytic activity. Paraoxon hydrolytic activity is greatest with purified PON1 from PON1 192*RR* individuals and least with PON1 192*QQ* individuals (Adkins *et al.*, 1993; Davies *et al.*, 1996; Mackness *et al.*, 1997b). On the other hand, 192*QQ* individuals have PON1 associated with the greatest protective capacity against oxidation of LDL (Mackness *et al.*, 1997c; Aviram *et al.*, 1998a; Mackness *et al.*, 1998d; Durrington *et al.*, 2001). Aviram and

colleagues (1998a) observed that the *Q* isoform is more efficient than *R* at protecting LDL from oxidation and that *Q* is more stable than *R*. Furthermore, this group found in sections of atherosclerotic human carotid artery that PON1 192*Q* reduces the lipid peroxide content by 27% after 24 hours of incubation compared to 16% for PON1 192*R* (Aviram *et al.*, 2000b). Also the incubation resulted in inactivation of PON1 192*Q* by 15% but PON1 192*R* by 45% (Aviram *et al.*, 2000b). These observations constituted the biological basis for the numerous population studies which investigated the *R* isozyme of PON1 as a risk factor for coronary and carotid vascular diseases. Cao and colleagues (1999) have reported, on the other hand, that protective capacity of HDL from *QQ* and *RR* diabetic patients towards peroxidation of LDL by Cu^{2+} were similar. In our opinion, to be more convincing such a study should have employed HDL from non-diabetic individuals. This is because increased glycosylation of the PON1 protein that occurs in type 2 diabetes (Hedrick *et al.*, 2000b; Mackness *et al.*, 2002b) might have had affected the result.

Nevertheless, the coding region 192Q/R polymorphism has been reported in some studies to be an independent risk factor for cardiovascular disease (Ruiz *et al.* 1995; Serrato and Marian, 1995; Zama *et al.*, 1997; Odawara *et al.* 1997; Garin *et al.* 1997; Sanghera *et al.*, 1997; Sanghera *et al.*, 1998b; Pati and Pati, 1998; Salonen *et al.,* 1999; Imai *et al.*, 2000; Senti *et al.*, 2000; Aubó *et al.*, 2000), although this could not be confirmed in other studies (Herrmann *et al.*, 1996; Suehiro *et al.*, 1996; Ko *et al.*, 1998; Hasselwander *et al.*, 1999; Aynacioglu and Kepekci, 2000; Heijmans *et al.*, 2000). Shih *et al.*, 2002 suggested that the failure to observe significant results could be explained by the complex nature of coronary heart disease. The failure to observe significant results could also be attributed to differences in ethnic populations examined. Allele frequencies differ considerably between populations, and there may well be gene-gene and gene-environment differences among these populations that influenced the results of the association studies. Most of the studies involved a fairly large number of individuals, but some negative results may have resulted from an insufficiently large sample size. Given the complexity of coronary heart disease, it is expected that a large sample size would be required to detect even major genetic factors (Shih *et al.*, 2002). However, there exists a difference in the constitution and

environment between coronary and cerebral arteries. Many factors affect the development of atherosclerosis, but these factors and the importance of each differ between cerebral and coronary arteries (Ueno *et al.*, 2003). Therefore, it would be better to compare our results with those carried out in stroke.

In this study, the coding region 192Q/R polymorphism of PON1 did not exhibit significant variability in its distribution between stroke patient (192*R*=0.337) and control groups (192*R*=0.324; *P*=0.536; Table 3.24). The frequency of 192*R* allele was previously found to be 0.361 in patients (n=108) and 0.341 in controls (n=78) in a smaller subgroup of the study population of the present investigation (Can Demirdöğen *et al*., 2008). In the present study, when both the patient and control populations were enlarged, neither the *RR* genotype, nor the *QR*+*RR*, was predictive of stroke vs. control status by use of logistic regression. Our results are consistent with the findings of Ueno *et al.* (2003) and Pasdar *et al.* (2006) that this gene polymorphism of PON1 did not have a role in the pathogenesis of stroke. In the Austrian stroke prevention study, Schmidt and coworkers (1998) also failed to detect a significant association between the 192Q/R polymorphism and carotid disease, although atherosclerotic lesions were more common in *RR* than in *QR* or *QQ* carriers. On the other hand, 192*RR* or 192*R*+ genotypes were identified as risk factors for ischemic stroke in other studies (Imai *et al.*, 2000; Voetsch *et al.*, 2002; Ranade *et al.*, 2005; Baum *et al.*, 2006). See Table 4.4 for comparison of 192*R* allele frequencies in stroke patients and controls found in the present study with other studies.

Table 4.4 Comparison of PON1 192Q/R, 55L/M and –107T/C allele frequencies of stroke patients and controls found in the present study to those found in other studies.

Allele	Patient	Control	*P*	Study
192*R*	0.337	0.324	0.536	Present
	0.361	0.341	0.483	Can Demirdöğen *et al.*, 2008
	0.290	0.290	0.890	Pasdar *et al.*, 2006
	0.410	0.280	0.0001	Ranade *et al.*, 2005
	0.620	0.600	0.578	Ueno *et al.*, 2003
	0.430	0.310	0.010	Voetsch *et al.*, 2002
	0.750	0.650	0.0002	Imai *et al.*, 2000
55*L*	0.690	0.628	0.003	Present
	0.690	0.628	0.003	Can Demirdöğen *et al.*, 2008
	0.640	0.630	0.73	Pasdar *et al.*, 2006
	0.660	0.637	0.538	Ranade *et al.*, 2005
	0.900	0.960	0.013	Ueno *et al.*, 2003
	0.680	0.680	0.920	Voetsch *et al.*, 2002
	0.930	0.920	0.729	Imai *et al.*, 2000
–107*T*	0.608	0.595	0.553	Present
	0.380	0.410	0.570	Voetsch *et al.*, 2004
	0.471	0.480	0.829	Ranade *et al.*, 2005

In 2001, Brophy and colleagues described a linkage disequilibrium between the –107*C* and the 192*R* alleles (Brophy *et al.*, 2001b). Although 192Q/R polymorphism is independently associated with variance in enzyme activity, it is possible that the –107*C* may partly compensate for the lower protection of PON1 192*R*, complicating the relationship between PON1 genotype and disease (Brophy *et al.*, 2001b; Deakin and James, 2004). It is thus possible that the differences in –107*C* allele frequency (Table 4.3), level of linkage disequilibrium with the 192*R* allele and the level of compensation for the lower protection of 192*R* between the populations studied vary around the world, which may account for the differences observed in the genetic association studies.

In the present work, frequency of the 55*L* allele of PON1 was significantly increased among patients (0.690) compared to controls (0.628; *P*=0.003; Table 3.24). Logistic regression analysis revealed PON1 55*LL* genotype to be associated with a 1.783-fold increase in the risk of ischemic stroke versus control status (Table 3.33; model 1). In the second model (model 2), when PON1 activity ratios were included instead of separate PON1 activities, PON1 activity ratio 1 (PON/ARE) was found to be a risk factor for stroke. Thus, in model 1, 55*LL* genotype seems to substitute arylesterase activity. This is quite plausible in that 55L/M polymorphism has been associated with the variability of PON1 levels present in plasma (Brophy *et al.*, 2002), like arylesterase activity. However, individuals carrying the *M* allele of 55L/M polymorphism, not the *L*, were found to have lower levels of PON1 activity (Blatter Garin *et al.*, 1997; Brophy *et al.*, 2000, 2001a, b), of circulating PON1 (Blatter Garin *et al.*, 1997; Mackness *et al.*, 1998c), and lower levels of PON1 mRNA (Leviev *et al.*, 1997). These observations have been suggested to be mostly due to the linkage disequilibrium between 55*L* allele and the –107 regulatory-region polymorphism (Suehiro *et al.*, 2000; Brophy *et al.*, 2001b), as well as due to the key role of 55*L* in correct packing of the protein (Harel *et al.*, 2004; Deakin and James, 2004). In the present study and in others as well, the reason of selecting the *L* isoform to be the risky allele was the observation by several researchers (Mackness *et al.*, 1997c; Aviram *et al.*, 1998a; Mackness *et al.*, 1998d) that 55*MM* individuals

have PON1 associated with the greatest protective capacity against LDL oxidation, and 55*LL* have the lowest.

PON1 55*LL* genotype was found to be associated with the extent and progression of white matter lesions and the presence and severity of carotid disease in the Austrian stroke prevention study (Schmidt *et al.*, 1998 and 2000). Fortunato and colleagues (2003) have also demonstrated that PON1 55(*LL*-*ML*) is associated with plaques both at the bifurcation and at the common carotid artery, and to the total number of plaques at any site. Surprisingly, a significant increase of the 55*M* allele in Japanese cerebral infarction subjects compared with controls was also found (Ueno *et al.*, 2003). On the other hand, in other studies (Voetsch *et al.*, 2002; Ranade *et al.*, 2005; Pasdar *et al.*, 2006) an association between the 55L/M polymorphism and stroke could not be found (Table 4.4). The inconsistency of results might be due to differences in the life styles (smoking, diet, exercise) and ethnicities of the populations studied (Table 4.2). For example, although PON1 genotype was not associated with intima media thickness in smokers (Van Himbergen *et al.*, 2004), in nonsmoking men, it was reported that a PON1 55*LL* genotype may represent a genetic risk factor for carotid artery atherosclerotic disease (CAAD; Malin *et al.*, 2001). The reverse effect in smokers implies that the ability of PON to protect against CAAD is influenced by cigarette smoking. The efficiency of this inhibition probably depends on the PON 55L/M genotype (Malin *et al.*, 2001).

The promoter polymorphism –107T/C was less studied in relation to ischemic stroke, when compared to the coding region polymorphisms. Given the importance of PON1 for the prevention of lipid peroxidation of LDL and HDL (Mackness *et al.*, 1991b; Mackness *et al.*, 1993b) and approximately 23% contribution of this polymorphism to the variances in PON1 levels (Brophy *et al.*, 2001b), we expected to observe a significantly higher prevalence of low expressor genotypes (–107*TT*) or low expressor allele (–107*T*) among stroke patients. However, the differences in the frequencies of low expressor allele –107*T* and the low expressor genotype –107*TT* between stroke patients and controls were insignificant (Table 3.24). Comparison of this allele frequency with that found in other studies is given in Table 4.4. No

significant differences were found between the –107*T* allele frequencies of stroke patients and controls in previous studies (Voetsch *et al.*, 2004; Ranade *et al.*, 2005). As will be given in the section "risk factors in the elderly" below, in the present study, PON1 −107*TT* genotype was found to be associated with approximately 2 times increased risk of ischemic stroke in the elderly group (60 years and older; Table 3.42). Leviev *et al.*, 2001b, however, reported that the high expressor genotype (–107*CC*) was associated with decreased risk of coronary disease in patients aged 60 years or under, but not in the elderly.

Distribution of double combined haplotypes in stroke patients and controls did not differ significantly (Table 3.25), however, prevalences of two risky genotypes together (*RRLL*, *RRTT* and *LLTT*) was increased among patients compared to controls. *RRLL* genotype was also more common among stroke patients compared to controls in a Brazilian (Voetsch *et al.*, 2002) and a Japanese cohort (Ueno *et al.*, 2003). In the present investigation, it was noteworthy to find that no individual exists carrying *R* and *M* alleles together, confirming the linkage disequilibrium observed in previous studies (Blatter-Garin *et al.*, 1997; Brophy *et al.*, 2000; Ferré *et al.*, 2002a). Voetsch and colleagues (2002), however, found 5 stroke patients carrying the *RRLM* genotype.

Prevalence of triple combined heterozygote haplotype (*QRLMTC*) was significantly lower in stroke patients (4.1%) when compared to controls (11.4%; *P*=0.019; Table 3.26). This parameter came out to be a significant protective factor against stroke by use of logistic regression. Having this haplotype was associated with a 4.587 fold increased protective effect against stroke (Table 3.38). When lipid parameters were added to the logistic regression analysis, protective effect of PON1 *QRLMTC* haplotype was increased from 4.587 to 5.650 (Table 3.39). When the analysis was restricted to individuals who did not use statin drugs, protective effect of PON1 *QRLMTC* genotype was increased from 5.650 to 6.944 (Table 3.40). This finding overlaps with our previous result (Can Demirdöğen *et al.*, 2008) where we have reported that the heterozygote genotype of the 192Q/R polymorphism (192*QR*), but not 192*QQ*, is more protective against stroke in risk groups. This suggestion

complies with the background that 192*QQ* individuals tend to have lower paraoxonase activity (Adkins *et al.*, 1993), although their paraoxonase 1 enzyme has high potency to neutralize oxidized lipids (Mackness *et al.*, 1998d; Aviram *et al.*, 1998a). Paraoxonase 1 of 192*RR* individuals, on the other hand, despite being at high levels due to linkage disequilibrium with the high expressor –107*C* allele (Blatter-Garin *et al.*, 1997; Brophy *et al.*, 2001b), has a lowered capacity to hydrolyze oxidized lipids (Mackness *et al.*, 1998d; Aviram *et al.*, 1998a). Thus, 192*QR* heterozygotes, having the advantage of both higher capacity to hydrolyze oxidized lipids (than *RR*) and chance of maintaining higher enzyme levels in the serum (than *QQ*), might have a higher degree of protection against ischemic stroke. Similar discussion would also be valid for the heterozygote genotypes of 55L/M and –107T/C polymorphisms. However, as will be stated below, a similar significant protective effect of heterozygote genotypes was not realized in the risk groups in the present study.

Further analysisof PON1 genetic polymorphisms in risk groups

We analyzed the effects of risk factors, hypertension, smoking and diabetes, on ischemic stroke among 192Q/R, 55L/M and –107T/C genotypes and the risky alleles. The proportion of stroke patients to controls was increased in hypertensive, smoker or diabetic individuals compared to normotensives, nonsmokers or nondiabetics, irrespective of the genotype group (Tables 3.27, 3.28 and 3.29). For hypertension, the proportion of hypertensive stroke patients to hypertensive controls compared to the proportion of normotensive stroke patients to normotensive controls (odds ratio) was highest in the 192*RR* group (OR=7). The risk of hypertensive individuals having ischemic stroke decreased to 2.182-fold in 192*QR* heterozygotes, and to insignificant levels in 192*QQ* individuals. PON1 192*R* allele represented 1.554 times increased risk for ischemic stroke in hypertensives compared to normotensives (Table 3.30). Ranade and coworkers (2005) also analyzed 192*R* allele frequency in hypertensives. Although they did not report odds ratio values, we calculated this parameter from their data. Accordingly, 192*R* allele represented a significant 1.909 fold risk (P=0.028) in hypertensives and did not represent a significant risk in normotensives

(Ranade *et al.*, 2005). Baum and coworkers (2006) reported that hypertension increased the association of *R*+ genotypes with stroke among hypertensive subjects compared to normotensive subjects. *R* alleles were marginally negatively associated with hypertension among controls. Among the 55L/M genotypes, the risk of stroke for hypertensive individuals was highest in 55*LL* (OR=2.66). 55*L* allele of PON1 was associated with a 1.66 times increased risk of stroke in hypertensives (Table 3.31). This allele was not, however, associated significantly to stroke risk in normotensives. As expected, the low expressor genotype for the –107T/C promoter polymorphism, –107*TT*, was found to be the most risky for stroke among hypertensives (OR=2.89). Although PON1 −107*T* allele also represented a small (1.345-fold) but significant (*P*=0.001) risk for stroke in hypertensives, this allele was not associated with stroke risk in normotensive individuals (Table 3.32). Thus, 192*RR*, 55*LL* and –107*TT* genotypes seem to be the most risky for stroke among hypertensive individuals. Furthermore, the risk associated with 192*RR* was highest, followed by –107*TT* and 55*LL*. There is evidence that hypertension may exert oxidative stress on the arterial wall (Alexander, 1995). This increased oxidative stress presumably constitutes an additional problem in hypertensive individuals carrying the 192*RR* genotype, due to the lower efficiency of the 192*R* isoform in preventing oxidation of LDL and its lower stability compared to 192*Q* (Aviram *et al.*, 1998a). Same argument would be valid for the 55*L* isoform, as it was found to have lower efficiency in preventing lipid peroxidation of LDL, and −107*T* allele, due to the lower expression levels of PON1 protein associated with this promoter polymorphism.

For diabetes, the only genotype associated with a significant risk of stroke was 192*QR* (Table 3.27). PON1 192*R* allele constituted a higher risk of stroke (1.546) in diabetics than in hypertensives (1.233; Table 3.30). 55*L* allele of PON1 was associated with a 2.6 times increased risk for stroke in diabetics compared to non-diabetics. Note that risk associated with the 55*L* allele of PON1 was higher than that of 192*R* allele in both hypertensives and diabetics. −107*T* allele of PON1 was not significantly associated with stroke in neither diabetics nor non-diabetics. Although

an increased proportion of stroke patients to controls were found among smokers in all of the genotype groups studied so far, none of the risks were significant.

Risk Factors in the Elderly

Ageing is associated with a shift in the redox equilibrium towards greater oxidative stress (Fukagawa, 1999) and LDL concentrations increase with age (Rifkind and Segal, 1983). PON is sensitive to oxidative stress and can be inactivated by the presence of excessive LDL peroxidation products (Aviram *et al.*, 1999). In this study, we measured lower paraoxonase, arylesterase and diazoxonase activities in the elderly group aged 60 years and older than in the respective younger group (Table 3.6). The differences in arylesterase and diazoxonase activites were significant. Leviev and coworkers (2001b) have also found significantly lower paraoxonase and arylesterase activities and PON1 concentrations in the older age group (61 years or over).

Interestingly, 192*RR* genotype frequency was diminished in the elderly population (11.6 %) compared to younger group (14 %). Obviously, there is reciprocity between age and 192*RR* prevalence. In other words, if age and 192*R* allele are considered as risk factors for stroke, one would expect to see increased frequency of 192*R* allele in the elderly. However, what we and others have observed is the complete reverse of this situation. One possible explanation for these confusing results comes from the so-called "age-risk paradox" (Baum *et al.*, 2006). Weakening of the association between disease and individual risk factors as the age progresses is well known (Leviev *et al.*, 2002). The greater the impact of a risk factor, the younger the age at which the risk factor starts to seem protective in a case-control study (Baum *et al.*, 2006). Such a reversal effect was also observed in our study. This age-risk paradox partly results from a survival bias in case-control studies. To circumvent this problem, further studies into the role of PON1 in ischemic stroke and any other disease for which age is a risk factor should be designed on a prospective basis.

Within a subgroup of elderly subjects who did not use statin drugs, in addition to hypertension, smoking, total cholesterol and LDL-cholesterol, PON1 −107*TT* genotype was found to be a significant predictor of ischemic stroke (Table 3.36). It was challenging to realize that the low expressor genotype −107*TT* was associated with a 1.973 times increased risk for stroke in elderly, although it was not associated with stroke in the overall population. When the statin users were included into the model, PON1 −107*TT* genotype was no more a predictor of stroke (data not given). Obviously, the fact that PON1 levels decrease with advancing age (Leviev *et al.*, 2001b), intensified the effect of the low expressor genotype. In addition, any effect of the low expressor PON1 107*TT* genotype would have on stroke risk might have been compensated for by the statins (Deakin *et al.*, 2003b) in the elderly group. However, what Leviev and coworkers (2001b) have observed was completely the reverse of our finding. They found that the high expressor −107*CC* genotype was associated with a decreased risk of coronary disease in patients less than 60 years. The −107 polymorphism was not associated with risk in older patients (61 years or over) (Leviev *et al.*, 2001b). In the present study, PON1 *QRLMTC* haplotype had the highest protective effect (10.4-fold) in this subgroup of statin non-user elderly subjects (Table 3.41). Despite finding smoking as a risk factor as in the overall population, the effect of this parameter was increased in the elderly group.

Lack of significant associations between ischemic stroke and certain PON1 genetic polymorphisms can be explained by the complex nature of ischemic stoke, which is clearly influenced by many different genetic factors. Another contributing factor to the lack of association in Turkish population might be differences in allele frequencies of this population and others. Gene-gene and gene-environment differences (Brophy *et al.*, 2002) might have been present between Turkish population and others that influenced the results of our study.

A potential limitation of the present investigation might be that PON1 mass was not measured, thus the influence of PON1 genetic polymorphisms on PON1 concentration has not been investigated. Nevertheless, a recent study showed that a higher PON1 concentration was not statistically related to coronary heart disease risk

whereas PON1 activity towards paraoxon was statistically related to this risk (Mackness *et al.*, 2003).

Conclusion

In conclusion, this work, for the first time, identified PON1 activity ratio and PON1 *QRLMTC* heterozygote genotype as significant protective factors for ischemic stroke. PON1 55*LL* genotype was associated with a 1.8 fold increase in stroke status. In addition, 55*L* allele of PON1 was associated with a 1.66 and 2.6 times increased risk of stroke in hypertensives and diabetics, respectively. PON1 192*R* allele represented around 1.5 times increased risk for ischemic stroke in hypertensives and diabetics. Furthermore, the low expressor genotype −107*TT* was associated with almost 2 times increased risk for stroke in elderly. Further studies of prospective nature employing larger populations are still warranted to confirm and extend the results.

CHAPTER V

CONCLUSIONS

Human serum paraoxonase (PON1) is a polymorphic esterase that is associated with HDL in plasma. Due to its role in preventing oxidation of HDL and LDL, serum PON1 has been extensively studied in relation to its role in cardiovascular diseases. Ischemic stroke, which has a similar pathology, was however less studied. Moreover, majority of studies in the field were genetic association studies and did not employ measurement of enzyme activity or concentration. Therefore in this study we tested three PON1 activities in addition to three genetic polymorphisms as risk factors for ischemic stroke.

The study population was comprised of 172 acute ischemic stroke patients and 105 symptom free controls. The two study groups did not differ significantly in terms of age and gender. PON1 activities towards all three substrates used in the present study (paraoxon, phenyl acetate and diazoxon) were lower in stroke patients compared to controls. Paraoxonase activity (PON) and PON1 activity ratio 1 (PON/ARE) were negatively associated with ischemic stroke by use of logistic regression analysis. PON/ARE was 1.26 times protective against stroke. Even though other researchers (Jarvik *et al.*, 2000 and 2003; Aydin *et al.*, 2006; Kim *et al.*, 2007) have also found low PON1 activity as a risk factor for vascular disease, this is the first time PON1 activity ratio is demonstrated to be a protective factor for ischemic stroke. In addition, PON1 enzyme activities differed in subgroups created within the study population. Arylesterase and diazoxonase activities of elderly subjects were

significantly lower than those of younger subjects. Arylesterase activity, which is associated with enzyme levels, was significantly lower in males than in females.

The allele frequencies of the so-called risky alleles of the coding region polymorphism 192Q/R and the promoter polymorphism −107T/C were found to be slightly higher in stroke patients compared to controls (192*R*=0.337 in patients and 0.324 in controls, *P*=0.536; −107*T*=0.608 in patients and 0.595 in controls, *P*=0.553). Frequency of the 55*L* allele of PON1 was significantly increased among patients (0.690) compared to controls (0.628; *P*=0.003). Logistic regression analysis revealed PON1 55*LL* genotype to be associated with a 1.783-fold increase in the risk of ischemic stroke versus control status. Prevalence of triple combined haplotype *QRLMTC* was significantly lower in stroke patients (4.1%) when compared to controls (11.4%; *P*=0.019). Triple combined heterozygote haplotype (*QRLMTC*) had around 7 times increased protective effect against stroke, within a subgroup of individuals who did not use statins.

192*R* allele of PON1 represented 1.554 times increased risk for ischemic stroke in hypertensives relative to normotensives. Furthermore, the risk of hypertensive individuals having ischemic stroke was highest in the 192*RR* group (OR=7). The risk decreased to 2.182-fold in 192*QR* heterozygotes, and to insignificant levels in 192*QQ* individuals. 192*R* allele of PON1 constituted a 1.546 times increased risk in diabetics. PON1 55*L* allele was associated with a 1.660 times increased risk of stroke in hypertensives. This allele was not associated with stroke risk in normotensives. 55*L* allele of PON1 constituted a 2.6 times increased risk for stroke in diabetics relative to non-diabetics. PON1 −107*T* allele also represented a significant risk for stroke in hypertensives. The low expressor genotype −107*TT* was associated with almost 2 times increased risk for stroke in elderly.

At one time, atherosclerosis was thought to be a degenerative disease that was an inevitable consequence of aging. Research in the last two decades has shown that atherosclerosis is neither a degenerative disease nor inevitable. Recently, it was suggested that adenovirus-mediated gene transfer of human PON1 may be a potential

and useful tool to prevent/retard atherosclerosis in humans (Mackness *et al.*, 2006). The importance of further work in this area is that dietary or pharmacological interventions which will significantly increase PON1 activity may be discovered. These may prove to have application in the prevention of arteriosclerosis and make it possible to test the oxidant theory of atherosclerosis (Durrington *et al.*, 2002).

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APPENDIX A

I) INFORMED CONSENT FOR ISCHEMIC STROKE 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 faktörlerin tespit edilmesinin öğrenilmesinde yararlı olacaktır. Şu anda bu çalışmanın hemen size bir fayda olarak dönüp dönmeyeceğini bilmiyoruz. Ancak ilgili hastalığın temelinde yatan nedenlerin öğrenilmesinde ve gelecekte yeni tedavi yaklaşımlarının geliştirilmesi, bu hastalık geçirme riski olan hastaların önceden tespit edilmesi ve belki de hastalık geçirmeden önce önlem alınmasında fayda sağlayacaktır.

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

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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

II) 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 hatalık 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 1ve benzeri enzimlerin aktivitesi ve genetik durumu incelenecektir. Yapacağımız çalışma daha önce temelde aynı mekanizmaya dayanan kalp krizi için yapılmış ve anlamlı sonuçlar bulunmuştur. Bu işlem için sizden 2 tüp 10 ml kan alınacak ve çalışmalar buradan yapılacaktır. Kan alımı sizin hastalığınızın klinik takibi sırasında alınacak kanlar ile birlikte alınacak ve size ek bir işlem yapılmayacaktır. Sizden 2 tüp kan alımı dışında her hangi bir işlem veya bu çalışmayla ilişkili ek bir tedavi yapılmayacaktır. Araştırma sırasında oluşabilecek herhangi bir zararlı durumu yoktur. Sizden sadece kan alınacaktır. Araştırmaya gönüllü olarak katılmaktasınız ve araştırmaya katılmakta tamamen serbestsiniz. Çalışmada yer alacak gönüllü sayısı yaklaşık 150 hasta ve 150 sağlıklı kişi olacaktır. Çalışmada yer aldığınız ve bilimsel gelişmelere katkılarınızdan dolayı teşekkür ediyoruz.

Açıklamaları Yapan Araştırmacının
Adı, Soyadı;
Görevi,
İmzası

Açıklamayı başından sonuna kadar tanıklık eden kişinin
Adı, Soyadı;
Adresi:
İmzası

Çalışmaya katılan gönüllünün
Adı, soyadı:
Adres:
İmzası

APPENDIX B

APPROVAL FROM ETHICAL COMMITTEE

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'den Prof. Dr. Oktay Varal'ın sorumlu araştırıcılığını yaptığı **"Paraoksonaz 1'in Aktivite ve Gen Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** başlıklı, çok merkezli, risk faktörü ve yetkinlik çalışması olan araştırma dosyası değerlendirildi. Araştırma dosyasının amaç, yöntem ve yaklaşım bakımından etik ilkelere UYGUN olduğuna karar verildi.

BAŞKAN
Hamdullah AYDIN
Prof.Tbp.Kd. Alb.

ÜYE
İsmet ARSLAN
Prof. Hv. Tbp. Kd. Alb.

ÜYE
Prof.Tbp.Kd.Alb.

ÜYE
Ali Oktay ÜRAL
Prof.Tbp.Kd.Alb.

ÜYE
Adnan ATAÇ
Doç.Dr.Ecz.Kd.Alb.

ÜYE
Turgay ÇELİK
Doç. Tbp. Alb.

ÜYE
Doç. Tbp.Alb.

ÜYE
Emin ÖZTAŞ
Doç. Hv. Tbp. Bnb.

ONAY

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

APPENDIX C

LIST OF CHEMICALS AND THEIR SUPPLIERS

Chemical	Catalog no	Supplier
Acetone	24201	Riedel de Haën, Seelze
Absolute ethanol	32221	Merck, Darmstadt, Germany
Agarose		Prona, Madrid, Spain
Borate	11607	Merck, Darmstadt, Germany
Bromophenol blue	B-5525	Sigma Chemical Company, Saint Louis, Missouri, USA.
BspPI (*AlwI*)	#ER1321	MBI Fermentas, USA
Diazinon-O-analog	MET90A	Chem Service, Inc. West Chester, PA, USA
dNTP mix	#R0191	MBI Fermentas, USA
Ethidium bromide	E-7637	Sigma Chemical Company, Saint Louis, Missouri, USA.
Ethylene diamine tetra acetic acid disodium salt (EDTA)	E-5134	Sigma Chemical Company, Saint Louis, Missouri, USA.
Gene RulerTM 50 bp DNA Ladder	#SM0371	MBI Fermentas, USA
Hin1II (*NlaIII)*	#ER1831	MBI Fermentas, USA
MbiI (*BsrBI*)	#ER1271	MBI Fermentas, USA
Paraoxon (Paraoxon-ethyl)	D9286	Sigma Chemical Company, Saint Louis, Missouri, USA.
Phenyl-acetate	108723	Aldrich Chemical Co., Milwaukee, USA.
Primers		Thermo Electron BmbH, Germany.
Sodium chloride (NaCl)	S-3014	Sigma Chemical Company, Saint Louis, Missouri, USA.
Sodium dodecyl sulfate (SDS)	L-4390	Sigma Chemical Company, Saint Louis, Missouri, USA.
2-amino-2(hydroxymethyl)-1,3-propandiol (Tris)	T-1503	Sigma Chemical Company, Saint Louis, Missouri, USA.
Taq DNA Polymerase	#EP0407	MBI Fermentas, USA

APPENDIX D

LIST OF STUDY POPULATION

Table A.1 List of study population composed of 172 stroke patients and 105 controls including demographic characteristics, lipid parameters, PON1 genotypes and PON1 activities. M: male; F: female; Y: yes; N: no; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; PON: paraoxonase activity; ARE: arylesterase activity; DIA: diazoxonase activity.

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	-107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
1	Patient	75	M	Y	Y	Y	N	N	124	114	72	24	QR	LM	TC	332.1	108	13927
2	Patient	57	F	Y	Y	N	N	N	210	136	132	51	QQ	LL	TT	89	60	12238
3	Patient	41	M	N	N	Y	N	100%	185	140	104	34	RR	LL	TT	353.6	74.5	8178.2
4	Patient	73	M	Y	N	N	N	N	143	64	74	57	QQ	MM	TT	58.7	49	7841.6
5	Patient	53	M	Y	Y	N	N	N	401	231	296	59	RR	LL	TT	467.2	108.7	10468.7
6	Patient	66	M	Y	Y	Y	N	N	130	126	85	20	RR	LL	TT	214.5	80	5676.6
7	Patient	84	F	Y	N	N	N	N	145	59	70	63	QQ	LL	TT	210.7	77	15313.6
8	Patient	56	F	Y	Y	N	N	N	138	212	68	38	QQ	LL	CC	118.3	137.4	16369.7
9	Patient	54	M	Y	N	Y	Y	50%	200	217	115	42	QR	LM	TC	298.1	129	10613.9
10	Patient	67	F	Y	N	N	N	N	209	106	139	49	QQ	LM	TC	104.2	103	12950.5
11	Patient	76	M	Y	N	Y	N	N	155	68	91	50	QR	LL	TC	253.7	123	11881.2
12	Patient	78	F	Y	Y	N	Y	50%	142	137	79	36	QR	LL	TC	420.1	94.5	15325.5

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
13	Patient	75	F	Y	N	N	N	N	182	104	127	34	QR	LL	CC	267	106	8052.8
14	Patient	74	F	Y	Y	N	N	50%	167	62	107	48	QR	LM	TT	160.5	60	3894
15	Patient	68	F	Y	N	Y	N	N	140	202	75	25	QQ	LL	TC	157.2	74	5676.6
16	Patient	72	F	N	N	N	N	N	256	253	165	40	QR	LL	TT	292.1	134.5	3498.3
17	Patient	84	M	Y	N	N	N	N	159	173	93	31	QR	LL	CC	330.3	128.5	7656.7
18	Patient	81	F	Y	N	N	N	70%	231	156	115	42	RR	LL	TC	317.7	91.5	7920.8
19	Patient	73	F	Y	Y	N	N	N	207	202	125	42	QR	LM	CC	379.2	160	18613.9
20	Patient	73	F	Y	N	N	N	N	180	88	107	55	QR	LM	TT	362.1	156.7	17821.8
21	Patient	67	F	Y	N	N	N	N	208	124	143	40	QQ	LM	TT	113.3	106	5564.4
22	Control	69	F	N	N	N	N	N	244	145	91	74	QR	MM	TT	118.3	57.3	8184.8
23	Control	71	M	Y	N	N	N	N	209	119	82	42	QQ	LL	CC	167.3	152	23168.3
24	Patient	61	M	Y	Y	N	N	100%	219	122	108	37	RR	LL	TC	439.2	129	7445.5
25	Patient	40	M	N	N	Y	N	N	187	104	78	47	QR	LL	CC	371.3	74	9670
26	Control	61	F	Y	N	N	N	N	242	83	46	68	QR	LL	CC	305.2	100	11841.6
27	Patient	60	F	Y	Y	N	N	90%	192	150	119	43	QR	LM	TT	282.5	74	10693
28	Patient	86	M	Y	N	Y	N	50%	177	90	113	46	QR	LL	TC	372.7	100	13729.4
29	Patient	75	M	N	N	N	N	50%	175	96	71	85	QQ	MM	TT	80.6	68.5	13095.7
30	Patient	76	F	Y	N	N	N	N	158	135	50	46	QR	LL	CC	347.6	172	18046.2
31	Control	76	M	Y	Y	N	N	50%	132	93	74	39	QR	LM	TC	162.8	68.5	7181.5
32	Control	51	M	N	N	Y	N	N	142	115	80	35	QR	LL	TC	312.8	169	18640.3

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	-107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
33	Control	50	F	N	N	N	N	N	164	180	73	30	QR	LL	CC	441.5	133	23551.2
34	Control	42	F	N	N	N	N	N	124	113	57	24	QR	LL	CC	495.6	177.5	25346.5
35	Control	45	F	N	N	Y	N	N	191	53	73	57	RR	LL	TC	437.2	123	14455.4
36	Patient	70	M	Y	N	N	N	40%	126	73	59	42	QQ	MM	TC	102.1	118	14204.6
37	Patient	76	M	N	N	Y	Y	N	128	124	73	30	QR	LM	TC	256.7	77	6534.6
38	Control	63	M	Y	Y	Y	N	40%	150	100	60	37	QR	LM	TC	287.7	106	11617.2
39	Patient	83	F	N	N	N	N	70%	279	210	182	55	QR	LM	TT	209.3	80	8633.6
40	Control	63	F	N	N	N	N	N	200	221	123	34	QQ	MM	TC	121.3	112	14442.2
41	Patient	86	F	N	N	N	N	100%	227	96	150	58	QQ	LM	TC	113.2	130	12224.4
42	Control	75	M	Y	Y	N	N	N	251	153	100	51	QQ	LM	TC	85.9	74	10759
43	Control	58	F	Y	N	N	N	N	186	97	43	42	QQ	MM	TT	89.5	86	12976.9
44	Control	78	F	Y	N	N	N	30%	139	95	71	49	QR	LM	TC	288.4	91.5	9993.4
46	Control	74	M	N	N	N	N	50%	182	82	100	51	QQ	LM	TC	48.1	43	5544.5
47	Patient	71	M	Y	N	N	N	50%	207	232	126	35	QR	LM	TC	336.5	80	9663.4
50	Control	61	M	N	N	N	N	N	228	166	155	40	RR	LL	TC	393.5	91.5	6732.6
51	Control	85	M	Y	N	N	N	50%	254	160	24	63	QQ	MM	TT	88.6	69	9504.9
52	Control	65	F	N	Y	N	N	50%	235	126	126	84	QQ	LL	CC	287.8	218	36303.6
53	Control	65	M	N	N	N	N	N	191	57	128	52	QR	LL	TT	334.3	100	11062.7
54	Control	58	M	N	Y	Y	N	N	229	107	169	39	QQ	LL	CC	146.7	100	7547.5
55	Control	61	F	N	N	N	N	N	262	163	170	59	QQ	LL	TC	257.4	166	20594.1

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	-107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
56	Control	80	F	Y	N	N	Y	50%	233	202	83	24	QR	LM	TT	308.2	89	11617.2
57	Control	80	F	N	N	N	N	N	244	63	46	71	QQ	LM	TC	146.1	94	17663.4
58	Control	67	F	Y	Y	N	Y	N	151	127	85	41	QR	LM	TT	223.2	77	8580.9
59	Control	76	M	N	N	N	N	50%	231	206	143	47	QQ	LM	TC	140.1	112	20726
61	Patient	61	M	N	Y	N	Y	50%	148	144	76	43	QR	LM	TT	166.4	66	7656.8
62	Control	69	F	Y	N	N	N	N	241	157	74	46	QR	LM	CC	450.4	166	20594
63	Control	66	M	N	Y	Y	N	N	268	349	160	38	QQ	LM	TT	87.7	94	16963.7
64	Control	60	F	Y	N	N	N	N	191	123	121	45	QR	LL	CC	392.8	140	17095.7
66	Patient	64	F	N	N	Y	N	50%	166	94	99	48	QQ	MM	TT	65.5	77	9782
67	Patient	58	F	Y	Y	N	N	N	350	360	228	50	QR	LM	TT	356.7	120	15049.5
68	Patient	74	F	Y	N	N	N	50%	241	154	162	48	QQ	LM	TT	130.7	154.5	19934
69	Control	71	M	N	N	N	N	50%	148	140	80	55	QR	LM	TC	277.5	109	11353
71	Patient	80	F	Y	N	N	N	100%	145	86	90	38	QR	LL	TT	185	103	13933.4
72	Patient	62	M	Y	Y	N	N	70%	188	107	128	39	QQ	LM	TT	117.5	115	15445
73	Control	68	M	N	N	N	N	N	210	115	123	64	RR	LL	TT	393.4	120	12198
74	Control	65	F	N	N	N	N	N	294	157	123	46	QR	LL	TT	309.7	146	24950.5
76	Control	72	F	Y	N	N	N	50%	234	149	158	46	QR	LL	TT	246.6	114.5	14521.4
77	Control	65	M	N	N	N	N	N	152	80	64	37	QQ	LM	TT	94.6	83	13663.4
78	Control	63	F	Y	Y	N	Y	50%	193	328	66	43	RR	LL	TT	489.6	138	15445.5
79	Control	70	F	N	N	N	N	50%	187	132	118	43	RR	LL	TT	168.3	132	3630.4

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	-107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
80	Control	70	F	Y	N	N	N	50%	202	105	130	51	QR	LL	TC	280.3	86	16884.5
81	Control	65	F	Y	N	N	N	N	229	157	147	51	QR	LM	TT	235.9	140	9901
82	Patient	63	M	Y	N	N	N	N	131	89	72	41	QR	LL	CC	358	169	12541
83	Control	65	F	Y	N	N	N	N	174	113	85	66	QQ	LM	CC	154.5	163	15313.6
84	Control	78	F	Y	N	N	N	50%	175	111	93	54	QR	LL	CC	355	177	17630.4
85	Patient	68	M	Y	N	N	N	N	178	80	95	60	QQ	MM	TT	91.2	100	13452.1
86	Control	47	M	N	N	N	N	N	241	272	130	57	QR	LL	TT	323.2	140	13940.6
87	Patient	77	F	Y	N	N	N	90%	195	95	137	39	QR	LM	CC	501.3	183	19577.6
88	Control	77	F	Y	Y	N	N	N	156	87	103	36	QR	LM	TT	273.2	114.5	9874.6
89	Patient	80	F	Y	N	N	N	N	201	94	120	62	RR	LL	TC	610.4	166	12897.7
90	Control	71	M	N	N	N	N	N	168	131	90	52	QR	LL	TT	282.8	140	18415.9
91	Patient	55	M	Y	N	N	Y	50%	157	124	95	37	QQ	MM	TT	94.5	89	9967
92	Control	73	M	Y	N	N	N	50%	140	220	76	20	QR	LL	CC	374.9	137	12396.1
93	Control	61	M	N	N	Y	N	N	180	35	114	59	QQ	MM	TT	155.9	117	14574.3
94	Control	37	M	N	Y	N	N	N	170	120	110	50	QQ	MM	TT	120.8	123	11406
95	Patient	62	M	N	N	N	N	50%	142	90	54	43	QR	LL	TT	182.5	95	8739.3
96	Patient	77	M	N	N	N	N	50%	113	54	68	68	QQ	LM	CC	103.3	97	12409.3
97	Patient	24	M	N	N	N	N	N	204	255	143	41	QQ	LL	TC	192.9	152	20574.3
98	Patient	53	F	N	N	Y	N	N	273	192	23	35	QR	LM	TT	249.9	114.5	-
99	Patient	61	M	N	N	N	N	N	186	142	121	37	QR	LL	TC	311.2	155	19643.6

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
101	Control	52	M	N	N	N	N	N	232	103	183	63	QR	LL	TC	232	103	15405.9
102	Patient	78	M	Y	N	Y	N	50%	201	183	120	44	QQ	LL	TC	96.4	77	17425.8
103	Control	65	M	N	N	N	N	N	205	76	113	32	QQ	LL	CC	137.4	123	25637
105	Patient	81	M	Y	N	N	N	70%	150	270	37	42	QR	LM	TT	80.4	69	7168.3
106	Patient	80	F	Y	N	N	N	70%	221	113	57	25	QQ	LL	CC	166	172	20943.9
107	Control	50	F	Y	N	N	N	N	167	76	84	24	RR	LL	TT	412.1	118	10943.9
108	Control	87	F	Y	N	N	N	50%	244	91	54	34	QR	LM	CC	348.9	175	19320.1
109	Control	67	M	N	N	N	N	N	168	102	82	6	QQ	LL	TT	529.4	143	10442.2
110	Patient	84	M	Y	N	N	N	70%	143	98	74	49	QQ	LM	TT	102.1	120	13155.1
111	Control	38	M	N	N	Y	N	N	184	50	111	63	QR	LM	TT	285.7	137	13023.1
112	Control	50	M	N	N	N	N	N	147	55	79	57	RR	LL	TT	413.9	152	10462
113	Patient	75	F	Y	N	N	Y	70%	110	58	52	46	QR	LM	TT	153.6	66	5610.6
114	Patient	26	M	N	N	N	N	N	162	95	80	63	QQ	MM	TC	117.9	126	18105.6
115	Patient	55	M	Y	N	N	N	N	170	83	123	54	QR	LM	TT	237.4	112	12957.1
116	Patient	26	M	N	N	N	N	N	156	64	114	29	QR	LL	TT	360.7	149	21941
117	Patient	73	F	Y	N	N	N	70%	154	101	99	35	QQ	LL	CC	110	106	15372.9
119	Control	80	F	N	Y	N	N	N	153	123	77	51	QQ	LL	CC	231.8	206	38231
120	Patient	36	M	N	N	N	N	N	187	140	116	43	QQ	LM	TC	145.6	106	25571
121	Patient	56	M	N	N	N	N	N	167	109	100	45	QQ	MM	CC	187.9	169	28231
122	Patient	47	F	N	N	N	N	N	184	123	123	36	QQ	LL	TC	152.3	114.5	21775.6

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
123	Patient	21	M	N	N	Y	N	N	101	38	46	47	QQ	LM	TT	159.7	135	28310.2
124	Patient	81	F	Y	Y	N	Y	N	139	193	69	31	QQ	LM	TC	68.9	72	12158.4
125	Patient	84	M	Y	Y	N	N	N	183	87	118	48	QR	LL	CC	408.1	148	22019.9
126	Patient	73	M	Y	Y	N	N	N	198	224	118	35	QR	LL	CC	421.2	149	22607.3
127	Patient	73	F	Y	N	N	N	70%	154	101	99	35	QQ	LL	CC	111.2	106	22330.1
128	Patient	66	F	N	N	N	N	70%	129	248	58	21	QQ	LL	TC	88.2	72	12818.5
129	Patient	74	F	Y	N	N	N	70%	230	127	154	51	RR	LL	TT	580.5	108.7	11907.6
130	Control	44	M	N	N	Y	N	N	200	212	96	62	QR	LM	TC	538.4	134.5	17386.1
131	Control	51	F	N	N	N	N	N	168	51	110	48	QQ	MM	TT	108.4	83	14950.5
132	Control	67	M	Y	N	N	N	N	172	148	101	41	QR	LL	TC	568.8	140	20468.6
133	Control	73	F	Y	Y	N	N	N	227	206	143	43	QR	LL	TC	403.5	132	21947.2
134	Control	88	F	Y	N	N	N	50%	154	71	100	40	QQ	MM	TT	48	77	6409.2
135	Patient	88	F	Y	N	N	N	70%	153	108	87	44	QR	LL	TC	236.7	126	14125.4
136	Patient	66	M	Y	Y	N	N	N	129	254	49	29	QQ	LM	TT	100.1	126	20198
137	Patient	89	M	N	N	N	N	70%	162	38	113	41	QQ	LL	CC	130.2	117	17788
138	Patient	61	F	Y	Y	N	N	50%	200	178	63	25	QQ	LL	TC	153.8	134.5	20924.1
139	Patient	78	M	N	N	Y	N	N	130	110	70	40	QQ	MM	TT	54.7	63	7366.4
140	Control	59	M	N	N	N	N	N	157	110	90	45	RR	LL	CC	529.5	149	19538
141	Control	69	F	N	Y	N	N	N	241	119	157	60	QR	LM	TT	204.9	77	11221.2
142	Patient	80	M	Y	N	N	N	N	304	74	239	50	RR	LL	TT	509.6	126	15973.6

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
143	Patient	76	F	Y	Y	N	N	90%	158	110	98	38	QR	LL	TC	315.8	137.5	17029.7
144	Patient	79	F	Y	N	N	N	N	202	136	129	46	QQ	LM	CC	216	174.5	23960.4
146	Control	51	F	N	N	N	N	N	110	90	54	38	QR	LL	TC	227.1	158	11287.1
147	Patient	21	M	N	N	N	N	N	157	83	100	32	QQ	LM	TT	90.3	135	13861.4
148	Patient	76	M	N	N	N	N	N	193	106	132	40	QQ	LM	TC	140.1	114.5	14039.6
149	Patient	68	F	Y	N	N	N	N	180	140	110	45	QR	LL	CC	585.1	155	19010
151	Patient	28	M	N	N	Y	N	N	151	146	78	44	QQ	MM	TC	91	109	13927.4
152	Patient	20	M	N	N	Y	N	N	180	80	100	50	QQ	LM	TT	89.5	97	12541.3
153	Patient	80	F	Y	N	N	N	N	193	251	86	57	QR	LL	TT	35	100	8778.9
154	Patient	64	F	Y	N	N	N	N	145	144	77	39	QR	LM	TT	176	52	3102.3
156	Patient	67	F	Y	Y	N	N	N	391	226	297	49	QR	LL	CC	350.3	195	14328.4
157	Patient	58	F	Y	Y	N	N	N	274	256	169	54	QQ	LM	TC	148.1	163	21524.8
158	Control	77	M	N	N	N	N	N	179	167	80	46	QQ	MM	TC	84.8	126	13538
159	Patient	49	M	N	N	Y	N	100%	226	204	139	46	RR	LL	TC	632.3	200	15815.2
161	Patient	78	F	N	N	N	N	50%	178	88	103	57	QQ	LM	TC	538.1	181	16699.7
162	Patient	65	M	N	N	N	N	100%	154	164	79	42	RR	LL	TC	116.1	154.5	19148.5
163	Patient	75	M	Y	N	N	N	90%	195	142	130	37	QR	LL	TT	176.2	106	12468.6
164	Patient	79	M	N	N	Y	N	50%	105	80	54	35	QQ	MM	TT	61.6	86	8316.8
165	Patient	73	F	Y	Y	N	N	90%	320	315	205	23	QQ	MM	TT	69.7	86	11313.5
166	Patient	84	F	Y	N	N	N	90%	258	78	170	72	RR	LL	TT	457.8	117	9901

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
167	Patient	25	M	N	N	N	N	N	180	140	107	43	QR	LL	TC	161.2	69	52145
168	Patient	73	M	Y	N	N	N	N	195	79	119	60	QQ	LL	CC	210.1	146	24831.7
169	Patient	74	M	Y	N	N	N	90%	128	118	70	34	RR	LL	CC	598.1	140	9986.8
170	Patient	78	F	Y	Y	N	Y	90%	181	138	109	44	QQ	LM	TC	119.1	89	8369.6
172	Patient	56	M	Y	Y	N	N	N	177	157	105	41	QR	LL	TC	380.4	114.5	14079.2
173	Patient	67	M	Y	N	N	N	N	150	154	89	43	RR	LL	TC	486.1	123	11141.9
174	Patient	74	M	Y	N	N	N	N	183	201	79	50	RR	LL	TT	392.9	94.5	7696.4
175	Patient	64	M	Y	Y	N	N	N	168	170	98	41	QR	LM	CC	360.1	134.5	11485.1
176	Patient	73	M	Y	Y	N	N	50%	192	173	100	43	QR	LM	TT	278.2	123	9075.9
177	Control	52	F	N	N	N	N	50%	120	100	73	54	QR	LM	TC	468.4	140	13861.4
179	Patient	57	M	N	N	Y	N	N	187	149	116	41	QR	LM	CC	287.9	106	12105.6
180	Patient	76	M	N	N	N	N	50%	161	171	94	33	QQ	LL	CC	139	123	17293.7
181	Patient	61	M	N	N	Y	N	100%	187	124	116	46	QR	LL	TC	281.8	129	13333.3
182	Patient	85	M	N	N	Y	N	N	87	73	31	41	QQ	LL	CC	98.7	92	10514.9
184	Control	77	F	Y	N	N	N	50%	162	178	79	47	QR	LM	TT	210	92	17194.6
185	Patient	62	F	N	Y	N	N	N	157	145	91	37	QQ	LM	TT	93.9	83	17835
186	Patient	73	M	N	N	Y	N	N	304	265	204	47	RR	LL	TC	420.9	112	13122.1
187	Patient	63	M	N	N	Y	Y	50%	133	103	66	46	QQ	LL	TC	132	109	14072.6
188	Patient	52	M	N	N	Y	N	100%	158	98	108	31	QQ	LM	TC	90.9	92	18217.8
189	Control	79	M	N	N	N	N	N	86	77	150	37	QQ	MM	TC	84.4	89	18231

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	-107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
190	Patient	61	F	Y	N	N	N	70%	121	124	62	34	QQ	MM	TT	57.6	74.4	10475.3
191	Control	46	M	Y	N	Y	N	N	304	252	207	47	QQ	MM	TT	99.8	112	23630.4
193	Patient	45	M	N	N	Y	N	N	151	333	51	33	RR	LL	TT	387.8	114.5	20660.1
194	Patient	64	M	N	N	Y	Y	90%	166	97	103	36	QQ	LM	TC	102.1	100	21247.5
195	Patient	56	F	N	Y	N	N	N	270	284	169	44	RR	LL	CC	381.7	152	26369.7
196	Patient	67	M	Y	Y	N	N	70%	266	253	171	44	QQ	LM	CC	293.7	137.5	20066
197	Patient	53	M	Y	N	N	N	N	284	302	176	48	RR	LL	TC	123.6	129	27221.1
200	Control	36	M	N	N	N	N	N	121	69	75	32	QQ	LM	TT	121.3	106	19141.9
201	Control	38	M	N	N	Y	N	N	169	129	100	52	QR	LL	TC	330.6	109	16303.6
202	Patient	80	M	N	N	N	N	70%	220	119	146	50	QR	LM	TT	154.6	75	6798.7
203	Patient	62	M	Y	Y	N	N	50%	200	110	143	46	QQ	LM	TT	75.5	74.4	11221.1
204	Patient	83	F	Y	N	N	N	100%	140	98	77	43	QQ	MM	TT	80.7	80	9109
207	Control	41	F	N	N	N	N	N	150	130	80	40	QR	LM	TT	180.5	86	9676.6
209	Patient	80	M	N	N	N	N	N	280	150	123	50	QQ	LL	TC	195.6	175	17689.8
210	Patient	61	M	N	N	Y	N	50%	279	120	75	34	RR	LL	TC	398.4	109	8778.9
211	Patient	67	F	N	Y	N	N	N	190	135	125	38	QQ	LM	TT	33.9	51.5	3366.3
212	Patient	64	M	N	N	Y	N	90%	166	97	103	44	QQ	LM	TT	98.4	92	10382.8
213	Control	48	F	N	N	N	N	N	100	86	55	28	QQ	LM	TT	62.2	72	5353.1
214	Patient	65	F	N	N	Y	N	50%	222	105	149	52	QQ	LM	TT	129.5	132	-
216	Patient	79	F	Y	Y	N	N	50%	164	150	103	31	QR	MM	TC	176.8	97	9188.1

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
217	Patient	61	M	Y	N	Y	N	50%	181	107	119	41	QR	LM	TT	223.6	83	9095.7
218	Patient	36	F	N	N	Y	N	70%	246	173	148	63	QQ	LL	CC	193.8	166	22607.3
219	Patient	80	M	Y	Y	N	N	70%	289	377	173	41	QR	LL	TC	249.3	109	11993.4
221	Patient	61	M	N	Y	Y	N	N	143	102	84	39	QQ	MM	TT	39.7	51.5	4996.7
224	Patient	69	F	N	N	N	N	50%	123	99	70	33	QQ	LM	TC	76.6	83	-
222	Patient	69	M	Y	N	N	N	100%	156	77	105	36	QQ	MM	TC	114.5	117	14851.5
223	Patient	76	F	Y	Y	N	N	70%	158	68	104	40	QR	LM	CC	319.5	120	11419.1
225	Control	43	F	N	N	Y	N	N	222	164	137	52	RR	LL	TC	372.1	112	8580.9
226	Patient	77	M	Y	Y	Y	N	100%	148	73	102	31	QQ	LM	TC	94	83	9544.6
227	Patient	82	M	N	N	N	N	50%	251	148	80	35	QQ	LM	TT	49.6	66	7643.6
229	Control	45	F	N	N	N	N	N	201	245	101	51	QR	LM	CC	358.7	144	15445.5
231	Control	38	F	N	N	N	N	N	220	115	139	58	QQ	MM	TT	85.8	112	12693.1
232	Patient	78	F	Y	N	N	N	N	188	62	124	52	QQ	MM	TT	80.6	94.5	9703
233	Control	64	F	Y	N	N	N	N	269	133	186	56	QR	LL	TC	416.5	149	16567.7
234	Patient	58	F	N	N	N	N	N	166	98	94	52	QR	LL	TC	70.4	77.5	9240.9
235	Control	66	M	N	N	N	N	50%	204	62	135	57	QQ	MM	TT	45.1	63	6006.6
239	Patient	53	M	N	N	Y	N	50%	139	33	86	46	QR	LM	TT	208.2	89	8191.4
240	Control	42	M	N	N	Y	N	N	178	244	96	33	QR	LM	TC	248.6	129	12125.4
241	Control	65	M	Y	N	N	N	N	148	115	76	49	QQ	MM	TT	46.8	69	6468.6
242	Control	87	M	Y	Y	N	N	50%	104	110	38	44	QQ	LM	TC	58.5	66	5604

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
243	Patient	87	F	Y	N	N	N	70%	127	130	110	22	QR	LM	TT	146.5	66	4752.5
244	Control	75	F	N	N	Y	N	50%	184	81	113	55	QR	LL	TC	315.1	112	10495
245	Patient	54	M	N	N	N	N	N	170	59	121	37	QQ	LL	CC	131	103	13181.5
246	Control	77	M	Y	N	N	N	N	139	60	95	32	QR	LM	TC	193.8	83	7874.6
247	Control	69	F	N	N	N	N	N	178	115	106	49	QQ	LM	TC	137.9	126	14125.4
248	Patient	75	F	N	Y	N	N	N	189	87	118	54	QQ	LL	TC	137.6	106	13927.4
249	Patient	87	F	Y	N	Y	N	50%	220	58	138	70	QR	LM	TC	314.3	106	12046.2
250	Patient	84	F	N	N	N	N	100%	175	71	102	59	QR	LL	TC	204.2	89	9042.9
251	Patient	78	M	Y	Y	N	Y	70%	163	135	100	36	QR	LL	TT	157.6	74.5	10244.2
252	Patient	40	F	N	N	Y	N	N	192	94	127	46	QQ	LM	TC	99.9	97	9914.2
253	Patient	48	M	Y	N	N	N	N	211	72	147	50	QR	LM	TC	278.1	126	5148.5
254	Patient	80	F	Y	Y	N	N	N	156	77	112	54	QR	LL	TC	212.3	106	11914.2
255	Patient	41	M	N	N	N	N	N	145	120	88	33	QQ	LL	CC	162.8	134.5	12475.2
256	Patient	77	F	Y	Y	N	N	70%	192	129	123	43	QR	LL	CC	347.6	129	21498.3
257	Patient	63	F	Y	Y	N	N	50%	151	69	100	37	QQ	LM	CC	151.6	154.5	14594.1
258	Patient	55	M	Y	N	Y	N	50%	277	163	207	37	QR	LL	TT	252.2	117	10217.8
259	Patient	79	M	Y	Y	N	N	70%	120	68	61	45	QQ	MM	TT	45.4	75	13432.3
260	Patient	67	F	Y	Y	N	Y	50%	197	127	114	58	QQ	MM	TC	103.6	103	17854.8
261	Patient	63	M	Y	Y	N	N	70%	145	157	83	31	QQ	LM	TT	84.3	74.5	12587.5
262	Patient	75	F	Y	N	N	N	50%	228	169	152	42	QR	LM	CC	508.7	160	23907.6

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
263	Control	65	M	N	N	N	N	50%	159	62	103	44	QR	LM	TT	167.9	74.5	8785.5
264	Control	78	M	N	N	N	N	N	158	82	87	55	QQ	LL	TT	90.2	83	10165
265	Control	81	M	Y	N	N	N	N	108	54	48	49	QR	LL	CC	358.1	131.5	21458.7
266	Control	56	M	N	Y	N	N	N	177	122	110	43	QQ	LM	TC	115.2	103	16561.1
267	Control	64	F	Y	Y	N	N	N	166	246	86	31	QQ	LM	CC	133.6	120	17221.1
268	Control	79	F	Y	N	N	N	N	181	110	110	49	QR	LM	TC	208	92	13062.7
269	Control	67	F	N	N	N	N	N	298	102	228	50	QQ	LM	TC	143.5	132	18521.5
270	Control	76	F	Y	N	N	N	50%	166	110	85	59	QR	LM	TC	204.5	106	10488.4
271	Control	64	M	N	N	Y	N	N	130	91	86	26	QQ	LM	TT	30.5	57	5630.4
272	Control	77	M	N	Y	N	N	N	338	158	243	63	QQ	MM	TT	131.7	120	13399.3
273	Control	75	M	N	N	N	N	N	191	149	109	52	QR	LM	TC	364.1	120	13736
274	Patient	54	F	N	N	N	N	100%	201	301	114	27	QR	LM	TT	233.9	100	20877.9
275	Patient	61	F	Y	N	N	N	50%	180	110	126	35	QQ	LL	TC	172.5	143	15392.7
276	Patient	71	M	Y	N	N	N	90%	125	71	73	38	QQ	MM	TT	58.6	71.5	5227.7
277	Control	68	F	Y	N	N	N	N	186	170	97	55	QR	LL	TC	288.1	123	25709.6
278	Patient	74	F	Y	N	N	N	70%	211	165	123	55	QQ	LL	CC	156.4	126	13953.8
279	Patient	59	M	N	N	N	Y	50%	128	127	60	23	QR	LL	TT	248.2	103	17650.2
280	Patient	62	M	N	N	Y	N	N	154	70	103	37	QR	LM	TT	212.3	108	10191.4
281	Patient	82	F	Y	Y	N	N	50%	179	105	120	38	RR	LL	TT	257.8	74	6033
282	Control	71	F	Y	N	N	N	50%	149	97	90	40	QQ	LL	TC	74.7	80	8580.9

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
283	Control	52	F	N	N	N	N	50%	154	71	100	40	QQ	LM	TC	181.2	154.5	23379.5
284	Control	78	M	Y	N	N	N	N	159	124	97	37	QQ	LL	CC	100.6	80	11742.6
285	Patient	70	M	Y	N	N	N	50%	198	76	134	49	QR	LM	TT	241.2	97	9749.2
286	Patient	69	M	Y	N	N	N	50%	170	83	111	42	QR	LM	TT	158.7	69	7696.4
287	Control	80	M	Y	N	N	N	50%	197	86	126	54	QR	LL	TC	144.2	80	6732.7
288	Patient	57	F	N	Y	N	N	100%	210	154	135	44	QQ	LM	TC	93.8	86	11947.2
289	Patient	58	F	Y	N	N	N	N	123	112	60	41	QR	LL	TC	183.9	86	8884.5
292	Patient	80	M	Y	N	N	N	70%	136	62	78	46	QR	LL	CC	426	132	19802
294	Control	78	F	N	N	N	N	50%	186	84	98	41	QR	LL	CC	93.2	92	17623.8
295	Patient	62	M	Y	Y	Y	Y	100%	131	192	57	36	QR	LM	TT	159	80	7458.7
296	Control	57	F	N	N	N	N	N	231	93	19	56	QQ	MM	TT	64.3	92	9703
297	Control	60	F	Y	N	N	N	50%	250	198	112	54	QQ	MM	CC	100.4	114.5	13135.3
298	Control	63	F	Y	N	N	Y	50%	257	246	160	48	QQ	MM	TT	75.4	92	11947.2
299	Patient	74	M	N	Y	N	N	50%	130	65	65	52	QR	LL	TT	177.5	80	11947.2
300	Patient	50	M	Y	N	N	N	50%	217	75	24	38	QR	LM	TT	182.7	80	9505
301	Control	58	M	Y	N	N	N	50%	190	200	80	30	QQ	LM	CC	98.4	108	15115.5
302	Control	54	M	Y	N	N	N	N	166	103	94	51	QQ	LM	TT	119.1	108	18019.8
303	Patient	86	F	Y	N	N	N	50%	162	149	53	79	QR	LL	TC	269.2	120	15049.5
304	Control	78	M	N	N	N	N	50%	130	60	80	50	QR	LL	TT	201.1	69	13531.4
305	Patient	57	M	N	Y	N	N	100%	193	127	139	29	QR	LM	TC	333.4	114.5	16699.7

Table A.1 (continued).

		Demographic Characteristics							Lipid Parameters				PON1 Genotypes			PON1 Activities		
no	Patient-Control	Age	Gender	Hypertension	Diabetes	Smoking	Statin Drug Use	Stenosis	Total Cholesterol	Triglycerides	LDL-C	HDL-C	192Q/R	55L/M	−107T/C	PON (U/L)	ARE (U/mL)	DIA (U/L)
306	Control	75	M	Y	Y	N	N	50%	185	71	115	56	QR	LM	TC	344	114.5	14257.4
307	Control	77	M	Y	N	N	N	50%	118	88	64	36	QQ	MM	TC	52.5	74	7656.8
308	Patient	85	M	N	N	N	N	50%	186	120	118	44	QR	LL	TT	89.5	92	13709.6
309	Patient	62	M	Y	N	N	N	50%	195	233	119	29	RR	LL	TC	184.9	63	8514.9
310	Patient	81	F	Y	Y	N	N	70%	161	85	96	48	QR	LL	TC	92.5	57.3	6996.7

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PUBLICATIONS

1. Thesis

"Sheep liver microsomal flavin-monooxygenases: characterization and effect of some modulators". Master of Science Thesis. Biochemistry METU 2002.

2. Science Citation Index Research Articles

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