EFFECT OF ATORVASTATIN ON PARAOXONASE (PON) GENE FAMILY AND OXIDATIVE STRESS IN HYPERCHOLESTEROLAEMIC THAI POPULATION

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EFFECT OF ATORVASTATIN ON PARAOXONASE (PON) GENE FAMILY AND OXIDATIVE STRESS IN HYPERCHOLESTEROLAEMIC THAI POPULATION

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ABSTRACT

The human paraoxonase (*PON)* gene family consists of three members, *PON1*, *PON2*, and *PON3*. Paraoxonase has been shown to reduce the oxidation of low density lipoprotein (LDL) and high density lipoprotein (HDL) by hydrolyzing lipid peroxides and using unknown mechanism(s), thus protecting against atherosclerosis. Various factors influence the PON activity including lipid-lowering agents such as atorvastatin. Atorvastatin has been shown to have a direct and indirect effect in the antioxidant system. In this study, we investigated the effect of 3 month atorvastatin treatment on PON activity and oxidative status in a hypercholesterolaemic Thai population. Atorvastatin significantly reduced total cholesterol, triglyceride, LDL, conjugated diene, total peroxide, and malondialdehy levels, whereas total antioxidant status level was significantly increased. Interestingly, atorvastatin significantly increased PON1 activity towards paraoxon and PON3 activity, but not PON2 activity. The gene distribution for the *PON1* L55M, Q192R, and T-107C polymorphisms were 73.68% LL, 21.05% LM, 5.26% MM; 42.11% QQ, 36.84% QR, 21.05% RR; 42.11% CC, 36.84% CT, and 21.05% TT, respectively and for the *PON2* C311S polymorphism, the gene distribution was 10.53% CC, 31.58% CS, and 57.89% SS. There were no significant differences of baseline PON1 activity towards paraoxon and PON2 activity towards *p*-nitrophenyl butyrate according to *PON1* and *PON2* polymorphisms, respectively. However, baseline PON1 activity towards phenyl acetate was significantly influenced by the *PON1* L55M polymorphism. Moreover, the *PON1* L55M and T-107C polymorphisms influenced the therapeutic response of PON1 activity to atorvastatin treatemnt. Taken together, these results indicate atorvastatin may have important antioxidant properties via increasing PON activity.

KEY WORDS: HYPERCHOLESTEROLEMIC / ATORVASTATIN / OXIDATIVE STATUS / PON / POLYMORPHISM

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ผลของยาอะทอวาสเตตินต่อระดับการทำงานของเอนไซม์ในกลุ่มพาราออกโซเนส และภาวะออกซิเดทิฟวสเตทในประชากรไทยที่มีภาวะไขมันสูงในเลือด (EFFECT OF ATORVASTATIN ON PARAOXONASE (PON) GENE FAMILY AND OXIDATIVE STRESS IN HYPERCHOLESTEROLAEMIC THAI POPULATION)

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บทคัดย่อ

เอนไซม์ในกลุ่มพาราออกโซเนส (PON) ประกอบด้วย PON1, PON2 และ PON3 ทำหน้าที่ป้องกันการเกิดออกซิเดชั่นของไขมันชนิด low density lipoprotein (LDL) และ high density lipoprotein (HDL) โดยการย่อย lipid peroxides และด้วยกลไกอื่นที่ยังไม่ทราบแน่ชัด จึงมีส่วนช่วยป้องกันการเกิดหลอดเลือดแดงแข็งตัว (Atherosclerosis) ระดับการทำงานของเอนไซม์ในกลุ่มพาราออกโซเนสขึ้นกับปัจจัยภายในและภายนอกร่างกายอาจรวมถึงยาที่ใช้ลดระดับไขมันอย่างเช่น อะทอวาสเตติน เชื่อกันว่ายาตัวนี้นอกจากลดระดับไขมันได้ยังอาจมีคุณสมบัติในการทำหน้าที่เป็นสารต้านอนุมูลอิสระทั้งทางตรงและทางอ้อม การศึกษานี้ได้ติดตามผลของยาอะทอวาสเตตินต่อระดับการทำงานของเอนไซม์ในกลุ่มพาราออกโซเนส และภาวะออกซิเดทิฟวสเตทในประชากรไทยที่มีภาวะไขมันสูงในเลือดหลังจากได้รับยาอะทอวาสเตติน 3 เดือน จากผลการทดลองพบว่ายาอะทอวาสเตตินลดระดับของไขมันโดยรวม,ไตรกลีเซอร์ไรด์, LDL, conjugated diene, total peroxide, และ malondialdehyde อย่างมีนัยสำคัญ ขณะที่ยาอะทอวาสเตตินเพิ่มระดับของ total antioxidant status อย่างมีนัยสำคัญ สิ่งที่น่าสนใจคือ ยาอะทอวาสเตตินเพิ่มระดับการทำงานทั้งของ PON1 เมื่อใช้ paraoxon เป็น substrate และ PON3 อย่างมีนัยสำคัญแต่ไม่มีผลต่อระดับการทำงานของ PON2 ลักษณะการกระจายตัวทางพันธุกรรมในกลุ่มประชากรนี้ของ *PON1* ที่ตำแหน่ง L55M พบว่าเป็นแบบ LL 73.68%, LM 21.05% และ MM 5.26%, *PON1* ที่ตำแหน่ง Q192R พบว่าเป็นแบบ QQ 42.11%, QR 36.84% และ RR 21.05%, *PON1* ที่ตำแหน่ง T-107C พบว่าเป็นแบบ CC 42.11%, CT 36.84% และ TT 21.05% และ *PON2* ที่ตำแหน่ง C311S พบว่าเป็นแบบ CC 10.53%, CS 31.58%, และ SS 57.89% โดยที่สภาวะก่อนการได้รับยาอะทอวาสเตติน ความแตกต่างของลักษณะทางพันธุกรรมของ *PON2* ไม่มีผลต่อระดับการทำงานของเอนไซม์ ขณะที่ความแตกต่างของลักษณะทางพันธุกรรมของ *PON1* ที่ตำแหน่ง L55M มีผลต่อระดับการทำงานของเอนไซม์เมื่อใช้ phenyl acetate เป็น substrate นอกจากนี้สิ่งที่น่าสนใจคือยาอะทอวาสเตตินมีผลต่อระดับการทำงานของ PON1 โดยขึ้นกับความแตกต่างของลักษณะทางพันธุกรรมของ *PON1* โดยเฉพาะที่ตำแหน่ง L55M และ T-107C จากการศึกษานี้ จะเห็นได้ว่ายาอะทอวาสเตตินมีคุณสมบัติในการทำหน้าที่เป็นสารต้านอนุมูลอิสระทางอ้อมโดยการเพิ่มระดับของ total antioxidant status โดยส่วนหนึ่งอาจมาจากการเพิ่มระดับการทำงานทั้งของ PON1 และ PON3 ทั้งนี้มีความแตกต่างของลักษณะทางพันธุกรรมเข้ามาเกี่ยวข้องด้วย

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LIST OF ABBREVIATIONS

α	=	Alpha
ABTS	=	2, 2’ azinobis (3-ethylbenzothiazoline-6-sulphate)
ALT	=	Alanine trasaminase
Apo A	=	Apoliportein A
Apo AI	=	Apoliportein AI
Apo AII	=	Apoliportein AII
Apo B	=	Apoliportein B
Apo C	=	Apoliportein C
AST	=	Aspartate transaminase
ATP	=	Adult Treatment Panel
BHT	=	Butylated hydroxytoluene
BMI	=	Body mass index
BW	=	Body weight
°C	=	Degree celcius
CAD	=	Cronary artery disease
CD	=	Conjugated diene
CETP	=	Cholesterolester transfer protein
CHD	=	Cronary heart disease
CVD	=	Cardiovascular disease
DBP	=	Diastolic blood pressure
dNTP	=	Deoxynucleotide triphosphate
dL	=	Deciliter
ε	=	Molar extinction coefficient
EDTA	=	Ethylene diamine tetraacetic acid
FBS	=	Fasting blood sugar
HDL	=	High density lipoprotein
HMG-CoA	=	3-Hydroxy-3-methylglutyral coenzyme A

LIST OF ABBREVIATIONS (CONTINUED)

hr	=	Hour
kDa	=	Kilo dalton
Kg	=	Kilogram
L	=	Liter
LACT	=	Leucithin cholesterol acetyltransferase
LDL	=	Low density lipoprotein
Lp	=	Lipoprotein
LPL	=	Lipoprotein lipase
µg	=	Microgram
µL	=	Microliter
µM	=	Micromolar
MCP1	=	Monocyte chemotactic protein1
MDA	=	Malondialdehyde
Hg	=	Mercury
mg	=	Milligram
MI	=	Myocardial Infarction
min	=	Minute
mL	=	Milliliter
mmHg	=	Milliliter mercury
mM	=	Millimolar
MM-LDL	=	Minimally modified low density lipoprotein
NCEP	=	National Cholesterol Education Program
OD	=	Optical density
OSI	=	Oxidative stress index
Ox-LDL	=	Oxidized low density lipoprotein
%	=	Percent
PCR	=	Polymerase chain reaction
PON	=	Paraoxonase
PBS	=	Phosphate buffer salin

LIST OF ABBREVIATIONS (CONTINUED)

RCT	=	Reverse cholesterol transport
PUFAs	=	Polyunsaturated fatty acids
SBP	=	Systolic blood pressure
sec	=	Second
SRBP	=	Sterol regulatory element binding protein
TAS	=	Total antioxidant status
TC	=	Total cholesterol
TG	=	Triglyceride
VLDL	=	Very low density lipoprotein
WHO	=	World Health Organization
WHR	=	Waist hip ration

CHAPTER I
INTRODUCTION

Atherosclerosis is the primary cause of cardiovascular disease (CVD) and coronary heart disease (CHD). It is a leading cause of global morbidity and mortality in the modern world (see rev. Lusis, 2000). Atherosclerosis is a complex multi-factorial disease. The oxidative modification theory for atherosclerosis postulates that oxidation of low density lipoprotein (LDL) is the key factor for this pathogenesis (see rev. Roland and keaney, 2003). Oxidized LDL (Ox-LDL), a chemo attractant for monocyte, transforms macrophage into foam cells. It exerts cytotoxic effects on endothelial cells, increases thrombocyte activation, and stimulates migration and proliferations of smooth muscles cells that lead to formations of atheroma lesions (see rev. Steinberg, 1889; Roland and keaney, 2003). Several potentially antiatherogenic mechanisms have been associated with HDL. These include both the protection of LDL against oxidation and attenuation of the biological activity of Ox-LDL (Hessler *et al*., 1979; Parthasarathy *et al*., 1990; Nofer *et al*., 2002). Antioxidant and antiatherogenic properties of a HDL are from HDL associated enzyme, paraoxonase1 (PON1). Numerous studies have indicated that PON1 is largely responsible for HDL's anti-oxidative property, the strength of which has been reinforced in the mouse model (Mackness M *et al*., 1993 and 1995; Mackness B *et al*., 1998; Shih *et al*., 1998; Durrington *et al*., 2001; Shih *et al*., 2002). Moreover, recent findings of two new PON members, PON2 and PON3 also showed the protection role in oxidative stress in tissues and plasma, respectively (see rev. Aviram *et al*., 2004; Ng *et al*., 2004). These three members of human *PON* gene family located adjacent to one another on the long arm of chromosome 7, which are highly similar to each other at the amino acid and nucleotide levels (Primo-Parmo *et al*., 1996; Mackness B *et al*., 2002).

Paraoxoanse 1 (PON1) is a calcium dependent ester hydrolase which is primarily synthesized in liver and associated with HDL in the plasma (Sorenson *et al*.,

1999; James *et al*., 2004; Ng *et al*., 2004). PON1 inhibits LDL and HDL oxidative modification and has the function of removing oxidized phospholipids from LDL (Watson *et al*., 1995; Mackness M *et al*., 1993 and 1995). Moreover, various clinical epidemiological studies demonstrated that PON1 enzyme activity was inversely related to the risk of CHD and CVD (Mackness M *et al*., 1991; Abbott *et al*., 1995; Boemi *et al*., 2001; Mackness B *et al*., 2001). PON1 activity was genetically determined and reported marked individual and racial variations (Adkins *et al*., 1993; Humbert *et al*., 1993; Richter *et al*., 1999). The *PON1* gene has two common polymorphisms in the coding region and five polymorphisms in the regulatory regions (see rev. Hong-Liang *et al*., 2003). Many evidences demonstrated that these polymorphisms may influence PON1 activity and expression as well as in response to various stimuli.

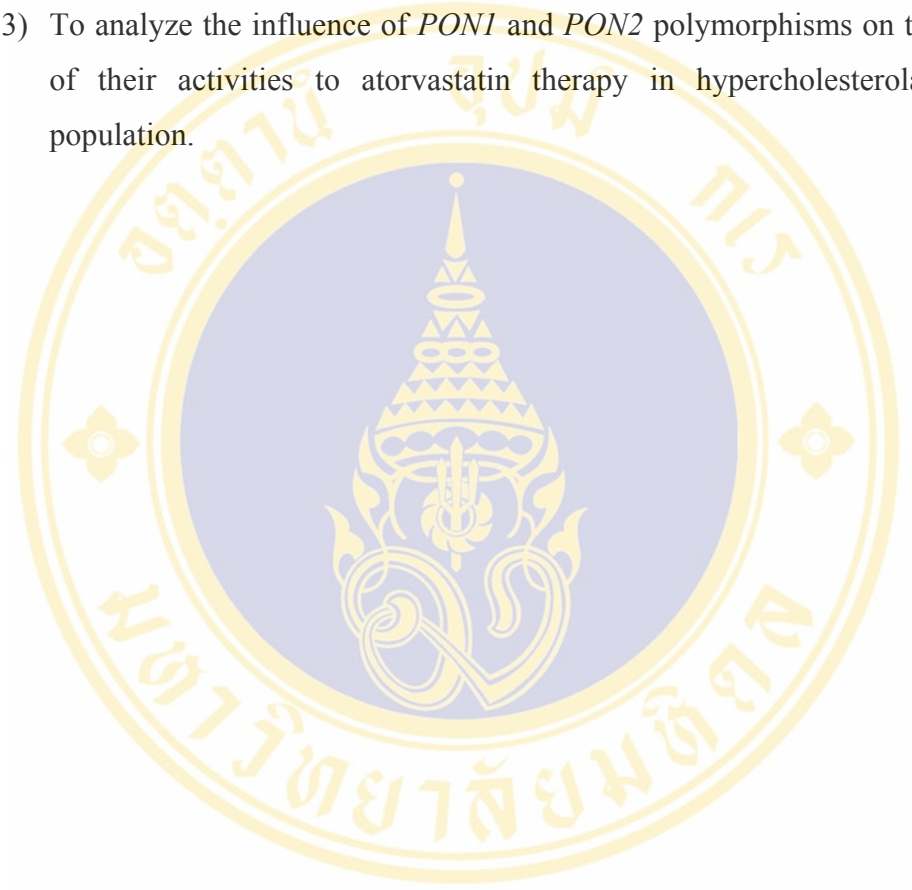
PON3 is synthesized primarily in liver and kidney, and associated with HDL in the plasma (Reddy *et al*., 2001; Ng *et al*., 2004). PON3 shows a high similarity in structure and functions with PON1. It was reported that rabbit PON3 is more potent than PON1 in protecting LDL oxidation (Draganov *et al*., 2000). Moreover, PON3 protein plays a role in lipoprotein metabolism of the kidney and metabolizes drugs that may be potentially important in the prevention of atherosclerosis (Draganov *et al*., 2000; La Du *et al*., 2001; Reddy *et al*., 2001). The polymorphism and information of PON3 are still under investigation. Unlike PON3, PON2 is a intracellular protein and found in several tissues including the heart, lung, kidney, placenta, small intestine, spleen, testis, stomach, cells of arteries, and macrophages but is not present in HDL and LDL (see rev. Ng *et al*., 2001). PON2 possesses antioxidant properties and play a role in reducing intracellular or local oxidative stress (see rev. Aviram *et al*., 2004). The human *PON2* gene has two common polymorphisms and some evidences showed that it may be associated with CHD (Leus *et al*., 2002; Pan *et al*., 2002; Chen *et al*., 2003).

Numerous clinical trails with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) demonstrated significant reduction of CHD and CVD development (Rubins *et al*., 1999; Marron *et al*., 2000). Clinical studies revealed that atorvastatin is the drug of choice in the statin group because of its pharmacokinetic profile and efficacy (Marron *et al*., 2000; Michael, 2004). Statins act

as antioxidants directly or indirectly, protect LDL and HDL oxidation, and exert additional beneficial effects on atherosclerosis apart from their effects on lipid profiles (Fuhrman *et al*., 2002; Paragh *et al*., 2004; Kural *et al*., 2004). However, there are few studies on the effect of atorvastatin on PON1 activity and the results are still unclear. Moreover, effect of atorvastatin on PON2 and PON3 is not clear. Based on the hypothesis that PON has a protective function against CHD and CVD development, the influence of PON activity may play a major protective function in these diseases. This study therefore makes an attempt to study the effect of atorvastatin on the activity of PON enzyme and oxidative stress status in hypercholesterolaemic subjects.

Objectives

1) To determine the effect of atorvastatin treatment on distribution of lipoproteins and oxidative stress in hypercholesterolaemic Thai population.
2) To determine the effect of atorvastatin treatment on the activity of PON in hypercholesterolaemic Thai population.
3) To analyze the influence of *PON1* and *PON2* polymorphisms on the response of their activities to atorvastatin therapy in hypercholesterolaemic Thai population.



CHAPTER II
LITERATURE REVIEW

1. ATHEROSCLEROSIS

1.1 Definition

Atherosclerosis is an underlying cause of about 50% of all death in Western countries and significant source of morbidity and mortality elsewhere in the world (Lusis *et al*., 2000). Atherosclerosis is a slow progressive multi-factorial disease, characterized by the accumulation of lipids and fibrous elements in subintima of middle to large size arteries that may even start since childhood. It affects the arteries of the brain, heart, kidneys, and the arms as well as legs.

The build-up of plaques begins when the innermost layer (endothelium or intima) of the artery is damaged by free radical attack (oxidation) and associated inflammation. Either as the body's attempt to repair this damage, or simply because they become trapped, fatty substances such as cholesterol and minerals collect at the site of the damage forming plaques that narrow the affected arterial openings, consequently, many serious diseases and complications follow. Coronary heat disease (CHD) and cerebrovascular disease (CVD) are among them (see rev. Roland and keaney, 2003).

1.2 Epidemiology

Coronary heart disease is the major source of morbidity and mortality in the western world**.** Coronary heart disease claimed 656,000 deaths in the United States in 2002 (American Heart Association, 2005). Approximately 1/6 of all who died from CVD are under the age of 65 and the estimated age-adjusted prevalence of CVD is 30% for men, and 24% for women (American Heart Association, 2002). In the black population, this number climbs to 41% for men, and 40% for women (American Heart Association, 2002). Using the United States economic costs, the estimated direct and

indirect medical cost related to CHD is $142.1 billion in 2005 (American Heart Association, 2005).

As a proportion of total deaths from all-causes, CVD in the Asia–Pacific region ranges from less than 20% in countries such as Thailand, Philippines, and Indonesia to 20–30% in urban China, Hong Kong, Japan, Korea, and Malaysia (Geok, 2001). Countries such as New Zealand, Australia, and Singapore have relatively high rates that exceed 30–35% with more than 150 deaths per 100,000 from CHD (Geok, 2001). Urbanization, globalization and industrializations as well as diet acquire from western habit lead to CHD/CVD becoming a major epidemic health problem also in developing countries.

1.3 Risk factors

Epidemiology studies had revealed numerous risk factors for atherosclerosis. These can be grouped into two factors, one with an important genetic component, and the other that is largely connected with environment. World Health Organization (WHO) major risk of atherosclerosis includes hypertension, diabetes, smoking, high blood cholesterol, physical inactivity, obesity, and positive family history (World health Organization, 2002). Smoking increases atherosclerotic disease by more than 50% and the incidence of CHD can be doubled as well (United State Department of Health and Human Services, 1989). This excess risk of smoking on CHD is readily reduced through smoking cessation. In fact, the risk of heart attack in ex-smokers declines to almost that of non-smokers over two years (Gaziano, 1996). Hypertension is defined as a systolic blood pressure (SBP) above 140 mmHg or a diastolic blood pressure (DBP) above 90 mmHg. There appears to be an approximately linear relation between blood pressure elevation and the increased incidence of atherosclerotic vascular disease, with an increase of 7 mmHg DBP corresponding to 27% increase in myocardial infarction (MI), and 42% increase in stroke (Macmahon *et al.*, 1990). Antihypertensive therapy has proven most effective in reducing stroke, with 5-6 mmHg reduction in blood pressure corresponding to 42% reduction in the risk of stroke, but only 14% reduction in the risk of MI (Collins *et al.*, 2000). In patients with diabetes, the risk of coronary atherosclerosis is 3-5 folds greater than non-diabetics despite controlling of other risk factors (Bierman, 1992). Although improved glucose

control in diabetes associated with a reduction in number of diabetic complications, coronary atherosclerosis is not reproducibly seen among these complications reduced by improved diabetic control. Epidemiological studies have been clearly demonstrated that hypercholesterolemia is a major risk factor for cardiovascular complications (Castelli, 1986; Korf, 1996).

1.4 Hypercholesterolemia

Hypercholesterolemia is a condition in which the level of cholesterol in the blood is higher than normal. Hypercholesterolemia in human is caused by genetic or acquired abnormalities in the synthesis or degradation of plasma lipoproteins that shuttle endogenous cholesterol between body tissues (Korf, 1996).

From a purely mechanistic endpoint, three basic abnormalities can contribute to hypercholesterolemia. The first mechanism is defective clearance of LDL by receptor or non receptor pathway. The second mechanism is due to over production of LDL, either through excessive hepatic synthesis of apoprotein B containing lipoproteins or an increased conversion of very low density lipoprotein (VLDL) remnants to LDL because of decreased hepatic uptake of these lipoproteins. The last mechanism is over loading of LDL particles with cholesterol ester without any increase in the number of particles (Hobbs *et al*., 1980).

The metabolic defects of Primary hypercholesterolemia may be caused by a single gene disorder such as familial hypercholesterolemia (FH) or may be polygenic and result from multiple genes interacting with environmental factors (Souter *et al*., 1980). Polygenic hypercholesterolemia is the most common cause of hypercholesterolemia and elevated LDL (Poledne *et al*., 1993). The FH has been most extensively studied. It is a genetic disorder of lipoprotein metabolism, caused by multiple mutations in the gene coding for the LDL receptor and characterized clinically by an elevated level of LDL particles and genetically by an autosomal inheritance (Vuorio *et al*., 1995; John *et al*., 2000).

1.4.1 Lipoproteins

Lipids are not soluble in aqueous solutions and don’t circulate freely in plasma. They are rendered water soluble by forming complexes with proteins, called

apoproteins. The resulting lipid-apoprotein complex is designated as lipoproteins. Lipoproteins are round shaped particles consisting of a hydrophobic core of lipids as esters and triglycerides (TG). They are surrounded by a single-layer shell composed of specific apoproteins and two polar lipids such as unesterified or free cholesterol, and phospholipids.

Two important pathways are involved in the lipoprotein transport of TG and cholesterol as shown in figure 1 (Merck and Dohme, 1989; Connor *et al*., 1990). The exogenous pathway is for transportation of dietary fat into the body while the endogenous pathway is for the transportation of lipid which is synthesized in the body. Ingested dietary cholesterol is absorbed through intestinal mucosa. Only 0.5 g/day of cholesterol is derived from diet. Within the mucosal cells of intestine, this dietary cholesterol is packaged into large TG particle which are called chylomicrons. Despite their short half-life, chylomicrons deliver dietary cholesterol to body tissue for energy and storage or supply dietary cholesterol to the liver, where it is either incorporated into very low density lipoprotein (VLDL) or converted to bile acids.

The endogenous pathway is the major pathway for cholesterol synthesis. Cholesterol in the body is mainly derived from the synthesis in liver. It can synthesize as much as, 1.5 gram per day**.** Cholesterol synthesis occurs in the cytoplasm and microsome from a two-carbon acetate group of acetyl-coenzyme A, where, hydroxymethyl-glutaryl coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34) is the rate limiting step enzyme (Ginsbeg et al., 1977). Once VLDL is secreted from liver into the plasma**,** VLDL derived apolipoprotein (apo) CII and apo E from high density lipoprotein (HDL) then the TG in VLDL is hydrolyzed by lipoprotein lipase (LPL) on capillary to form intermediate density lipoprotein by releasing free fatty acid and finally LDL is generated (Merck and Dohme, 1989; Connor *et al*., 1990).

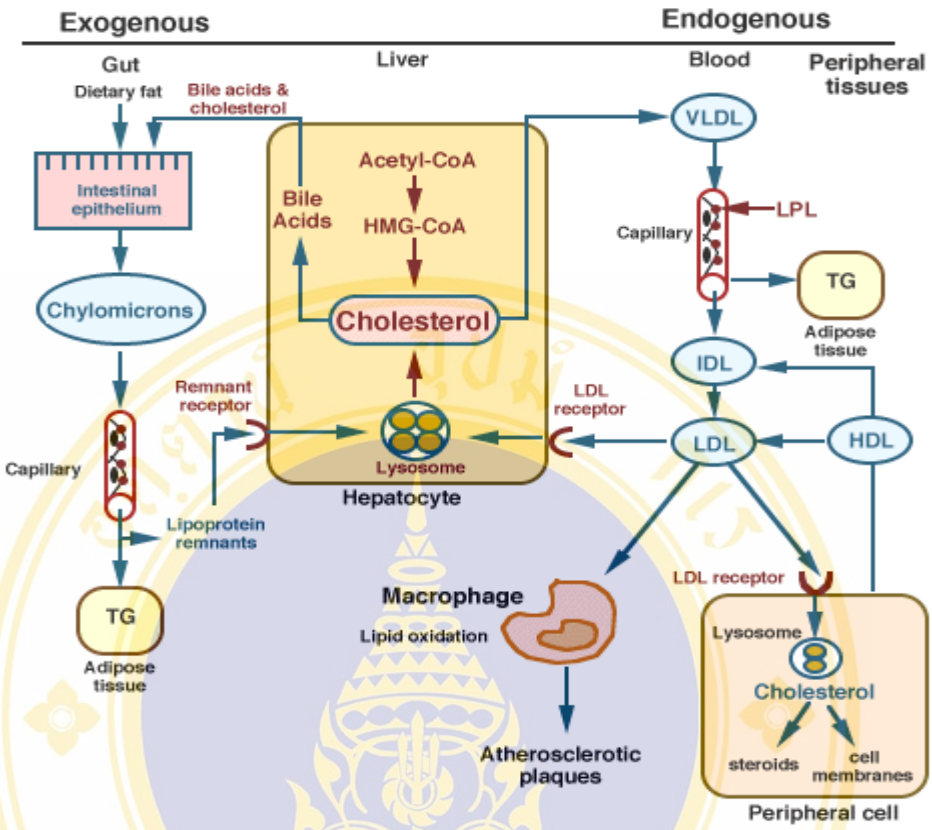


Figure1. Lipoprotein metabolism pathway (http://www.ovc. uoguelph.ca/ BioMed / Courses/Public/Pharmacology/pharmsite/98-409/Blood/hyperlipidemia.html).

Lipoproteins are classifies on the basis of their buoyant densities into four groups. The characteristics of major lipoproteins are shown in table 1.

1.4.2 Chylomicron

Dietary cholesterol and triacylglycerols are assembled in the intestinal mucosa for transport to other parts of the body. The predominant lipids of chylomicrons are triacylglycerols. Apolipoproteins that predominate before the chylomicrons enter the circulation include apo B48 and apo AI, apo AII, and apo AIV. The apo B48 combines with chylomicron (Hussain *et al*., 1996).

During the removal of fatty acids, a substantial portion of phospholipid, apo A and apo C are transferred to HDL. The loss of apo CII prevents LPL from further degrading the chylomicron remnants. Chylomicron remnants containing primarily

cholesterol, apo E, and apo B48 are then delivered to, and taken up by the liver through interaction with the chylomicron remnant receptor (Hussain *et al*., 1996).

Table 1. Characteristics of major plasma lipoproteins

Lipoprotein classes	Density g/mL	Diameter nm	Major lipid classes	Major apolipoprotein	Electrophoresis mobility
Chylomicron and remnant	<1.006	500-80	Dietary triglycerides	apo B48 apo AI apo AII apo AIV apo CII/CIII apo E	Remains at origin
VLDL	<1.006	80-30	Endogenous Triglycerides	apo B100 apo E apo CII/CIII	Pre-β
LDL	1.019-1.063	25-18	Cholestrol esters	apo B100	β
HDL	1.063-1.210	12-5	Cholestrol esters, Phospholipids	apo AI apo AII apo CII/CIII	α

1.4.3 Very low density lipoprotein (VLDL)

Triacylglycerols are packaged into VLDL and released into the circulation for delivery to the various tissues (primarily muscles and adipose tissues) for storage or production of energy through oxidation. In addition to triacylglycerols, VLDL contains some cholesterol, cholesteryl esters, apo B100, apo CI, apo CII, apo CIII, and apoE. Like nascent chylomicrons, newly released VLDL acquires apo C and apo E from circulating HDL.

The fatty acid portion of VLDL is released to adipose tissue and muscle in the same manner as the chylomicrons through the action of LPL. The action of LPL coupled to a loss of certain apoproteins (the apo C) converts VLDL to intermediate density lipoproteins which are also called VLDL remnants.

1.4.4 Low density lipoproteins (LDL)

In the circulation, VLDL is converted to LDL through the action of LPL. LDL is the primary plasma carriers of cholesterol for delivery to all tissues. The exclusive apolipoprotein of LDL is apo B100. Low density lipoprotein is taken up by cells via LDL receptor-mediated endocytosis. The uptake of LDL occurs predominantly in liver (75%), adrenals, and adipose tissues. The endocytosed membrane vesicles (endosomes) fuse with lysosomes, in which the apoproteins are degraded and the cholesterol esters are hydrolyzed to yield free cholesterol.

1.5 Oxidative modification: a hypothesis for atherosclerosis

Mechanisms of LDL oxidation

With respect to the lipid classes within LDL particle, a prototypical particle contains 2,700 fatty acid molecules, about half of these fatty acids are polyunsaturated fatty acids (PUFAs) (Esterbauer *et al*., 1990). Low density lipoprotein, lipid peroxidation are generally restricted to PUFAs. With its relatively high content of PUFAs, LDL oxidation does occur spontaneously (Gurd *et al*., 1960). Low density lipoprotein can be oxidized by metal ions and reactive nitrogen species (Ray *et al*., 1954). Small and dense LDL is more prone to oxidation. Initial oxidation of LDL within the arterial wall forms a minimally modified form of LDL (MM-LDL) that has a number of properties in and of itself which include stimulates adjacent endothelial cells and smooth muscle cells to synthesize and secrete monocyte chemotactic protein 1 (MCP 1) (Cushing *et al*., 1990). Monocyte chemotactic protein 1 as well as the expression of endothelial leukocyte adhesion molecules are responsible for the recruitment of monocytes that undergo activation-differentiation in the subendothelial space to become macrophages, which are subsequently transform into foam cells that agglutinate forming the core of atheromatous plaque (Cybulsky *et al*., 1991; Navab *et al*., 1991).

It is strongly believed that the level of serum LDL positively correlate with the incidence of atherosclerosis. One convincingly accepted theory for this phenomenon is oxidation of LDL which is the key factor in pathogenesis of atherosclerosis (Steingberg, 1989). Framingham heart study shows that for every 1% increase of LDL there is an increase of 5% risk for CHD (Castelli, 1986). Plasma LDL target level of less than 100 mg/dL was recommended (Ebrahim and Balbisi, 2006). The reduction of LDL levels in individuals with and without pre-existing CHD and elevated LDL levels has been shown to reduce cardiovascular and total mortality rates (Long-term Intervention with Pravastatin Ischaemic Disease study group, 1998).

1.6 High density lipoproteins (HDL)

High density lipoprotein is synthesized in liver and small intestine, primarily protein-rich disc-shaped particles. The newly formed HDL is nearly devoid of any cholesterol and cholesteryl esters. The primary apoproteins of HDL are apo AI, apo CI, apo CII, and apo E.

Several genetic and acquired factors contribute to low level of HDL. Low level of HDL is an important risk factor for CHD, which has been found in more than 40% of patients expressing MI (Genest *et al*., 1991; Rubins *et al*., 1995). Numerous epidemiologic studies showed inverse relation between the level of HDL and occurrence of CHD (Wilson *et al*., 1988; Gordon *et al*., 1989). Epidemiological data showed 1% decrease in HDL increases the risk for CHD by 2-3%. Cardiovascular mortality was 36% higher in man with low HDL compare with normal HDL. This effect was amplified in subjects with combination of low HDL and diabetes producing 65% increase in CHD mortality (Wilson *et al*., 1988; Genest *et al*., 1991; Rubins *et al*., 1995).

Numerous studies both *in vitro* and *in vivo* support the concept of reverse cholesterol transport (RCT) (Eckardstein *et al*., 2001). In addition to RCT, several other potentially antiatherogenic activities are exerted by HDL (Nofer *et al*., 2002). Since oxidation of LDL is commonly consider as major event in the initiation and development of atherosclerosis (Diaz et al., 1997; Yla-Herttuala et al., 1999), several studies have been shown that HDL significantly reduces the oxidative modification of LDL (Hessler *et al*., 1979; Parthasarathy *et al*., 1990; Nofer *et al*., 2002). Some

protein associated with HDL has enzymatic activity and the best known are Lecithin cholesterol acetyltransferase (LACT), Cholesterolester transfer protein (CETP), and paraoxonase (PON). The antioxidant property of HDL is attributed from the PON enzyme. It has been suggested that PON inhibits LDL oxidation by hydrolyzing lipid peroxides (Mackness *et al*., 1993). The potential of PON to protect against LDL oxidative modification was reinforced by studies using mouse model (Shih *et al*., 1998; Tward *et al*., 2002).

2. Paraoxonase (PON)

PON gene family

The *PON* gene family consists of there members *PON1*, *PON2*, and *PON3*, located adjacent to each other on the long arm of chromosome 7 in humans and on chromosome 6 in mouse between q22.3 and q23.1 (Primo-Parmo *et al*., 1996). These three genes appear to be clustered (Fig. 2) (Hong-Liang *et al*., 2003).

Figure 2. *PON* gene family (Hong-Liang *et al*., 2003).

The genes share considerable structural homology and appear to have arisen by gene duplication from common evolutionary precursor**.** The three human *PON* genes share approximately 65% similarity at the amino acid level and approximately 70% similarity at the nucleotide level (Mackness B *et al*., 2002). From an evolutionary standpoint, *PON2* appears to be the oldest member, followed by *PON3* and *PON1* (see rev. Draganov *et al*., 2004).

2.1 Paraoxonase 1 (PON1)

PON1 is a calcium–dependent esterase which is highly conserved in mammals but absent in fish, birds, and invertebrates such as arthropods (La Du, 1996). It

consists of 354 amino acids with a molecular mass of 43 kDa and distributed among tissues such as liver and kidney (Fig. 3) (Durrington *et al*., 2001; Ng *et al*., 2004).

Figure 3. Expression of PON1 (Ng *et al*., 2004).

PON1 is associated with HDL in plasma. It is believed that HDL stimulates PON1 secretion from the liver and stabilizes the secreted peptide by apo AI (Sorenson *et al*., 1999; James *et al*., 2004). PON1 has an extremely hydrophobic N-terminal end that anchor it to HDL, however, PON1 is also shown to be associated with the triglycerides-rich lipoproteins, chylomicrons, and VLDL but not with LDL (Sorenson *et al*., 1999; Deakin *et al*., 2005; Fuhrman *et al*., 2005). As shown in figure 4, PON1 consists of six bladed (1-6) beta propellers with a unique active site and each blade contains four strands (A, B, C and D) and two calcium ions (Ca^{+2} 1 and Ca^{+2} 2) in the central tunnel of the propeller.

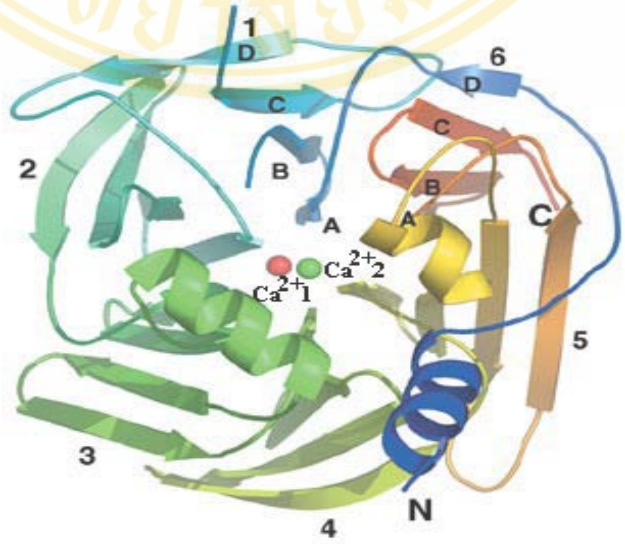


Figure 4. Overall structure of PON1 (Harel *et al*., 2004).

PON1 has three cysteine residues; cystein-42 and cystein-353 form an intramolecular disulfide linkage, while the remaining free cysteine-284 is essential for the action of PON1 to inhibit LDL oxidation (Fig. 5) (Aviram *et al*., 1998; 1999). PON1 contains two calcium binding sites, one of which is needed for its hydrolytic activity. Chelation of calcium inactivates PON1 activity and decreases its stability but not interfere PON1 to protect LDL oxidative modification (Aviram *et al*., 1998; 1999).

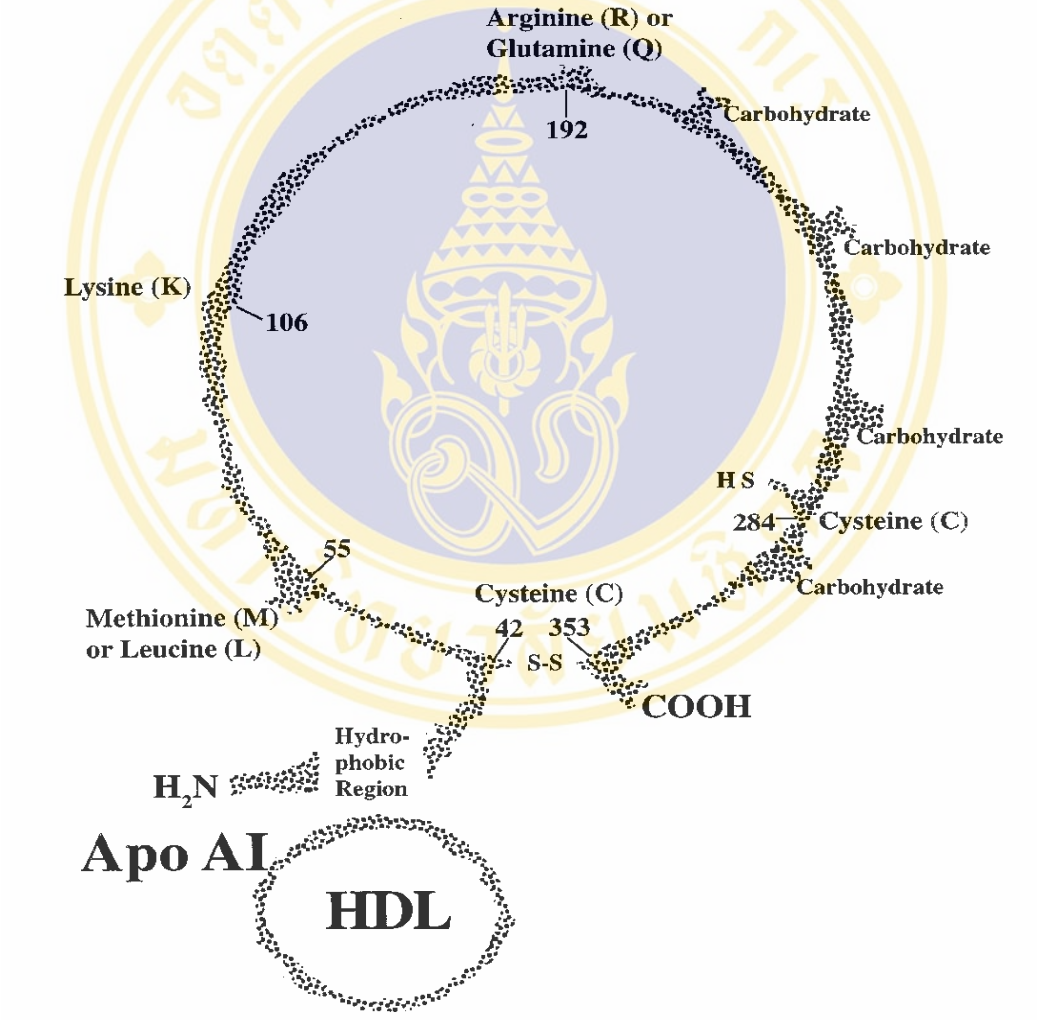


Figure 5. PON1 structure characteristics (La Du *et al*., 1999).

PON1 is primarily synthesized in liver and secrete into the plasma where it is associate with HDL. PON1 activity increases from birth to 15-25 months of age in human and reaches a plateau in adults but is not influenced by sex (Mackness B *et al*., 1998; Cole *et al*., 2003). However, wide variation (up to 13 folds) in PON1 concentration and activity between individuals even within genotypes group were reported (Richter and Furlong, 1999).

Initially, PON1 is characterized for its ability to hydrolyze organophosphates. The name paraoxonase reflects its ability to hydrolyze paraoxon, a metabolite of the insecticide parathion. In addition, PON1 has been shown to hydrolyze metabolites of number of other insecticides such as oxon and diazoxon, to detoxify various nerve agents such as sarin and soman, and also capable to hydrolyze aromatic esters preferably those of acetic acid (see rev. Mackness M *et al*., 1996). Paraoxon is the most commonly used substrates for determine PON1 enzyme activity in serum. More recently, PON1 has been shown to catalyze the hydrolysis of variety of aromatic and aliphatic lactones (Biggadike *et al*., 2000; Billecke *et al*., 2000). The specific enzymatic activities of the purified recombinant human PON are shown in table 2.

Table 2. Specific enzymatic activities of the purified human PON (Dragomir *et al*., 2005)

Substrate	PON1	PON2	PON3
Organophosphatase activity (U/mg)			
Paraoxon	1.94 ± 0.11	ND	0.205 ± 0.05
Chlorpyrifos oxon (0.32 mM)	40.9 ± 0.9	ND	ND
Diazoxon	113 ± 5	ND	ND
Arylesterase activity (U/mg)			
Phenyl acetate	1,120 ± 50	0.086 ± 0.0013	4.1 ± 0.3
p-NO_2-acetate	15.0 ± 0.03	0.7 ± 0.07	39.0 ± 4.1
p-NO_2-propionate	13.6 ± 0.04	0.96 ± 0.06	20.7 ± 3.2
p-NO_2-butyrate	1.3 ± 0.015	1.4 ± 0.03	11.4 ± 0.7
Lactonase activity (U/mg)			
Dihydrocoumarin	129.9 ± 8.30	3.1 ± 0.2	126.1 ± 12

Substrate	PON1	PON2	PON3
Homogentisic acid lactone	329.5 ± 13.1	ND	ND
γ-Butyrolactone	32.1 ± 2.73	ND	0.81 ± 0.1
γ-Valerolactone	45.0 ± 3.7	ND	6.2 ± 0.4
γ-Hexalactone	51.7 ± 4.2	ND	23.9 ± 3.2
γ-Heptalactone	57.2 ± 2.3	ND	27.7 ± 2.7
γ-Octalactone	69.2 ± 4.3	ND	25.6 ± 3.2
γ-Nonalactone	144.7 ± 11.3	ND	30.9 ± 2.7
-Decanolactone	173.8 ± 14.7	ND	45.6 ± 3.6
γ-Undecanolactone	127.6 ± 10.5	ND	71.4 ± 3.1
α-Angelica lactone	183.0 ± 16	ND	20.7 ± 3.2
γ-Phenyl-γ-butyrolactone (0.5 mM)	63.0 ± 3.1	0.68 ± 0.08	11.4 ± 0.7
α-Valerolactone	671 ± 14	ND	14.5 ± 0.7
δ-Hexalactone	72 ± 2.3	ND	11.7 ± 1.2
δ-Nonalactone	150 ± 12.3	ND	11.1 ± 0.9
δ-Decanolactone	251 ± 13	ND	44.3 ± 3.2
δ-Undecanolactone	287 ± 17	ND	84.4 ± 2.7
δ-Tetradecanolactone (0.5 mM)	154 ± 24	ND	22.7 ± 2.2
5-HETEL (10 μM)	75.4 ± 8.36	1.83 ± 0.08	27.5 ± 3.6
DL-3-Oxo-hexanoyl-HSL (250 μM)	0.0334 ± 0.0031	0.2683 ± 0.0384	ND
L-3-Oxo-hexanoyl-HSL (250 μM)		0.5080 ± 0.0661	
DL-Heptanoyl-HSL (25 μM)	0.0036 ± 0.0004	0.0311 ± 0.0026	0.0049 ± 0.0023
DL-Dodecanoyl-HSL (25 μM)	0.0167 ± 0.0005	0.4588 ± 0.0371	0.0877 ± 0.0014
DL-Tetradecanoyl-HSL (25 μM)	0.0035 ± 0.0013	0.4239 ± 0.0204	0.0255 ± 0.0003
Lovastatin (25 μM)	ND	ND	0.0266 ± 0.022
Spironolactone (25 μM)	0.0035 ± 0.0013	0.4239 ± 0.0204	0.0255 ± 0.0003
Canrenone (25 μM)	ND	ND	0.266 ± 0.022

The precise physiological role of the PON1 is still unknown. One natural physiological function of PON1 appears to be the metabolism of toxic oxidized lipids of both LDL as well as HDL particles. PON1 association with HDL in serum led to the suggestion that the enzyme might have a role in lipid metabolism and protect against atherosclerosis. The ability of HDL to prevent LDL oxidation has been found to come from PON1 (Mackness M *et al*., 1991 and 1995; Watson *et al*., 1995; Aviram *et al*., 1998 and 1999). Mackness and colleague were the first to demonstrate that purified human PON1 inhibit LDL oxidation *in vitro*. PON1 is also able to protect HDL oxidation and preserve its functions (Aviram *et al*., 1998). In the mean time, various studies demonstrated that PON1 both prevent the formation of oxidized LDL (Ox-LDL) and inactivates oxidized LDL once they are formed (Mackness M *et al*., 1991; Watson *et al*., 1995; Mackness B *et al*., 1998). The antiatherogenic role of PON1 is further supported by the study in mouse model. *PON1* knockout mice are more susceptible to develop atherosclerosis than wild type mice, and their HDL, fails to prevent LDL oxidation (Shih *et al*., 1998). High density lipoprotein isolated from mice over-expressing PON1 was more resistant to lipid peroxidation and developed smaller atherosclerotic lesion compared to control mice (Shih *et al*., 2002). These findings suggest a protective role of PON1 in CVD and atherosclerosis.

2.1.1 PON1 as antiatherogenicity

Although it has been recognized that plasma PON1 plays an important role in organophosphate metabolism, the role of PON1 in lipid metabolism is a relatively new area of investigations. The demonstrations of PON1 inhibit LDL oxidation led to an explosion of interest in the enzyme's possible role in atherosclerosis. Both *in vitro* and *in vivo* studies have demonstrated the capacity of PON1 to prevent LDL and HDL oxidation by variety of pro-oxidant factors, including cell induced LDL oxidation. In *in vitro* study, supplementation of human HDL or whole serum with purified PON1 significantly inhibit copper induced lipoprotein oxidation in a concentration dependent manner, while adding PON1 specific and non competitive inhibitor enhanced HDL oxidation that induced by either copper or free radical generating system (Aviram *et al*., 1998; Mackness M *et al*., 1998). Both HDL associated and purified PON1 significantly protect human LDL oxidative modification as well as HDL from

oxidation and prevent its other antiatherogenic properties (Aviram *et al*., 1998; Mackness *et al*., 1998). It is believes that PON1 in HDL protect against the induction of inflammatory responses in cells of arterial wall by destroying biologically active lipids in mildly Ox-LDL. It has been shown that purified PON1 prevents proinflammatory effects of Ox-LDL when incubated in a vascular cell coculture system. This may be probably due to the metabolism of oxidized arachidonic acid derivatives in the LDL phospholipids (Watson *et al*., 1995; Shih *et al*., 1998; Aviram *et al*.1998, 2000). The precise mechanism by which PON1 prevent lipoprotein oxidative modification is still unclear. However, it is convincingly believed that PON1 prevents LDL oxidation by hydrolyzing lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxides (Mackness M *et al*., 1993). This prevention may be made possible by the free sulphydryl group at cysteine 284 of PON1 (Watson *et al*., 1995; Aviram *et al*.1998). As shown in figure 6, it has been proposed that PON1 is capable of blocking each and every step in the formation of macrophage foam cell by hydrolysis of macrophage oxidized lipid, reducing macrophage-mediated oxidation of LDL, and reducing Ox-LDL level (Navab *et al*., 2002; Aviram *et al*., 2004). Therefore, PON1 is believed as antiatherogenic.

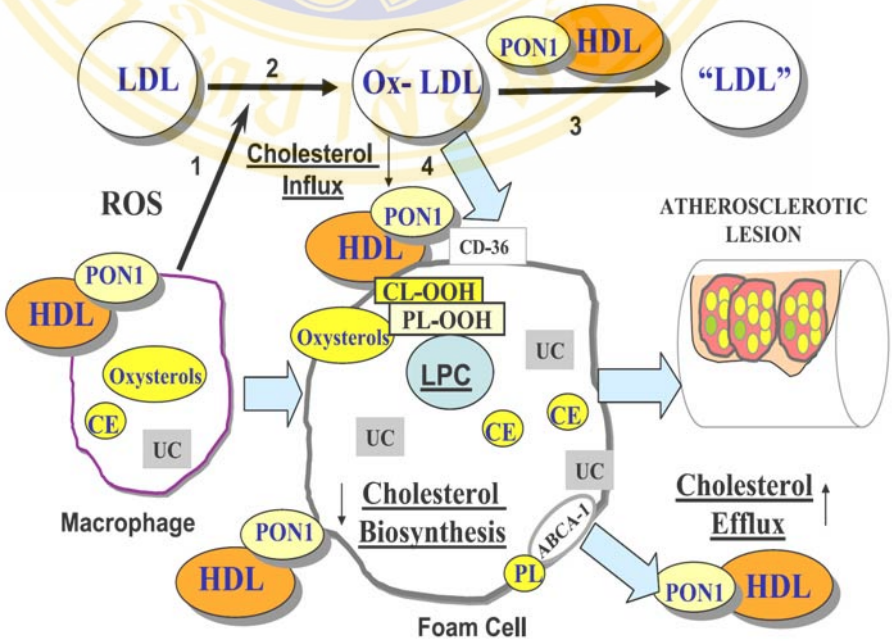


Figure 6. PON1 as antiatherogenicity (Aviram *et al*., 2004).

2.1.2 *PON1* polymorphisms

The *PON1* gene contains several polymorphisms in both coding and regulatory region as demonstrated in figure 7.

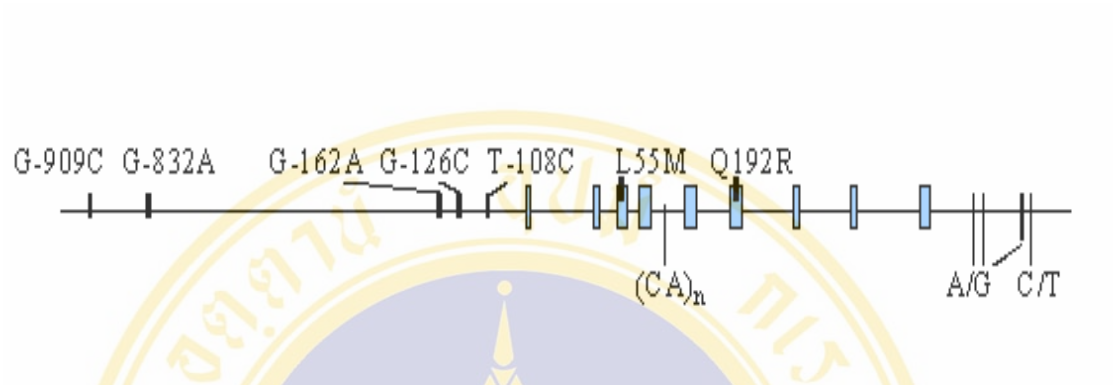


Figure 7. *PON1* polymorphisms structure (Brophy *et al*., 2002).

The *PON1* gene has two common polymorphisms in the coding region, glutamine to arginine substitution at position 192 (Q192R) and leucine to methionine substitution at position 55 (L55M) (Adkins *et al*., 1993; Humbert *et al*., 1993). These two polymorphisms independently influence PON1 activity and has been defined as the molecular basis for the inter-individual variability (Adkins *et al*., 1993; Humbert *et al*., 1993). Individuals with *PON1* 55M have been found to have lower level of PON1 activity than *PON1* L55 (Blatter *et al*., 1997; Brophy *et al*., 2001). The *PON1* Q192 R polymorphism has been found to affect the PON1 activity in different degree towards various substrates including paraoxon, diazoxon, soman, and serin (Davies *et al*., 1996). The frequencies of the *PON1* alleles vary greatly across human populations. *PON1* 55M allele was higher in white than blacks, whereas the frequency of the *PON1* 192R allele was higher in blacks than white (Leviev *et al*., 2000, 2001).

The five polymorphisms of *PON1* in the regulatory region are -106/-108, -126, -160/-162, -824/-832, and -907/-909 (Leviev *et al*., 2000; Suehiro *et al*., 2000).These polymorphisms exert different effects on PON1 expression. The variants T-107, G-824, and C-907 were found to be associated with lower serum PON1 levels, while -107C, -824A and -907G were correlated with the highest PON1 concentration and activity (Leviev *et al*., 2000; Suehiro *et al*., 2000).

2.1.3 Association of PON1 with CHD/CVD

There has been an explosion of interest in the PON1 role in atherosclerosis and large numbers of population association study involving *PON1* gene polymorphisms and PON1 activity have been continuously reported. The CHD risk associated with the *PON1* Q192R and L55M polymorphisms remain unclear. In recent years, many studies had reported the results of their case control studies conducted in patient groups of different ethnic origin on the relationship between the *PON1* Q192R polymorphism and the presence of CHD. Some studies indicated significant association with CHD (Osei-Hyiaman *et al*., 2000; Ito 93 *et al*., 2002), however, other studies did not find any association between *PON1* Q192R polymorphism and CHD risk (Hangel *et al*., 1999; Watzinger *et al*., 2002). Few studies reported an association between *PON1* L55 allele and CHD but others found no significant association (Mackness *et al*., 2004). *PON1* T-107C polymorphism in the regulatory region significantly affects PON1 levels, and found to be a risk factor of CHD (Mackness M and Mackness B, 2004). Moreover, a large meta-analysis of 43 genetic association studies had elucidated an association between *PON1* 192R and CHD, while there was no significant association between *PON1* 55M as well as T-107 and CHD (Wheeler *et al*., 2004). A prospective study involved 3,052 men ages 50 to 60 years with CHD events of 6 years found no direct positive association between the *PON1* L55M and *PON1* Q192 R polymorphisms and CHD (Robertson *et al*., 2003). A few studies on PON1 activity and concentration found that phenotype but not genotype is associated with CHD. In addition, Mackness and colleague suggested that PON1 concentration and activity is better predictor of the risk for atherosclerosis than the *PON1* genotype (see rev. Mackness M *et al*., 2004).

Various clinical epidemiological studies reported human serum PON1 activities were shown to be inversely related to the risk of CHD and low PON1 activities were observed in atherosclerotic, hypercholesterolemia, and diabetic patients (Mackness M *et al*., 1991; Abbott *et al*., 1995; Boemi *et al*., 2001; Mackness B *et al*., 2001 and 2003). A study in a large population found that the activity and concentration of PON1 were significantly lower in the population with CVD and none of the *PON1* polymorphisms, whether considered single or in combination, were associated with CVD (Jarvik *et al*., 1995). So far, the only one prospective study

reported on the relationship between PON1 activity and CHD, is Caerphilly prospective study (Mackness B *et al*., 2003), which involved 1,338 men ages 50-65 years for 10 years study. PON1 activity was 30% lower in men who had a new coronary event than in those who did not. PON1 activity predicted coronary events independent of all other coronary risk factors, including HDL (Mackness B *et al*., 2003).

2.1.4 Influences and modulation of PON1

As the PON1 activity is inversely correlated to the risk of atherosclerosis, the factors affecting PON1 activity and expression level were vital for a better understand. *PON1* gene promoter polymorphisms account for approximately 25% of variation in serum PON1 concentration. In addition, PON1 levels can also be modified by acquired factors such as diet, life style, and diseases. Numerous studies shown that PON1 activity decreases with age and activity is reduced in diabetic patients (James and Leviev, 2000; Boemi *et al*., 2001; Kordonouri *et al*., 2001; Mackness *et al*., 2001; Senti *et al*., 2003; Xia *et al*., 2003; Seres *et al*., 2004) with some dissension (Sozmen *et al*., 1999; Kopprasch *et al*., 2003), in patients with metabolic syndrome, vascular dementia, Alzheimer's disease, renal failure, liver cirrhosis, chronic hepatitis, and anxiety (Dantoine *et al*., 1998; Ferre *et al*., 2001; Scacchi *et al*., 2003; Senti *et al*., 2003; Sklan *et al*., 2004). Besides these, various environmental and pharmacological compounds also affect the PON1 level.

2.1.4.1 Environmental factors

Diets

Diets with a high *trans*-unsaturated fat content reduce PON1 activity (De Roos *et al*., 2002). In contrast, oleic acid from olive oil associates with increase PON1 activity (Tomas *et al*., 2001; Wallace *et al*., 2001). Meals rich in used cooking fat, which contains a high content of oxidized lipids, were followed by significant fall in PON1 activity when fed to healthy man (Sutherland *et al*., 1999). Administration of an omega-3-polysaturated fatty acid to a group of 14 patients with familial combined hyperlipidaemia for 8 weeks, resulted significant increase in PON1 concentration (Calabersi *et al*., 2004). Studies had shown that consumption of pomegranate juice,

rich in polyphenol, and other antioxidants raises PON1 activity up to 20% in human (Kaplan *et al*., 2001). A study in white man found a positive correlation between the dietary and medicinal intakes of vitamin C and vitamin E with the level of serum PON1 activity (Jarvik *et al*., 2002). In contrast to this finding, two earlier studies in Finish population reported that high intake of vegetables, possibly rich in vitamin C and E, were negatively correlated with serum PON1 activity (Kleemola *et al*., 2002; Rantala *et al*., 2002). In addition, another study among Spanish population also reported no association between vitamin intake and serum PON1 level (Ferre *et al*., 2003).

Smoking

Cigarette smoke inhibits PON1 activity (Nishio *et al*., 1997). Compounds suggested to be responsible for inhibition of PON1 activity are various reactive aldehydes, as well as aromatic hydrocarbons (Nishio *et al*., 1997). Four studies in human confirmed that smoking reduced serum PON1 activity (James *et al*., 2000; Jarvik *et al*., 2002; Ferre *et al*., 2003; Senti *et al*., 2003). The effect of smoking appears to reverse within a relatively short time. Interestingly, smokers who also drank moderately or exercised regularly had PON1 levels similar to those of non smokers (Senti *et al*., 2003; Boemi *et al*., 2004).

Alcohol

It has been found that human PON1 is inhibited by ethanol and other aliphatic alcohols (Debord *et al*., 1998; James *et al*., 2000; Ferre *et al*., 2003). Two studies in healthy men showed an increase in PON1 activity following moderate alcohol consumption as wine or beer (Debord *et al*., 1998). Another study suggested that light drinkers had 39% higher, whereas heavy drinkers had 45% lower serum PON1 activity compared to non drinkers (Vandergaag *et al*., 1999; Sierksma *et al*., 2002) but there were no association between alcohol consumptions and serum PON1 activity were found in another study (Sarandol *et al*., 2003).

Environmental toxins

PON1 protect animals against organophosphate (OP) poisoning. Two recent publications had suggested that exposure to environmental toxins can also have an impact on serum PON1 activity (Sozmen *et al*., 2002; Serhatlioglu *et al*., 2003). Twenty eight patients exposed to OP poisoning were found to have lower PON1

activity than controls. At 6 month after poisoning, the levels of PON1 return to normal. This suggests that the toxins exert a temporary inhibition of PON1 (Sozmen *et al*., 2002). PON1 activity was 30% lower in workers exposed to radiation than in the non-exposed group (Serhatlioglu *et al*., 2003).

2.1.4.2 Pharmacological compounds

A number of clinical studies had been carried out to examine the effect of lipid lowering drugs on PON1 activity and concentrations. Such studies with statin and fibrates yielded somewhat conflicting results however, recently, more detailed studies of their potential role in the regulation of *PON1* gene expression has been undertaken. Simvastatin is found to up-regulate *PON1* promoter activity in hepatocellular carcinoma cells (HepG2) (Deakin *et al*., 2003). In another study**,** two oxidized metabolites of atorvastatin and gemfibrozil, but not the parent compounds were found to increase PON1 activity (Aviram *et al*., 1998). Studies in human had provided contrasting results**.** Increased in serum PON1 activity was found in patients treated with simvastatin, atorvastatin, gemfibrozil, and micronized fenofibrate (Paragh *et al*., 2000, 2003; Tomas *et al*., 2000) whereas, no significant change on PON1 activity was reported by other studies in patients treated with simvastatin (Balogh *et al*., 2001), bezafibrate, and gemfibrozil (Durrington *et al*., 1998).

2.2 PON 3

Paraoxonase 3 (*PON3*) gene is interposed between *PON1* and *PON2* in the *PON* gene cluster. PON3 is most recently identified member with 40 kDa protein that associated with HDL in the circulation but absent in LDL **(**Reddy *et al*., 2001; Ng *et al*., 2004**)**. PON3 shows high similarity in structure and functions with PON1, however, it is not clear whether the PON1 and PON3 proteins coexist on the same or different HDL particles (Reddy *et al*., 2001). PON3 is synthesized primarily in liver, however, significant *PON3* mRNA level is also detectable in kidney (Reddy *et al*., 2001). Mature human PON3, as like PON1, contains the N-terminal hydrophobic peptide and share there conserved cysteine residues: Cystein-42, Cystein-284 and Cystein-353. The Cystein-42 and Cystein-353 form an intramolecular disulphide bond

and Cystein-284 is free in active PON. The free sulphydryl group is not required for PON1's arylesterase activity but is very important for its antioxidant activity (Aviram *et al*., 1998). So, it is believes that PON3 acts as an antioxidant like PON1. Previous report showed that PON3 possesses antioxidant properties and can hydrolyze a variety of lactones, cyclic carbonates esters, and metabolize the drugs (Draganov *et al*., 2000; La Du *et al*., 2001; Reddy *et al*., 2001).

Reddy and colleague previously reported that LDL incubated with stably transfected cells over-expressing human *PON3* had significantly less lipid hydroperoxide and less ability to induce monocyte chemotactic activity (Reddy *et al*., 2001). Draganov and colleague reported that purified rabbit PON3 inhibits copper-induced LDL oxidation *in vitro* and PON3 is approximately 100 times more potent than PON1 in protecting LDL against oxidation (Draganov *et al*., 2000).

In another study, Rosenblat and colleague demonstrated that presence of PON3 in mouse macrophage which was affected directly by oxidative stress but PON3 was absence in human macrophage and suggested it may act like PON1 in circulation. They found that under oxidative stress, *PON3* mRNA levels keep unchanged but activity was significantly increased (Rosenblat *et al*., 2003).

In contrast to PON1, PON3 has very limited arylesterase and no paraoxonase activities but rapidly hydrolyzes variety of lactonases such as statin prodrug. PON3 lactonase activity has been described by Draganov *et al.* (2000) in rabbit and hypothesized by Reddy *et al.* (2001) in humans. The data suggested that PON3 also protect vascular damage by hydrolyzing potentially toxic endogenous lactones. In addition, the presence of significant amounts of *PON3* mRNA in kidneys suggests that PON3 protein may play a role in the lipoprotein metabolism of the kidney (Hong-Liang *et al*., 2003). These observations indicate human PON3 possesses potent antioxidant capabilities and prevent formation and progression of atherosclerotic lesion which may distinct mechanism from PON1.

Polymorphism on *PON3* has not been found up to this time. Recently, Wang and colleague reported a study on *PON3* gene in samples of Chinese population, but they did not found any missense polymorphisms (Wang *et al*., 2003), however, Campo and colleague identified serine to threonine substitution at codon 311 (S311T) and a glycine to aspartic acid substitution at codon 324 (G324D) in population of

southern Italy (Campo *et al*., 2004). Even though, to date, the functional consequence of these two polymorphisms has not been elucidated.

2.3 PON 2

Human PON2 is widely expressed intracellular protein with molecular mass 44 kDa and found in variety of tissues including heart, kidney, liver, lung, placenta, small intestine, spleen, stomach, testis, in the cells of artery wall, and macrophages. PON2 is not presence either in HDL or LDL but express in tissue throughout the body, so it is likely that PON2 play a role in reducing intracellular or local oxidative stress (Ng *et al*., 2001). Phylogenetic analysis reveals *PON2* to be the oldest member of the *PON* gene family, while very little has known about the physiologic or pathophysiologic role of this recently identified protein (Hassett *et al*., 1991). The specific substrate and exact physiological function of PON2 is not known, however, dihydrocoumarine and some arylester substrates are reported to date (Dragomir *et al*., 2005).

Since PON2 shows high similarity in their structural characteristic with PON1 it was speculated that PON2 may act as antioxidant like PON1 and has been reported that PON2 possesses antioxidant property. Rosenblat and colleague demonstrated that purified recombinant PON2 protects against LDL oxidation (Rosenblat *et al*., 2003). Low density lipoprotein incubated with stably transfected cells over-expressing *PON2* had lower levels of lipidperoxide and was less able to induced monocyte transmigration through endothelial cells. They clearly demonstrated that the presence of *PON2* expression, protein, and enzymatic activity in macrophages (Ng *et al*., 2001; Rosenblat *et al*., 2003). Both *in vitro* and *in vivo* study found that under oxidative stress *PON2* expression and enzymatic activity increased. Stably transfected cells over-expressing *PON2* also exhibit significantly lower level of intracellular oxidative stress when expose to oxidized phospholipids (Ng *et al*., 2001). Furthermore, it had shown that PON2 was able to retard the oxidation of preformed mildly Ox-LDL (Ng *et al*., 2001). Since increased oxidative stress was observed in hypercholesterolaemic patients and consequently *PON2* expression was lower by 100% and activity was lower by 40% in patient's macrophage than healthy subject's macrophage, it could be because of increased macrophage cholesterol content (Aviram and Rosenblasst, 2004).

Treatment of mouse peritoneal macrophage with various oxidative stress inducing agents resulted in an increase in *PON2* expression and lactonase activity. Also in human, atorvastatin therapy reduced both cellular oxidative stress and cholesterol content leading to up-regulated macrophage *PON2* expression and activity (Rosenblat *et al*., 2004). Thus, it is believed that one function of PON2 may be act as a cellular antioxidant, protecting cells from oxidative stress and its consequent in prevention of atherosclerosis development.

2.3.1 Association of PON2 with CHD and other diseases

PON2 is the second member of the *PON* gene cluster on chromosome 7 q21.3-22.1. The human *PON2* gene has two common polymorphisms, both of which give rise to amino acid substitute with an alanine or glycine at codon 148 (A148G) and cysteine or serine at 311 position (C311S) (Fig. 8) (Mochizuki *et al*., 1998).

Figure 8. *PON2* polymorphisms structure.

The *PON2* A148G polymorphism is associated with variations in total and LDL cholesterol levels, fasting plasma glucose levels, and birth weight. In addition, the polymorphism at position *PON2* C311S is associated with CHD, ischemic stroke in patients with type 2 diabetes mellitus, late onset Alzheimer's disease, and reduced bone mass in postmenopausal women (Janka *et al*., 2002; Kao *et al*., 2002; Pan *et al*., 2002; Chen *et al*., 2003; 2003; Wang *et al*., 2003; Yamada *et al*., 2003).

There are few published reports of an association of *PON2* gene polymorphisms and CHD. Recently, three reports showed that the *PON2* 311S polymorphism associated with CHD (Leus *et al*., 2002; Pan *et al*., 2002; Chen *et al*., 2003) but one report revealed that *PON2* polymorphism was not associate with CHD (Pinizzotto *et al*., 2001). In one study, CHD risk associated with *PON2* 311S allele was confined to *PON1* 192R allele carriers (Sanghera *et al*., 1978), while in another study, CHD risk associated with the *PON2* 311S allele was confined to *PON1* Q192

allele carrier subjects (Leus *et al*., 2002). Based on observation found in these studies lead to speculate that the *PON2* gene is responsible gene rather than the *PON1* gene for the association with CHD.

Several studies have reported the relationship between *PON2* polymorphism and disease other than CHD. Hangel and colleague found that homozygous for the *PON2* allele encoded A148 and 311S had significantly high plasma cholesterol and apoB than subjects with the other two genotypes (Hangel *et al*., 1999). They also found that codon 148 polymorphism of *PON2* was significantly associated with variation in fasting plasma (Hangel *et al*., 1999). Buesh and colleague found that the *PON2* A148 and 311S was associated with lower birth weight in neonates of South Asian origin but not in neonates of African origin (Busch *et al*., 1999). Pinizzotto and colleague found strong positive association between *PON2* polymorphism and the presence of diabetic complication leading nephropathy and retinopathy (Pinizzotto *et al*., 2001). In contrast, Kao and colleague reported no association of *PON2* genotype with retinopathy (Kao *et al*., 2002).

3. Atorvastatin

On the basis of clinical trial evidence, the most commonly prescribed lipid modifying therapies are the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, more commonly known as the statin. Hydroxymethylglutaryl coenzyme A reductase catalyses the conversion of HMG-CoA to mevalonate, the rate-limiting step in *de novo* cholesterol synthesis (Marron *et al*., 2000). Competitive inhibition of this enzyme by the statin decreases hepatocyte cholesterol synthesis (Fig. 9) (Michael *et al*., 2004).

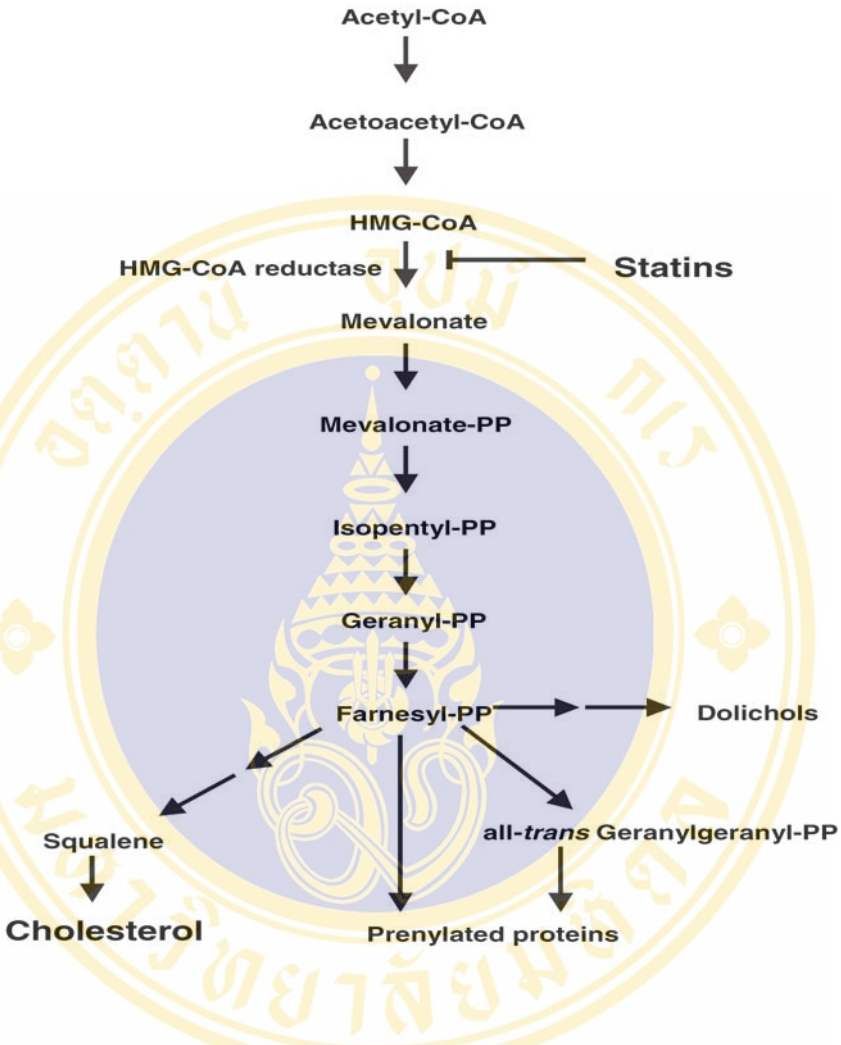


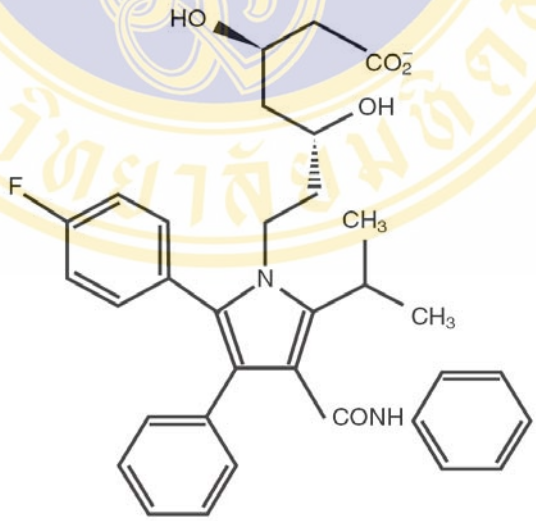
Figure 9. Site of action of statin on *de novo* cholesterol synthesis (Michael *et al*., 2004).

Statin also shows beneficial effect on other lipid parameters, including increases in HDL and decreases in TG. In general, statin is regarded as a remarkably safe and well tolerated class of drugs. Seven statins are now approved for clinical uses, which are atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, simvastatin, rosuvastatin, and pitavastatin (Davidson, 2002). Although all statins share a common mechanism of function, they differ in terms of their chemical structures, pharmacokinetic profiles, and lipid modifying efficacy (Michael, 2004). Clinical studies have demonstrated that atorvastatin is the choice of stain because of its pharmocokinetc profile and efficacy (Michael, 2004).

Atorvastatin is a synthetic lipid-lowering agent which inhibits HMG-CoA reductase, an early and rate-limiting step in cholesterol biosynthesis. Atorvastatin is used to reduce high level of cholesterol and other fat-like substances in the blood by reducing the production of cholesterol and increasing its rate of removal from the body (Istvan *et al*., 2001).

3.1 Chemistry and biological properties

Atorvastatin calcium is a fully synthesized compound with a white to off-white crystalline powder of pH 4. Atorvastatin is a relatively lipophilic compound, very slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile, slightly soluble in ethanol, and freely soluble in methanol (Michael, 2004). Atorvastatin contain a complex hydrophobic ring structure that is covalently linked to the substrate analogue and is involved in binding of the statin to the reductase enzyme, while the side group of atorvastatin on the ring defines solubility properties of the drug (Gaw *et al*., 2000; Istvan *et al*., 2001; Michael, 2004) (Fig. 10).



Atorvastatin

Figure 10. Chemical structure of atorvastatin (Michael, 2004).

3.2 Pharmacodynamic properties

Atorvastatin as well as some of its metabolites are pharmacologically active in human. The lipid modifying effects of atorvastatin are characterized by a reduction of TC, LDL, and TG levels and an increase in HDL levels. Atorvasttain also exerts pleiotropic effects on vascular cells. Statin acts as antioxidants, either directly or indirectly and may have beneficial effect on atherosclerosis apart from their effect on plasma cholesterol level, which may involve mechanisms that modify endothelial function (Egashira *et al*., 1994; Straznicky *et al*., 1995; Treasure *et al*., 1995; Aengevaeren, 1999; Feron *et al*., 2001), inflammatory response, plaque stability, and thrombus formation (Notarbartolo *et al*., 1995; Vaughan *et al*., 1996; Rosensen *et al*., 1998). Since oxidative stress was positively related with atherosclerosis, recently, various studies showed that atorvastain increases total antioxidant status with increasing some HDL associated antioxidant enzyme such as paraoxonase and reduced the oxidative stress in hypercholesterolaemic patients (Fuhrman *et al*., 2002; Kural *et al*., 2004; Paragh *et al*., 2004). Atorvastatin is predominantly metabolized by the cytochrome P3A4 (CYP3A4), family of enzymes in the cytochrome P450 (Bottorff *et al*., 2000). The major active metabolites of atorvastatin are 2-hydroxy- and 4-hydroxy-atorvastatin acid (Jacobsen *et al*., 2000).

3.3 Pharmacokinetic properties

Atorvastatin administrate orally as the active hydroxyl acid (Corsini 200 *et al*., 1999), is absorbe rapidly reaching peak plasma concentration within 4 hours (Cilla *et al*., 1996). Presently available statins including atorvastatin possesses low systemic bioavailability, i.e. 12 % (see rev. Lennerna *et al*., 2003). Since liver is the target organ for statin, efficient first-pass uptake may be more important than high bioavailability for its effect. Food intake has an effect on atorvastatin absorption causing the reduction in its bioavailability (Radulovic *et al*., 1995).

All statin including atorvastatin are highly bound to plasma proteins and as a result systemic exposure to unbound, pharmacologically active drug is relatively low (Corsini 200 *et al*., 1999). It is bound more than 98% to plasma proteins. Based on observation in rats, atorvastatin is likely to be secreted in human milk. The predominant route of elimination of atorvastatin is via the bile after metabolism by

liver (Knopp *et al*., 1991). The elimination half-life of atorvastatin is approximately 14 hours (Cilla *et al*., 1996). Thus, hepatic dysfunction is a risk factor for statin induced myopathy. Less than 2% of a dose of atorvastatin is recovered in urine following oral administration (see rev. Maron *et al*., 2000).

3.4 Clinical efficacy

Atorvastatin is highly efficacious at lowering TC and reduces LDL in patients with homozygous FH, a population that rarely responds to other lipid-lowering medication(s) (Gaw *et al*., 2000). The effect of atorvastatin on inhibition of cholesterol synthesis has been demonstrated in primary rat hepatocytes, exhibit a 50% inhibitory concentration (IC_{50}). In heterozygous FH in pediatric patients, atorvastatin significantly reduced TC of 31.4%, LDL 39.6%, TG 12%, apoB 34%, and increase HDL 2.8% (Michael, 2004). Edwards and colleague found 26% reduction of total cholesterol, 36% reduction of LDL, 17% reduction of TG whereas 7% increase of HDL in hypercholesterolemia patients (Edward *et al*., 2003).

Beside reduction of TC and LDL, atorvastatin reduced the morbidity and mortality by CHD and CVD among the patients with normal lipid levels, suggesting atorvastatin may involve mechanisms that modify endothelial function, inflammatory responses, plaque stability, and thrombus formation (Mayer *et al*., 1992; Lacoste *et al*., 1995; Notarbartolo *et al*., 1995; Vaughan *et al*., 1996; Rosensen *et al*., 1998; Feron *et al*., 2001). Moreover, atorvastatin and its metabolite exert pleiotropic properties including increasing antioxidant capacity, reduced oxidative stress (Fuhrman *et al*., 2002; Kural *et al*., 2004; Paragh *et al*., 2004), and increased PON activity (Fig. 11) (Rosenblat *et al*., 2004; see rev. Michael *et al*., 2005).

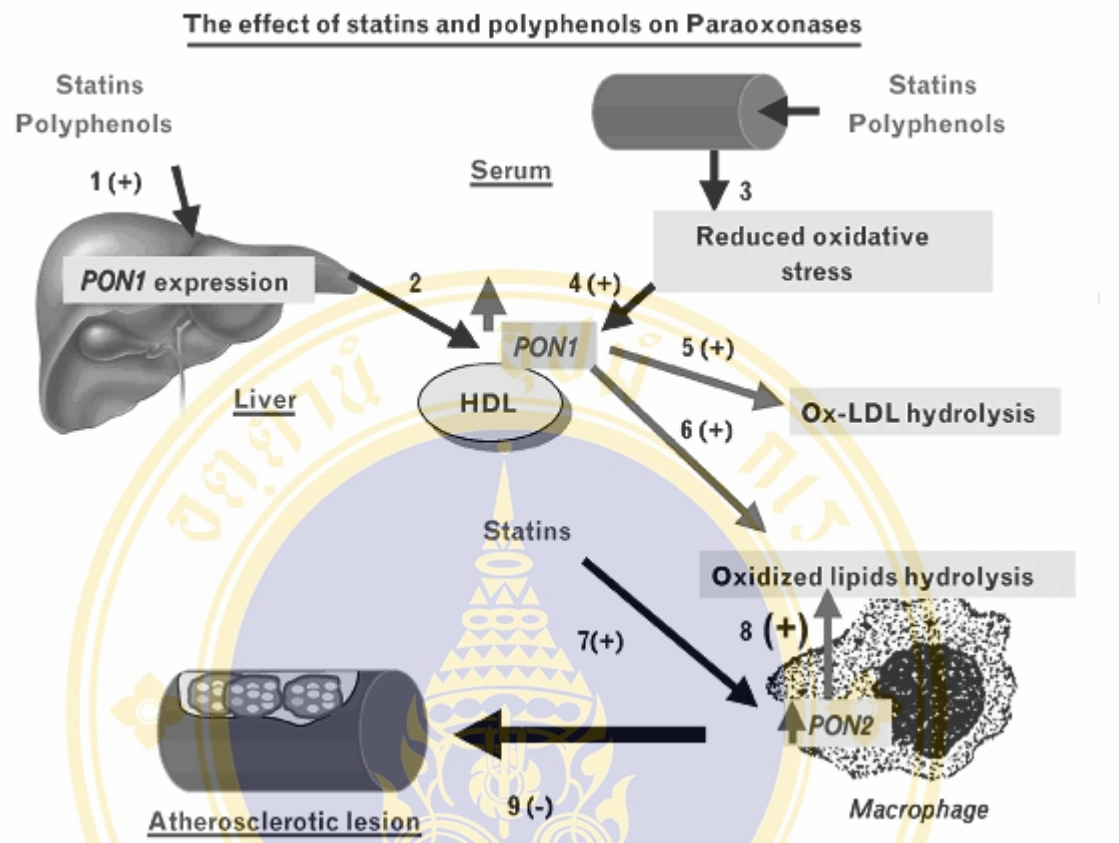


Figure 11. The effect of statin on PON (see rev. Michael *et al*., 2005).

3.5 Tolerability

In general, atorvastatin is well tolerated and serious adverse events are rare (Black *et al*., 2002). The most serious adverse effect associate with atorvastatin is myopathy, which may progress to fatal or nonfatal rhabdomyolysis. Statin-induced myopathy was seen more commonly in patients with hypothyroidism or myopathic conditions. Other conditions that may predispose to the occurrence of myopathy and rhabdomyolysis include electrolyte disorders, major trauma, seizures, hypothermia, metabolic acidosis, viral infections, and drugs abuse (Black *et al*., 2002; Ballantyne *et al*., 2003). The incident of myopathy is low (approximately 1 in 1000 patients treated), dose related, and increased when used in combination with agents that share common metabolic pathway (Ballantyne *et al*., 2003). A recent clinical trial among 83,585 patients randomly assign to statin or placebo in a total of 30 studies, 49 cases of

myositis and seven cases of rhabdomyolysis were reported in a statin group compared with 44 and 5 cases, respectively, in the placebo groups (Thompson *et al*., 2003). An analyzed pooled data from 44 clinical trials of atorvastain, involving 16,495 patients demonstrated serious adverse event was rare and reported less than 1% of patients treated atorvastatin. No deaths were considered related to treatment (Brewer *et al*., 2003; Newman *et al*., 2003).

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Chemicals

Chemicals used in determination of conjugated diene (CD)

- Methanol [Merck]
- Chloroform [Merck]
- Potassium Chloride (KCl) [Merck]
- Cyclohexane [Merck]

Chemicals used in determination of total peroxide

- Butylated hydroxytoluene (BHT) [Sigma]
- Sulphuric acid (H_2SO_4) [Merck]
- Ammonium ferrous sulphate [Sigma]
- Xylenol orange [Sigma]
- Hydrogen peroxide (H_2O_2) [Carloerba]

Chemicals used in determination of malondialdehyde (MDA)

- n-Butanol [Merck]
- Hydrochloric acid (HCl) [Fluka]
- Sodium hydroxide (NaOH) [Merck]
- Trichloroacetic acid (TCA) [Merck]
- Thiobarbituric acid (TBA) [Sigma]
- 1,1,3,3-Tetramethoxypropane (TMP) [Sigma]

Chemicals used in determination of total antioxidant status (TAS)

- Sodium acetate (CH_3COONa) [Merck]
- Glacial acetic acid [Merck]

- 2, 2’ azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) [Sigma]
- Hydrogen peroxide (H_2O_2) [Carloerba]

Chemicals used in determination of paraoxonase 1 (PON1) activity

- Phenyl acetate [Merck]
- Diethyl *p*-nitrophynyl phosphate (paraoxon) [Sigma]
- Sodium chloride (NaCl) [Merck]
- Calcium chloride ($CaCl_2$) [Merck]
- Tris [hydroxymethyl] aminomethane hydrochloride [Sigma]

Chemicals used in determination of paraoxonase 2 (PON2) activity

- *p*-nitrophenyl butyrate [Sigma]
- Bovine serum albumin (BSA) [Sigma]
- Brilliant Blue G-250 [Sigma]
- Ethanol [Merck]
- Methanol [Merck]
- Phospheric acid [Merck]
- Histopaque [Sigma]
- Disodium phosphate (Na_2HPO_4) [Merck]
- Potassium dihydrogen phosphate (KH_2PO_4) [Fluka]
- di-Potassium hydrogen phosphate (K_2HPO_4) [Fluka]
- Sodium chloride (NaCl) [Merck]
- Calcium chloride ($CaCl_2$) [Merck]
- Tris [hydroxymethyl] aminomethane hydrochloride [Sigma]

Chemicals used in determination of paraoxonase 3 (PON3) activity

- *P*-nitrophenyl butyrate [Sigma]
- Disodium phosphate (Na_2HPO_4) [Merck]
- Potassium dihydrogen phosphate (KH_2PO_4) [Fluka]
- Sodium chloride (NaCl) [Merck]
- Calcium chloride ($CaCl_2$) [Merck]
- Tris [hydroxymethyl] aminomethane hydrochloride [Sigma]

Chemicals used in DNA extraction

- Ethylene diamine tetraacetic acid disodium salt (EDTA) [Merck]
- Sodium dodecyl sulphate (SDS) [Sigma]
- Sodium citrate [BDH]
- Sodium chloride (NaCl) [Merck]
- Sodium hydroxide (NaOH) [Merck]
- Hydrochloric acid (HCl) [Merck]
- Tris base [Sigma]
- Boric acid [BDH]
- Ethanol [Merck]
- Isoamyl alcohol [Merck]
- Chloroform [Merck]
- Proteinase K [Sigma]

Chemicals and primers used in determination of *PON1* and *PON2* genotypes by restriction fragment length polymorphism (RFLP)

- Magnesium chloride ($MgCl_2$) [Fermentas]
- Taq polymerase [Fermentas]
- Agarose gel [Bio-Active]
- dNTP [Fermentas]
- Ethedium bromide (EtBr) [sigma]
- 6X loading dye [Fermentas]
- 10X Taq buffer with $(NH_4)_2SO_4$ [Fermentas]
- 50 base pair (bp) DNA marker [Fermentas]
- Primers (Gibco / Invitrogen) see table 3.
- Restriction endonuclease (NEB) see table 3.

1.2 Drugs

- Atorvastatin 10 mg and 20 mg

1.3 Water

We used autoclaved 18 Ω deionized water for preparing the reagents used in DNA extraction and genotype determination. The reagents for the all other tests were prepared by using 18 Ω deionized water without autoclaved.

Table 3. Primers and restriction enzymes used for *PON* genotype

Genotype	Primers	Restriction Endonucleases
PON1 L55M	Forward primer 5' CCT GCA ATA ATA TGA AAC AAC C 3' Reverse primer 5' GAA AGA CTT AAA CTG CCA G 3'	Nla III 5'…CATG▼…. 3' 3'…▲GTAC…5'
PON1 Q192R	Forward primer 5' TAT TGT TGC TGT GGG ACC TGA G 3' Reverse primer 5' CCA CTA CAT TTC AGA GAG TTC AC 3'	Alw I 5'…GGATC(N)▼…3' 3'…CCTAG(N)▲…5'
PON1 C-107T	Forward primer 5' GCT AGC TGC GGA CCC GGC GGG GAG GA 3' Reverse primer 5' GCT GCA GCC CTC ACC ACA ACC 3'	BsrB I 5'….CCG▼CTC…. 3' 3'…GGC▲GAG... 5'
PON2 C311S	Forward primer 5' CAA CAG CAT GTC CCC TTA ATC 3' Reverse primer 5'GGC TAC AGA ACT TCC TTG GAG 3'	Dde I 5'…C▼TNAG…..3' 3'…..GANT▲C... 5'

1.4 Instruments

The following listed equipments were used in this study.

- Chemistry auto-analyzer [Olympus]
- UV-spectrophotometer [UV-2450, Shimadzu]
- Backman spectrophotometer [S250, Secomam]
- Refrigerator microcentrifuge [Thermo electron]
- Centrifuge [Dynac]
- Polymerase chain reaction (PCR) machine [Bio-Rad]
- Gel electrophoresis [Home made]
- UV translluminator gel documentation [Syngene]
- Freezer -80 ˚C, -20 °C [Thermoelectron]
- Refrigerator [Mitsubishi]
- pH meter [Therma]
- Water bath [Julabo]
- Magnetic stirrer plate [Chiltern Scientific]
- Analytical balance [Precisa]
- Vortex [Scientific Industries]
- Auto pipette: 10, 20, 100, 200, 1000 microliters (μL) [Bio-Rad]
- Volumetric flask: 50, 100, 250, 500, 1000 milliliters (mL)
- Beaker: 50, 100, 250, 500, 1000 mL
- Cylinder: 50, 100, 250, 500, 1000 mL
- Conical tube 10 mL
- Disposable plastic conical tube 15 mL
- Screw capped test tube 15 mL
- Blood collection tube: plain 7 mL, 10 mL, and EDTA 3 mL
- Disposable Pasteur pipette 3 mL
- Microcentrifuge tube (0.7 mL and 1.7 mL)

2. Methods

Specimen used in this study were obtained from hypercholesterolaemic Thai subjects with total cholesterol (TC) and low density lipoprotein (LDL) levels more than 240 mg/dL and 170 mg/dL, respectively. All subjects who undergo medical check up at the Division of Cardiology, Chest Disease Institute, Nonthaburi, were screened for medical history, physical examination, and laboratory tests. Physical examination included weight, height, waist, hip, and blood pressure measurements. Laboratory tests included a complete blood count, blood urea nitrogen, creatinine, fasting blood sugar, liver function tests, and lipid profile. A questionnaire was used to collect medical history, smoking, and alcohol habits from the subjects.

The study was approved by the ethic committee of Mahidol University. All patients signed the informed consent form before being enrolled.

2.1 Study design

Atorvastatin 10 or 20 mg/day treatment, in hypercholesterolaemic subjects with TC more than 240 mg/dL and LDL more than 170 mg/dL levels was used. After screening visit to determine possible eligibility, patients entered three months active treatment period to receive atorvastatin 10 or 20 mg/day. The outcomes were analyzed at 0 and 3 months after receiving medication. After 3 months of atorvastatin treatment, hypercholesterolaemic subjects were interviewed, physically examined, and laboratory tests including liver function tests (alanine aminotransaminase, ALT and aspartate aminotransaminase, AST) were measured for any possible side effects. The drug would be discontinued if ALT or AST levels were more than 3 times the normal values. The study design was shown in figure 12.

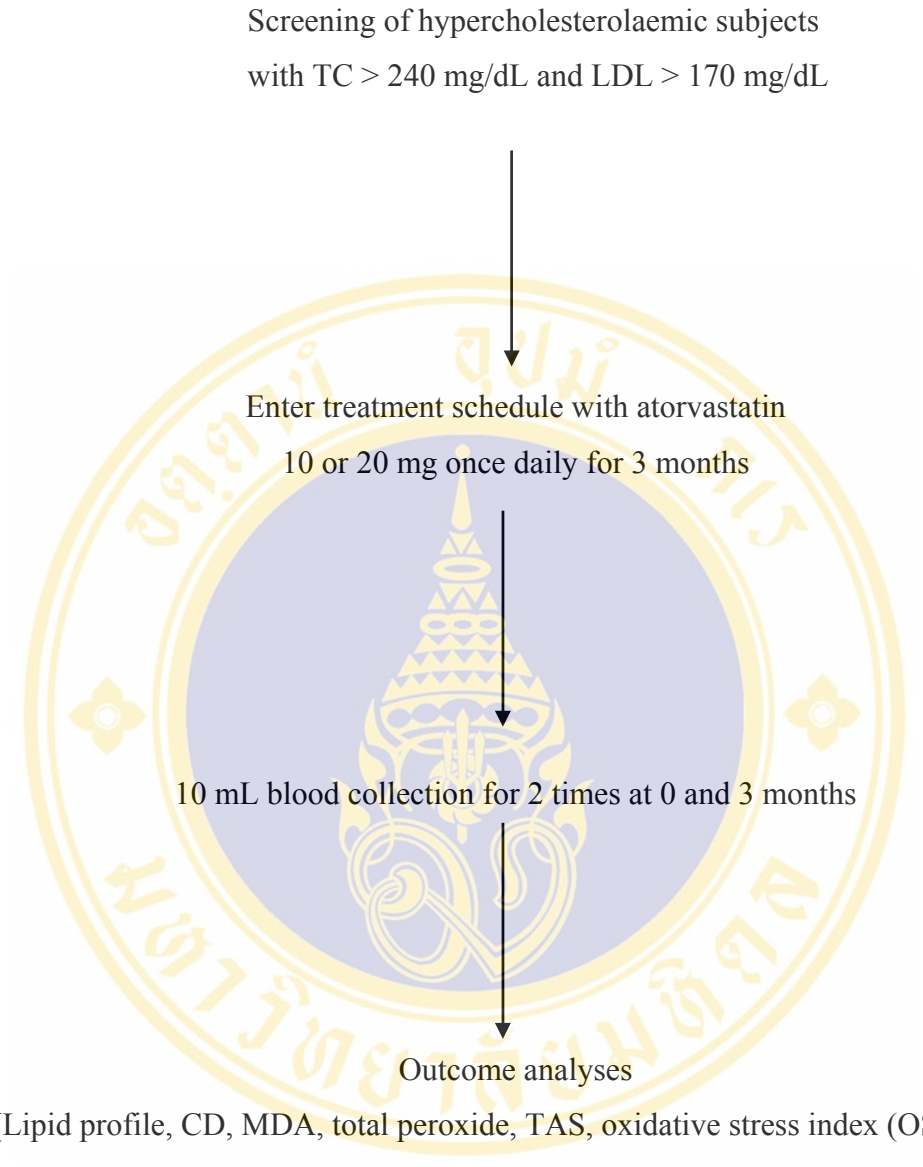


Figure 12. Experimental design of the effect of atorvastatin on PON and oxidative stress in hypercholesterolaemic subjects.

2.2 Subjects

Twenty two hypercholesterolaemic volunteers with age more than 30 years, who were under atorvastatin treatment for at least 3 months at Chest Disease Institute, Nonthaburi, were included in this study.

The patients with diabetes, uncontrolled high blood pressure, hyperthyroidism, liver disease, renal dysfunction, malignant, previous myocardial infraction symptom, coronary heart disease, cardiovascular disease, alcoholism, smoking habit, drug addiction, pregnancy, lactation, previously received lipid reducing therapy, and currently taking medication known to affect lipid levels or antioxidant vitamins were excluded from the study.

2.3 Blood sample collection

All volunteers were properly instructed to fast for 12 hours before the day appointed for venipuncture. Venous blood was collected two times, before and after 3 months atorvastatin treatment. Four milliliter of blood was collected into EDTA tube and 5 mL blood was collected in a plain tube without anticoagulant.

Blood collected in plain tube was allowed to clot at room temperature then centrifuged at 3,500 ×g for 10 min. Serum was aliquoted for total peroxide, TAS, PON1, and PON3 activity determinations in microtube without BHT and for CD and MDA determinations in a glass tube with BHT. EDTA whole blood was used for genomic DNA extraction and mononuclear cell separation. Mononuclear cell was separated by using histopaque then the cell pellet was resuspended in 0.1 M Potassium Phosphate Buffer. Serum, mononuclear cell, and EDTA whole blood were kept at –70 ˚C until analysis.

2.4 Lipid and lipoprotein measurement

Serum TC and triglyceride (TG) were measured by using commercial available enzymatic kits [Audit diagnostic]. High density lipoprotein (HDL) was determined by the phosphotungustic acid precipitation method and LDL was calculated by using the

Friedewald formula. All tests were performed by using automation (Olympus) at Laboratory of Chest Disease Institute, Nonthaburi.

2.5 Lipidperoxidation

Lipid peroxidation was measured for 3 markers; CD, total peroxide, and MDA, as corresponding to lipid peroxidation process.

2.5.1 Determination of conjugated diene (CD)

Conjugated diene was assayed as previously described by Buege and Aust (Buege and Aust, 1978). Lipid was extracted by an organic solvent and evaporated to dryness, then dissolved in cyclohexane. A volume of 0.2 mL of serum was diluted with 0.3 mL water and protein was precipitated by adding 1 mL of methanol. Lipid was extracted by adding 2 mL of chloroform and 3.0 mL of 0.05 M KCl. The mixture was separated by centrifuged at 3,150 ×g for 10 min then 1 mL of bottom layer was collected and left for air dry at room temperature for overnight. Conjugated diene was dissolved in 1.5 mL cyclohexane and measured absorbance at 233 nm by UV-2450 spectrophotometer.

2.5.2 Determination of total peroxide

Reagent preparation:

Solution A:

A volume of 180 mL methanol was used to dissolve 158.4 mg of BHT and thereafter 15.2 mg xylenol orange was added.

FOX_2 Reagent:

A quantity of 9.8 mg ammonium ferrous sulphate was dissolved in 10 mL of 250 mM H_2SO_4 and mixed with 90 mL of solution A.

Reagent Blank:

The blank reagent was prepared by mixing 90 mL of solution A with 10 mL 250 mM H_2SO_4.

Procedures:

Total peroxide in serum sample was determined by FOX2 method as previously described (Mustafa *et al*., 2004). Oxidation of ferrous ion to ferric ion by various types of peroxides contained within the serum produced colored ferric-xylenol orange complex whose absorbance was measured. A 100 µL of serum was mixed with 900 µL of FOX2 reagent. After incubation for 30 min at room temperature, the mixture was centrifuged at 12,000 ×g for 10 min. Total peroxide was determined against FOX_2 blank at 560 nm by UV-2450 spectrophotometer.

2.5.3 Determination of malondialdehyde (MDA)

Reagent preparation:

1. 1.22 M TCA in 0.6 M HCl

   This solution was prepared by first dissolving 39.8 g of TCA with 80 mL of water then made up to final volume of 200 mL with 1 M HCl.

2. 4.6 mM TBA

   A quantity of 500 mg of TBA was dissolved in 6 mL of 1 M NaOH and made up to final volume of 69 mL with water.

Procedures:

Malondialdehyde determination was carried out spectrophotometrically by measuring the levels of TBA reactive substances in serum as previously described (Flemming *et al*., 1997). Thiobarbituric acid reactive substance formed in serum sample after a calibrated sample pretreatment procedure primarily consist of MDA by a nucleophilic attack involved carbon-5 of TBA onto carbon-1 of MDA, followed by dehydration and a similar reaction of the intermediate MDA-TBA adduct with a second molecule of TBA which formed a red adduct with two molecules of TBA ($MDATBA_2$). A volume of 0.5 mL serum was mixed with 2.5 mL TCA and incubated 15 min at room temperature. Then 1.5 mL TBA was added and mixed for 30 sec and incubated at 95 ˚C for 30 min**.** The reaction was stopped by incubate on ice bath for 3 hr. Then 4.0 mL n-butanol was added and mixed vigorously for 3 min. The

MDA-TBA adduct was centrifuged at 950 ×g for 10 min and absorbance was taken at 532 nm. TMP was used as standard.

2.6 Total antioxidant status (TAS)

Reagent preparation:

Reagent 1

Solution A:

A quantity of 32.8 g CH_3COONa was mixed with 1000 mL water.

Solution B:

A volume of 22.8 mL glacial acetic acid was mixed with 987.2 mL water.

(Reagent 1 was prepared by mixing 940 mL of solution A with 60 mL of solution B then adjusted pH 5.8).

Reagent 2:

Solution C:

This solution was prepared by dissolving 2.46 g of CH_3COONa in 1000 mL water.

Solution D:

This solution was prepared by mixing 1.7 mL of glacial acetic acid with 998.3 mL water.

Solution E:

Solution E is prepared by mixing 75 mL of solution C with 925 mL solution D then adjusted pH 3.6.

Solution F:

Solution F is prepared by adding 278 µL of H_2O_2 (35%) in 1000 mL solution E.

(Reagent 2 is prepared by dissolving 0.549 g of ABTS was in 100 mL solution F).

Procedures:

Total antioxidant status was determined as previously described by Ozacan (Ozacan, 2004). The reduced ABTS molecule is oxidized to $ABTS^{\cdot +}$ by hydrogen peroxide. Deep green $ABTS^{\cdot +}$ molecules remains more stable in 30 mM acetate buffer pH 3.6, while it was diluted with a more concentrated acetate buffer at high pH (0.4 M acetate buffer pH 5.8), the color was spontaneously and slowly bleached. Antioxidants in the serum accelerated the bleaching rate to a degree proportional to their concentrations. The bleaching rate was inversely proportional related with TAS of the serum. The mixture of 1000 µL of reagent 1 and 25 µL of serum was used to set zero absorbance at 660 nm and then 100 µL reagent 2 was added to determine serum TAS by measuring absorbance at 660 nm after 5 min incubation.

2.7 Determination of PON1 activity

PON1 activities were determined by using paraoxon and phenyl acetate as substrates (Eckerson *et al*., 1983; La Du *et al*., 1984).

2.7.1 Paraoxon assay

Paraoxonase activity was determined by measuring the increased in absorbance of *p*-nitrophenol at 405 nm. The activity was measured by adding 20 µL of serum to 1 mL Tris buffer (100 mM, pH 8.0) containing 2 mM $CaCl_2$ and 1.1 mM paraoxon. The rate of hydrolysis of paraoxon was followed by measuring the liberation of *p*-nitrophenol at 405 nm at 37 ˚C over 50 sec after 1 min lag time with UV-2450 spectrophotometer.

$$\text{Activity} = \frac{\text{(OD/min) x assay volume}}{\varepsilon \text{ x sample volume x path length}}$$

The enzyme activity was calculated from the molar extinction coefficient (ε) of 18,700 M^{-1} cm^{-1} at 405 nm, pH 8.0. Activity was expressed as catalytic concentration (U/L) which corresponds to the product of 1 µM of *p*-nitrophenol per minute per liter of serum.

2.7.2 Arylesterase activity assay

The reaction mixture contained 1.0 mM phenylacetate and 0.9 mM calcium chloride in 10 mM Tris-HCl buffer pH 8.0. The reaction was initiated by adding 20 µL serum which was prediluted 1:20 ratio with 10 mM Tris-buffer pH 7.4. Increased in absorbance of phenol was followed at 270 nm at 37 ˚C using UV-2450 spectrophotometer.

$$\text{Activity} = \frac{\text{(OD/min) x assay volume}}{\varepsilon \text{ x sample volume x path length}}$$

The enzyme activity was calculated from the molar extinction coefficient of 1,310 M^{-1} cm^{-1}. Activity was expressed as catalytic concentration (U/L) which corresponds to the product of 1 mM of phenol per minute per liter of serum.

2.8 Determination of PON3 activity

PON3 activity was measured by measuring the arylesterase activity, using *p*-nitrophenyl butyrate as a substrate, with minor modification of previously reported method (Cuo *et al*., 1995; Dragomir *et al*., 2000 and 2005). PON3 activity was measured by adding 10 µL serum to 1 mL Tris-HCl buffer (50 mM pH 8.0) containing 1 mM $CaCl_2$ and 1 mM *p*-nitrophenyl butyrate. The rate of *p*-nitrophenyl butyrate hydrolysis was followed by measuring the librations of *p*-nitrophenol at 405 nm at 37 ˚C over 50 sec after 1 min lag time with UV-2450 spectrophotometer.

$$\text{Activity} = \frac{\text{(OD/min) x assay volume}}{\varepsilon \text{ x sample volume x path length}}$$

The enzyme activity was calculated from the molar extinction coefficient of 18,700 M^{-1} cm^{-1}. Activity was expressed as catalytic concentration (U/L) which corresponds to the product of 1 µM of *p*-nitrophenol per minute per liter of serum.

2.9 Determination of PON2 activity

PON2 activity was measured as arylestrase activity in monocytes by using *p*-nitrophenyl butyrate as a substrate (Cuo *et al*., 1995; Dragomir *et al*., 2000 and 2005).

Separation of mononuclear cells from whole blood

Mononuclear cells were separated by using histopaque reagents. The centrifugal force together with polysucrose aggregates the erythrocytes and granulocytes whereas the lymphocytes and other mononuclear cells remained at the interface of plasma and histopaque. Four milliliters of whole blood was carefully layered onto the 3.0 mL histopaque in 15 mL conical tube. Mononuclear cells were separated by centrifuge at 3,800 ×g for 30 min at room temperature. The opaque interface containing mononuclear cells were carefully transferred into clean conical tube then cells were washed at least 3 times with 6.0 mL 0.1 M Potassium Phosphate Buffer and centrifuged at 3,200 ×g for 10 min. The cell pellet was resuspended in 0.3 mL 0.1 M Potassium Phosphate Buffer and stored at -70 ºC until assay.

p-nitrophenyl butyrate assay

Mononuclear cells were homogenized by hand homogenizer. PON2 activity was measured by adding 10 µL serum to 1 mL Tris-HCl buffer (50 mM pH 8.0) containing 1 mM $CaCl_2$ and 1 mM *p*-nitrophenyl butyrate. The hydrolysis rate of *p*-nitrophenyl butyrate was followed by measuring the librations of *p*-nitrophenol at 405 nm at 37 ˚C by using UV-2450 spectrophotometer.

$$\text{Activity} = \frac{\text{(OD/min) x assay volume}}{\varepsilon \text{ x sample volume x path length}}$$

The enzyme activity was calculated from the molar extinction coefficient of 18,700 M^{-1} cm^{-1}.

Total protein estimation

Total protein in monocyte was determined by using Bradford method.

Reagent preparation:

A quantity of 50 mg Brilliant Blue G-250 was dissolved in 25 mL of 95% ethanol and 50 mL of 80% arthophospheric acid then made up final volume of 500 mL with water.

Procedures:

Bradford protein assay is a dye binding assay in which a differential color change of dye occurs in response to various concentrations of proteins. A volume of 25 µL sample was mixed with 1 mL of Bradford reagent and incubated at room temperature for 5 min. Total protein was determined at 595 nm by UV-2450 spectrophotometer. Bovine serum albumin was used to make a standard curve.

2.10 Extraction of genomic DNA

Reagent preparation:

1. 20X SSC (standard saline citrate)

   A quantity of 17.53 g of NaCl and 8.82 g of sodium citrate were dissolved in 80 mL water and adjusted pH 7.0 with 1 M HCl and then made up final volume of 100 mL with water.

2. TE buffer pH 8.0

   This buffer was prepared by mixing 1 µL of 1 mM Tris-HCL with 0.2 mL of 0.5 M EDTA and then made up final volume of 100 mL with water.

3. 0.5 M EDTA

   A quantity of 18.61 g EDTA dihydrate salt was dissolved with 80 mL water then 2-3 tablet of NaOH was added and adjusted pH 8.0 with 1 M HCl and made up final volume of 100 mL with water and autoclaved.

4. 10 X TBE buffer

A quantity of 54 g Tris-base and 27.5 g boric acid were dissolved in 400 mL of water and then 20 mL 0.5 M EDTA was added. The solution was made up final volume of 500 mL with water.

Procedures:

Genomic DNA from whole blood was extracted as previously performed in Roe's laboratory with minor modifications. Red blood cells were lysed by standard saline citrate. The nuclei pellet was digested in SDS and proteinase K. The DNA was extracted by chloroform/isoamyl alcohol and precipitated by ethanol. The frozen 0.7 mL whole blood was thaw and lysed by adding 0.6 mL 1X SSC**.** The nuclei pellet was precipitated by centrifuged at 950 ×g for 1 min at 4 °C. The contaminated red blood cells were removed by adding 1 mL of 1X SSC buffer, mixing, and centrifuging at 950 ×g for 1 min at 4 °C. This step was repeated until no color of red blood cell contamination. The nuclei pellet was washed with 200 µL TE buffer pH 8.0 and was broken by using wide-bore tip. Then 10 µL of 10 % SDS and 4 µL of proteinase K (2 mg/mL) was added and incubated at 55 ºC for 1-2 hr. After incubation, DNA was extracted by adding 400 µL TE buffer pH 8.0 and 1 volume of isoamyl alcohol/chloroform (1:24). The mixture was mixed by inverted tube and centrifuged at 950 ×g for 2 min at room temperature. The chloroform/isoamyl alcohol extraction was performed until DNA is cleaned. DNA was precipitated by adding 0.1 volume of 3 M NaOAc pH 5.2 and 1 mL of cold absolute ethanol and then incubated for 6-12 hr at -20 ºC. DNA was centrifuged at 950 ×g for 2 min at 4 ºC. DNA pellet was washed with 1 mL of cold 70 % ethanol and centrifuged at 950 ×g for 10 min at 4 ºC. The DNA pellet was resuspended in 100-200 µL of water and incubated 6-8 hours at 55 ºC followed by 10 min at 60 ºC. Finally, the DNA concentration and purity was determined by measuring OD at 260 and 280 nm and stored at -20 ºC for genotype determination.

2.11 *PON1* genotypes determination

The *PON1* genotypes, L55M, Q192R and T-107C polymorphisms, were determined by RFLP (Sardo *et al*., 2005). Restrction fragment is generated when a DNA molecule is treated with a restriction endonuclease that binds and cut DNA molecule at a specific sequence. The DNA fragments are revealed by agarose gel electrophoresis.

2.11.1 *PON 1* L55M genotype

Polymerase chain reaction mixture contained 300-500 ng genomic DNA, 0.5 µM of each primer, 200 µM dNTP, 3 µL of 10X reaction buffer, 1.25 mM $MgCl_2$, and 1.5 U of Taq polymerase in 30 µL reaction. After the DNA was denatured at 94 ˚C for 5 min, the reaction mixture was subject to 40 cycles, each cycle comprising denaturation 95 ˚C for 30 sec, annealing at 55 ˚C for 45 sec, and extension at 72 ˚C for 50 sec, with a final extension time of 10 min. PCR products were digested with Nla III. The digestion mixture contained 1.5 µL H_2O, 1.5 µL of 10X buffer # 4, 1.5 µl BSA (1 mg/mL), 10 µL of PCR product and 5 U NIa III. The mixture was incubated at 37 ˚C for 5 hr. After digestion, 10 µL digested PCR product was mixed with 2 µL of 6X loading dye and separated on 2% agarose gel containing 25 µL EtBr (1 mg/mL) at 90 volts for 2 hr. Then result was visualized on UV transilluminator gel documentation. Nla III will cut PCR product and resulted in 66 bp and 106 bp fragments for the MM genotypes and in a non-digested 172 bp fragments for the LL genotypes. Each genotype was read by two independent observers with out knowledge of the clinical status of the subjects.

2.11.2 *PON 1* Q192R genotype

Polymerase chain reaction mixture contained 300-500 ng genomic DNA, 0.5 µM of each primer, 200 µM dNTP, 5 µL of 10X reaction buffer, 1.25 mM $MgCl_2$, and 1.5 U of Taq polymerase in 30 µL reaction. After the DNA was denatured at 94 ˚C for 5 min, the reaction mixture was subject to 40 cycles, each cycle comprising denaturation 95 ˚C for 30 sec, annealing at 59 ˚C for 45 sec, and extension at 72 ˚C for

50 sec, with a final extension time of 10 min. PCR products were digested with Alw I. The digestion mixture contained 3.0 µL H_2O, 1.5 µL of 10X buffer # 2, 10 µL of PCR product and 5 U Alw I. The mixture was incubated at 37 ˚C for 5 hr. After digestion, 10 µL digested PCR product was mixed with 2 µL of 6X loading dye and separated on 2% agarose gel containing 25 µL EtBr (1 mg/mL) at 90 volts for 2 hr. Then result was visualized on UV transilluminator gel documentation. Alw I will cut PCR product and resulted in 66 and 172 bp for the RR genotype and in a none-digested 238 bp for the QQ genotype. Each genotype was read by two independent observers with out knowledge of the clinical status of the subjects.

2.11.3 *PON1* T-107C genotype

Polymerase chain reaction mixture contained 300-500 ng genomic DNA, 0.5 µM of each primer, 200 µM dNTP, 5 µL of 10X reaction buffer, 1.25 mM $MgCl_2$, and 1.5 U of Taq polymerase in 30 µL reaction. After the DNA was denatured at 94 ˚C for 5 min, the reaction mixture was subject to 40 cycles, each cycle comprising denaturation 94 ˚C for 30 sec, annealing at 69 ˚C for 45 sec, and extension at 72 ˚C for 50 sec, with a final extension time of 10 min. PCR products were digested with BsrB I. The digestion mixture contained 1.5 µL H_2O, 1.5 µL of 10X buffer # 2, 1.5 µl BSA (1 mg/mL), 10 µL of PCR product and 5 U BsrB I. The mixture was incubated at 37 ˚C for overnight. After digestion, 10 µL digested PCR product was mixed with 2 µL of 6X loading dye and separated on 3% agarose gel containing 25 µL EtBr (1 mg/mL) at 90 volts for 3 hr. Then result was visualized on UV transilluminator gel documentation. BsrB I will cut PCR product and resulted in 28 bp and 212 bp for the CC genotype and in a non-digested 240 bp fragments for the TT genotype. Each genotype was read by two independent observers with out knowledge of the clinical status of the subjects.

2.12 *PON2* genotype determination

Polymerase chain reaction mixture contained 300-500 ng genomic DNA, 0.5 μM of each primer, 200 μM dNTP, 3 μL of 10X reaction buffer, 1.25 mM $MgCl_2$, and 1.5 U of Taq polymerase in 30 μL reaction. After the DNA was denatured at 94 ˚C for 5 min, the reaction mixture was subject to 40 cycles, each cycle comprising denaturation 95 ˚C for 45 sec, annealing at 55 ˚C for 50 sec, and extension at 72 ˚C for 50 sec, with a final extension time of 10 min. PCR products were digested with Dde I. The digestion mixture contained 2.75 μL H_2O, 1.5 μL of 10X buffer # 2, 10 μL of PCR product and 5 U Dde I. The mixture was incubated at 37 ˚C for overnight. After digestion, 10 μL digested PCR product was mixed with 2 μL of 6X loading dye and separated on 2.5% agarose gel containing 25 μL EtBr (1 mg/mL) at 90 volts for 2.30 hr. Then result was visualized on UV transilluminator gel documentation. Dde I will cut PCR product and resulted in 68 and 36 bp for the SS genotype whereas the CC genotype resulted in a none-digested 104 bp fragments. Each genotype was read by two independent observers with out knowledge of the clinical status of the subjects.

2.13 Statistical analysis

Statistical analysis was performed by using the SPSS version 11.0 for windows software. A *P* value of less than 0.05 was considered statistically significant. The continuous parameters were expressed as mean ± standard deviation and median (range). The normality of the sample distribution of each continuous parameter was tested with Kolmogorov-Smirnov test. The parameters with normal distribution were performed by Student's paired t-test for comparisons. Mann-Whitney U test was used to compare the differences of non-normally distributed parameters. Changes from baseline outcomes were analyzed using the Student's paired t-test for outcomes with the normal distribution and the Wilcoxon Rank test for outcomes with the non-normal distribution. Pearson and Spearman correlation tests were used to test the strength of associations of parameters with normal distribution and non-normal distribution, respectively.

Allele frequencies were calculated by the Gene Counting method. The Chi-square test was used to determine both the observed genotype frequencies deviated

from Hardy-Weinberg equilibrium expectations. One-way ANOVA and Kruskal Wallis tests were used for comparison of normally and non-normally distributed parameters among genotype, respectively.

CHAPTER IV
RESULTS

A total of 22 hypercholesterolaemic subjects were recruited into the study from Chest Disease Institute, Nonthaburi. Nineteen of 22 patients (86.4%) completed the study. The withdrawal rate in this study was 13.6% (3/22). Atorvastatin treatment was well tolerated and none of the patients experienced serious adverse effects in this study. Three patients discontinued treatment due to loss of follow up.

1. Demographic characteristics

The demographic characteristics of hypercholesterolaemic subjects are presented in table 4. The mean age was 57.7 years. The study population had a greater proportion of female than male. Out of 22 patients, males were 8 (36.4%) and females were 14 (63.6%). The mean of body mass index (BMI), waist hip ratio (WHR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and fasting blood sugar (FBS) of baseline were 25 ± 3.7 kg/m^2, 0.85 ± 0.1, 132 ± 17.8 mmHg, 80 ± 11.9 mmHg, and 103 ± 9 mg/dL, respectively. As shown in table 4, there were no significant changes on demographic characteristics after 3 months of atorvastatin treatment.

2. Baseline lipid, Conjugated diene (CD), total peroxide, malondialdehyde (MDA), total antioxidant status (TAS), and oxidative stress index (OSI) levels

The baseline levels of lipid, lipid peroxidation parameters, TAS, and OSI of the study subjects are summarized in table 5 and table 6. The mean levels of total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) at baseline were 254 ± 40, 185 ± 20, 50.1 ± 12.5 and 158 ± 32 mg/dL, respectively. The mean levels of CD, total peroxide, MDA, TAS, and OSI at baseline

were 0.38 ± 0.06, 18.4 (13.6-26.6) μmol H_2O_2/L, 17.6 ± 2.7 mmol/L, 0.81 ± 0.14 mmol Trolox equiv/L, and 2.42 ± 0.73, respectively.

3. Effect of atorvastatin on lipid, Conjugated diene, total peroxide, malondialdehyde, total antioxidant status, and oxidative stress index levels

As shown in table 5, table 6, and figure 13, atorvastatin treatment significantly decreased TC, TG, LDL, CD, total peroxide, MDA, and OSI levels by 24.5%, 24.4%, 22.4%, 4.4%, 13.0%, 15.2%, and 24.0%, respectively. Meanwhile, the mean of TAS level significantly increased by 27.3%, however, there was no significant difference in level of HDL at 3 month treatment of atorvastatin (50.4 ± 9.2 mg/dL) when compared with the baseline level (50.1 ± 12.5 mg/dL).

The percent (%) change of LDL was positively correlated with % change of TC ($R^2 = 0.63$, $P > 0.05$, Fig. 14). There was no correlation between % change of TAS and % change of LDL ($R^2 = 0.15$, $P > 0.05$, Fig.15). High density lipoprotein level did not increase significantly by atorvastatin treatment and the % change of HDL level was not correlated with change of lipid peroxidation parameter ($R^2 = 0.05$, Fig. 16).

Table 4. Demographic details of study subjects

Parameters	Hypercholesterolaemic subjects (n = 19)		
	Baseline	3 month	*P*
Age (years)	57.7 ± 7.4	58.0 ± 7.4	NS
BMI (kg/m^2)	25 ± 3.7	26.6 ± 3.4	NS
WHR	0.85 ± 0.1	0.85 ± 0.9	NS
SBP (mmHg)	132 ± 17.8	132 ± 25.4	NS
DBP (mmHg)	80 ± 11.9	74 ± 11.8	NS
FBS (mg/dL)	103 ± 9	108 ± 10	NS

Values are shown as mean ± SD.

$P < 0.05$ is considered significant.

NS = non significant.

Table 5. Serum lipid parameters at baseline and 3 month after atorvastatin treatment

Parameters	Hypercholesterolaemic subjects (n = 19)	
	Baseline	3 month
TC (mg/dL)	254 ± 40	199 ± 36***
% Change▼		- 24.5
TG (mg/dL)	185 ± 20	141 ± 15*
% Change▼		- 24.4
HDL (mg/dL)	50.1 ± 12.5	50.4 ± 9.2
% Change▼		+ 3.31
LDL (mg/dL)	158 ± 32	118 ± 27***
% Change▼		- 22.4

Values are shown as mean ± SD.

▼% Change is the mean of % change from individual patients.

Significant differences from baseline are given: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 6. Serum CD, total peroxide, MDA, TAS, and OSI levels at baseline and 3 month after atorvastatin treatment

	Hypercholesterolaemic subjects (n = 19)	
Parameters	Baseline	3 month
CD	0.38 ± 0.06	0.34 ± 0.05*
% Change▼		- 4.4
Total peroxide [a] (μmol H_2O_2/L)	18.4 (13.6-26.6)	15.9 (11.1-24.0) **
% Change▼		- 13.0
MDA (mmol/L)	17.6 ± 2.7	14.8 ± 2.9**
% Change▼		- 15.2
TAS (mmol Trolox Equiv/L)	0.81 ± 0.14	1.01 ± 0.17***
% Change▼		+ 27.3
OSI	2.42 ± 0.73	1.79 ± 0.56***
% Change▼		- 24.0

Values are shown as mean ± SD. [a] Values are shown as medium (range).

▼% Change is the mean of % change from individual patients.

Significant differences from baseline are given: * $P < 0.05$, $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

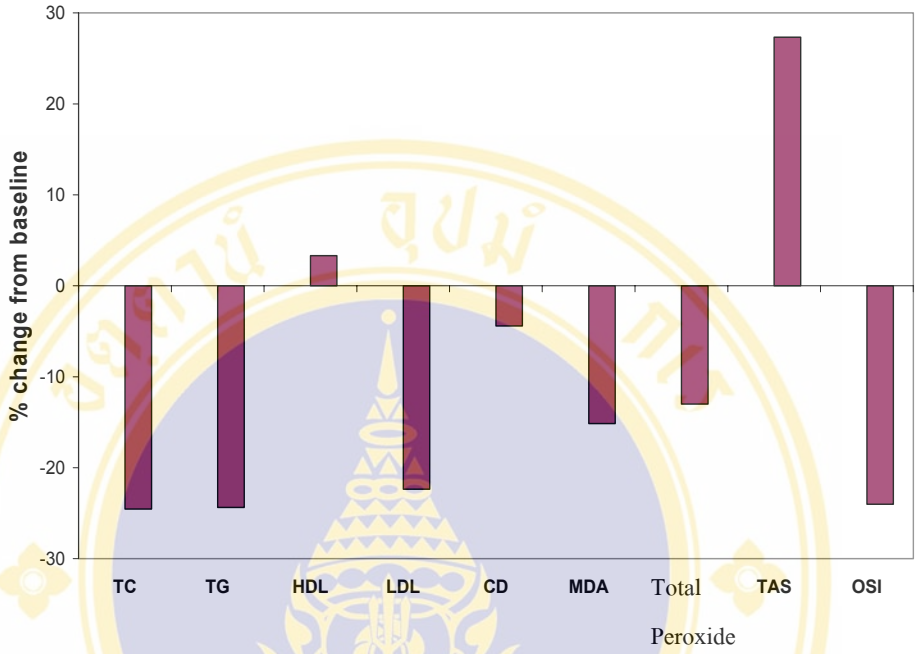


Figure 13. Effect of atorvastatin on lipid, lipid peroxidation, TAS, and OSI.

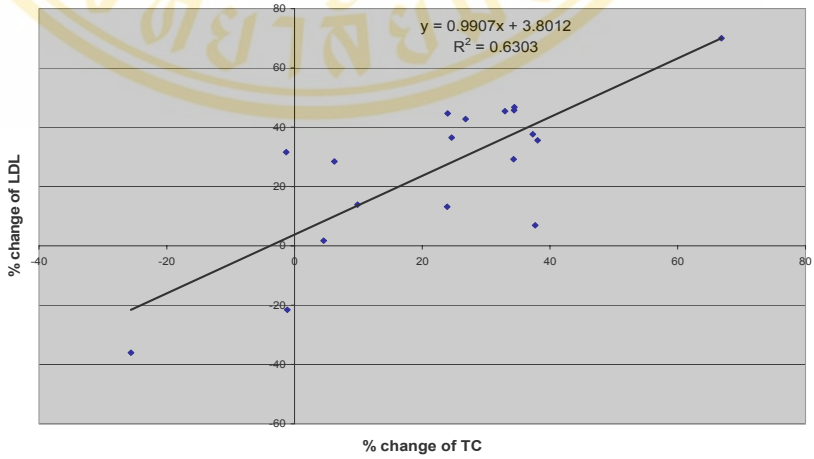


Figure 14. Relationship between % change of TC and % change of LDL.

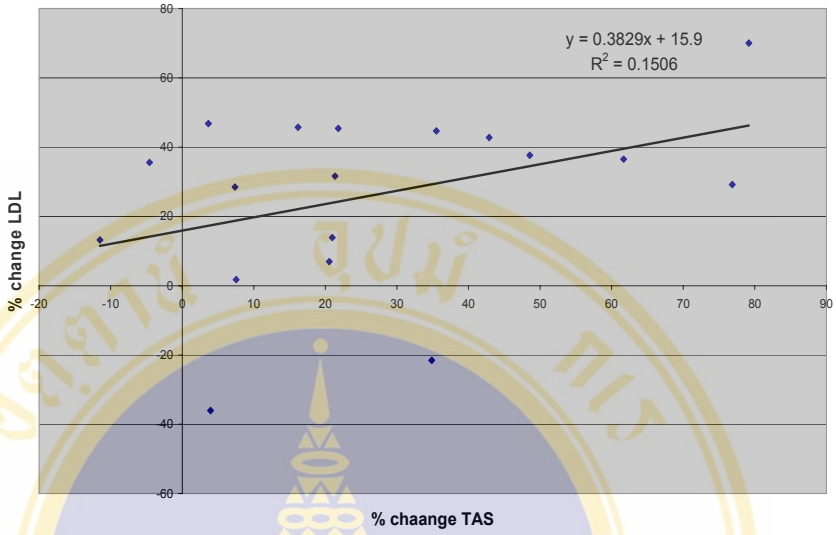


Figure 15. Relationship between % change of TAS and % change of LDL.

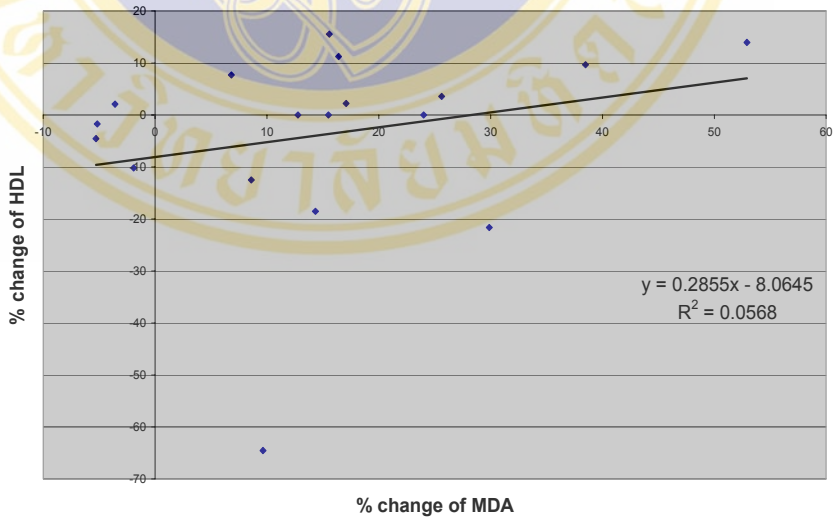


Figure 16. Relationship between % change of MDA and % change of HDL.

4. Baseline levels of PON1, PON2, and PON3 activities

PON1 activities towards paraoxon and phenyl acetate, PON2, and PON3 activities towards *p*-nitrophenyl butyrate at baseline were summarized in table 7. The mean levels of PON1 activities towards paraoxon and phenyl acetate were 178 ± 77 μmol/min/mL and 142 ± 25 mmol/min/mL, respectively. The mean levels of PON2 and PON3 activities toward *p*-nitro phenyl butyrate were 355 ± 102 U/mg protein and 2,164 ± 281 μmol/min/mL, respectively.

5. Effect of atorvastatin on PON1, PON2, and PON3 activities

As shown in table 7, table 8 and figure 17, there was significantly increased in PON1 activity towards paraoxon (+13.4%, $P < 0.05$) but PON1 activity towards phenyl acetate did not change significantly (+1.35%, $P > 0.05$) after 3 month of atorvastatin treatment. However, in male slightly significant increased in PON1 activity toward phenyl acetate was detected ($P = 0.04$, Table 8). As shown in table 7, table 8, and figure 19, PON2 activity did not change significantly (-0.87%, $P > 0.05$) but PON3 activity was significantly increased (+13.2%, $P < 0.05$) after 3 month of atorvastatin treatment.

In this study, we found non-significant difference of baseline PON1 activity towards paraoxon, PON2, and PON3 activities towards *p*-nitrophenyl butyrate between male and female but, PON1 activity towards phenyl acetate was significantly different between male and female ($P = 0.03$, Table 8).

The percent change of PON1 activity towards paraoxon was positively correlate with % change of TAS ($R^2 = 0.30$, Fig. 18). The very strong positive correlation between % change of PON1 activity towards paraoxon and % change of PON3 activity was observed ($R^2 = 0.71$, Fig. 19) and % change of PON3 activity was positively correlated with % change of TAS ($R^2 = 0.30$, Fig. 20).

Table 7. PON1, PON2, and PON3 activities at baseline and 3 month after atorvastatin treatment

Parameters	Hypercholesterolaemic subjects (n = 19)	
PON activity	Baseline	3 month
PON1 levels		
Paraoxon hydrolysis (µmol/min/mL)	178 ± 77	196 ± 90*
% Change▼		+ 13.4
Phenylacetate hydrolysis (mmol/min/mL)	142 ± 25	145 ± 33
% Change▼		+ 1.35
PON2		
p-nitrophenyl butyrate hydrolysis (IU/mL protein)	355 ± 102	316 ± 113
% Change▼		- 0.87 %
PON3		
p-nitrophenyl butyrate hydrolysis (µmol/min/mL)	2164 ± 281	2444 ± 456*
% Change▼		+ 13.2

Values are shown as mean ± SD.

▼% Change is the mean of % change from individual patients.

Significant differences from baseline are given: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 8. PON1, PON2, and PON3 activities in men and women

PON activity	Male (n = 5)			Female (n = 14)		
	Baseline	3 month	P_m	Baseline	3 month	P_f
PON1 (Paraoxon)	181 ± 43	214 ± 42	0.19	178 ± 19 *Ps* = 0.57	190 ± 24	0.10
PON1 (Phenyl acetate)	152 ± 8	157 ± 11	0.04	137 ± 7 *Ps* = 0.03	138 ± 8	0.76
PON2 (*p*-nitrophenyl butyrate)	333 ± 57	310 ± 54	0.78	357 ± 29 *Ps* = 0.53	298 ± 31	0.18
PON3 (*p*-nitrophenyl butyrate)	2090 ± 90	2478 ± 153	0.02	2190 ± 81 *Ps* = 0.49	2432 ± 131	0.03

Values are shown as mean ± SEM.

$P < 0.05$ is considered significant.

P_m and P_f is the P value of male and female PON activity before and after treatment, respectively.

Ps is the P value of baseline PON activity between male and female.

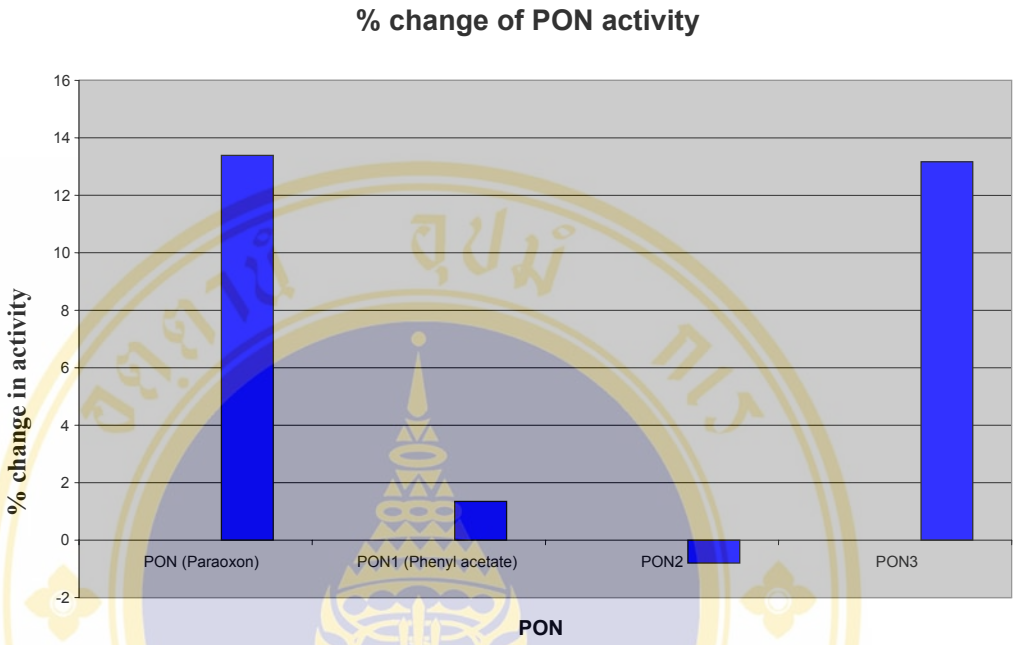


Figure 17. Effect of atorvastatin on PON1, PON2, and PON3 activities.

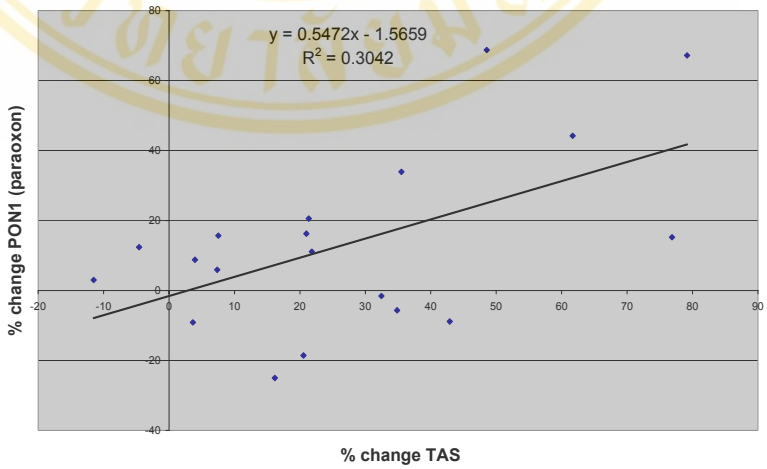


Figure 18. Relationship between % change of TAS and % change of PON1 activity towards paraoxon.

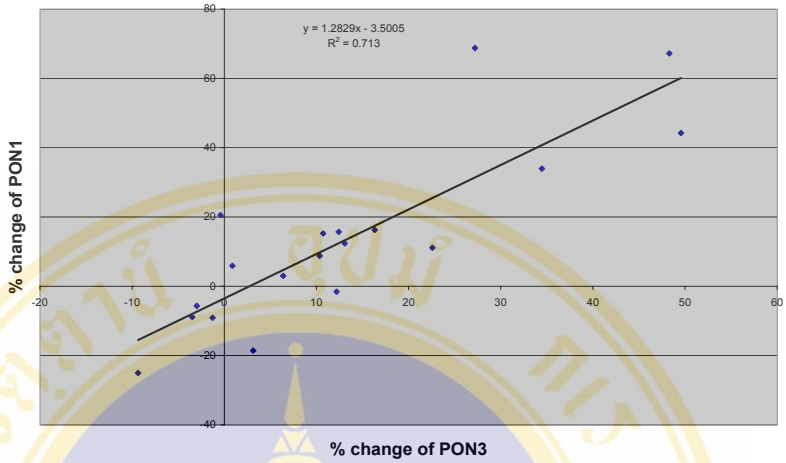


Figure 19. Relationship between % change of PON1 activity towards paraoxon and % change of PON3 activity.

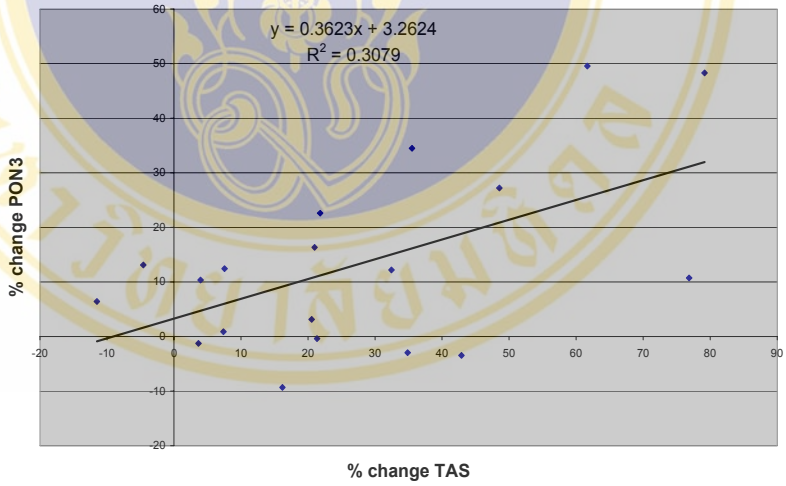


Figure 20. Relationship between % change of TAS and % change of PON3 activity.

6. Genotype and allele frequencies of the *PON1* in study subjects

The genotype and allele frequencies of three *PON1* polymorphisms are shown in figure 21A, 21B, and 21C, and table 9. Genotype and allele frequencies for the *PON1* L55M, Q192R and T-107C polymorphisms were 73.68% LL, 21.05% LM, 5.26% MM (L = 0.84, M = 0.16), 42.11% QQ, 36.84% QR, 21.05% RR (Q = 0.61, R = 0.39), 42.11% CC, 36.84% CT, and 21.05% TT (C = 0.61, T = 0.39), respectively. The LL genotype was the most common and LM was the rarest genotype found in the population.

As shown in table 11, a good agreement was found between the observed and expected genotype frequencies of *PON1* at position 55, 192, and -107, according to Hardy-Weinberg Equilibrium (P = 0.45, 0.60, and 0.60, respectively). These genotype frequencies did not deviate from Hardy-Weinberg Equilibrium expectations using Chi-square test. As presented at table 12**,** chi-square test showed no significant linkage disequilibrium between *PON1* polymorphisms ($P > 0.05$).

7. Genotype and allele frequencies of the *PON2* in study subjects

The genotype and allele frequencies of *PON2* C311S polymorphism are shown in figure 22 and table 10. Genotype and allele frequencies for the *PON2* C311S polymorphism are 10.53% CC, 31.58% CS, and 57.89% SS (C = 0.26, S = 0.74).

As shown in table 11, a good agreement was found between the observed and expected genotype frequencies of *PON2* at position 311, according to Hardy-Weinberg Equilibrium (P = 0.71). Genotype frequencies did not deviate from Hardy-Weinberg Equilibrium expectations using Chi-square test. As presented in table 12**,** Chi-square test showed no significant linkage disequilibrium between *PON1* and *PON2* polymorphisms ($P > 0.05$).

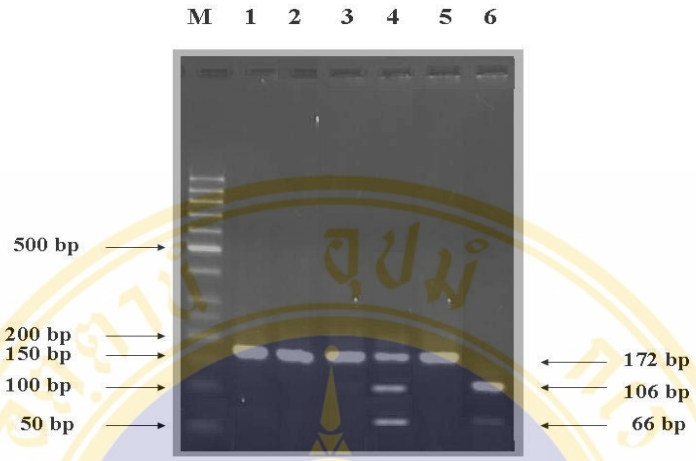


Figure 21A. Separation of restriction fragment length polymorphism (RFLP) product of *PON1* L55M polymorphism after digestion with Nla III on 2.5% agarose gel electrophoresis. Lane M represents the 50 bp DNA ladder. Lane 1, 3, and 5 represent uncut PCR products of lane 2, 4, and 6, respectively. Lane 2, 4, and 6 were PCR product digestion with Nla III. Lane 2, 4, and 6 represents LL, LM, and MM genotypes, respectively.

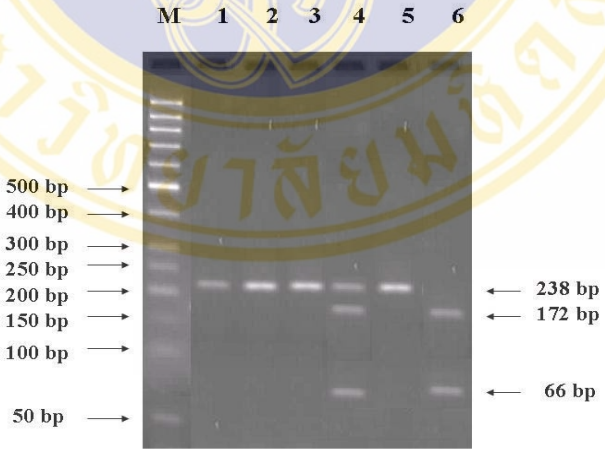


Figure 21B. Separation of RFLP product of *PON1* Q192R polymorphism after digestion with Alw I on 2.5% agarose gel electrophoresis. Lane M represents the 50 bp DNA ladder. Lane 1, 3, and 5 represent uncut PCR products of lane 2, 4, and 6, respectively. Lane 2, 4, and 6 were PCR product digestion with Alw I. Lane 2, 4, and 6 represents QQ, QR, and RR genotypes, respectively.

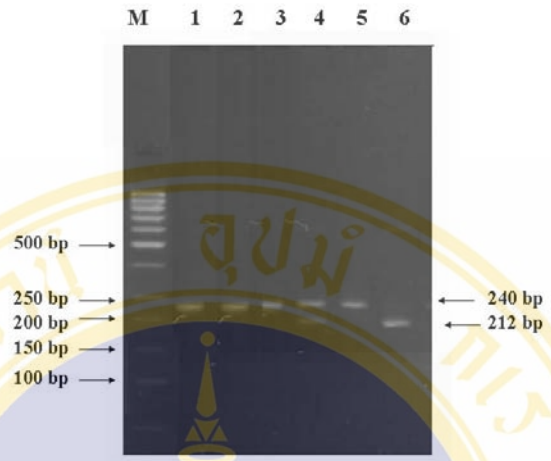


Figure 21C. Seperation of RFLP product of *PON1* T-107C polymorphism after digestion with BsrB I on 3.0% agarose gel electrophoresis. Lane M represents 50 bp DNA ladder. Lane 1, 3, and 5 represent uncut PCR products of lane 2, 4, and 6, respectively. Lane 2, 4, and 6 were PCR product digestion with BsrB I. Lane 2, 4 and 6 represents TT, CT, and CC genotypes, respectively.

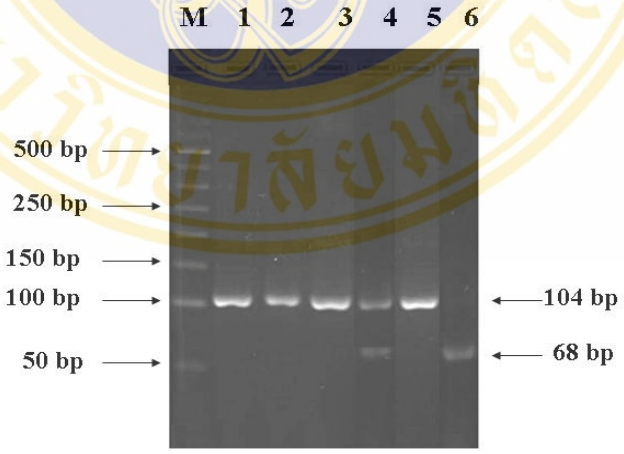


Figure 22. Seperation of RFLP product of *PON2* C311S polymnorphism after digestion with Dde I on 2.5% agarose gel electrophoresis. Lane M represents 50 bp DNA ladder. Lane 1, 3, and 5 represent uncut PCR products of lane 2, 4, and 6, respectively. Lane 2, 4, and 6 were PCR product digestion with Dde I. Lane 2,4 and 6 represents CC, CS, and SS genotypes, respectively.

Table 9. Genotype and allele frequencies of the *PON1* polymorphisms

	Polymorphic sites		
	L55M	Q192R	T-107C
Genotype frequencies	LL : 73.68% LM : 21.05% MM : 5.26 %	QQ : 42.11% QR : 36.84% RR : 21.05%	CC : 42.11% CT : 36.84% TT : 21.05%
Allele frequencies	L : 0.84 M : 0.16	Q : 0.61 R : 0.39	C : 0.61 T : 0.39

Table 10. Genotype and allele frequencies of the *PON2* polymorphism

	Polymorphic site
	PON2 C311S
Genotype frequencies	CC : 10.53% CS : 31.58% SS : 57.89%
Allele frequencies	C : 0.26 S : 0.74

Table 11. Hardy-Weinberg Equilibrium for the *PON1* and *PON2* polymorphisms

	Genotype frequency			
	Observed	Expected	Chi-square value	*P*
PON1 L55M				
LL	14	14.06		
LM	4	5.13	1.57	0.45
MM	1	0.47		
PON1 Q192R				
QQ	8	7.06		
QR	7	9.04	1.02	0.60
RR	4	2.88		
PON1 T-107C				
CC	8	2.89		
CT	7	9.04	1.01	0.60
TT	4	7.06		
PON2 C311S				
CC	2	1.28		
CS	6	7.31	0.67	0.71
SS	11	10.4		

P > 0.05 indicates no deviation from Hardy-Weinberg Equilibrium.

Table 12. Linkage disequilibrium of *PON1* and *PON2* polymorphisms

	P		
	PON1 L55M	*PON1* Q192R	*PON1* T-107C	*PON2* C311S
PON1 L55M	……..	0.50	0.22	0.67
PON1 Q192R		……	0.71	0.52
PON1 T-107C			..……	0.09
PON2 C311S				. ……

Values of *P* are obtained by Chi-square test.

P > 0.05 indicates no linkage disequilibrium.

8. Influence of *PON1* polymorphisms on baseline lipid profile and PON1 activity

Baseline lipid profile according to the *PON1* polymorphisms are shown in table 13. There were no significant differences in baseline lipid profile according to *PON1* L55M, Q192R, and T-107C polymorphisms.

Table 14 showed PON1 activity towards paraoxon and phenyl acetate in each genotype according to three *PON1* polymorphisms. There was no significant difference level of baseline PON1 activity towards paraoxon according to *PON1* polymorphisms. However, PON1 activity towards phenyl acetate was significantly different according to *PON1* L55M polymorphism ($P = 0.03$).

9. Influence of the *PON1* polymorphisms on its activity in response to atorvastatin treatment

There was no significant influence in the change of PON1 activity towards paraoxon by the *PON1* L55M and Q192R polymorphisms. However, *PON1* T-107C polymorphism significantly influenced the difference in changes of PON1 activity towards paraoxon after 3 months of atorvastatin treatment (30.1% for CC genotypes, 3.15% for CT genotypes, and -2.09% for TT genotypes, Table 15). Meanwhile, *PON1* L55M polymorphism also significantly influenced the difference in changes of PON1 activity towards phenyl acetate after 3 month of treatment (0.9% for the LL genotypes, 1.3% for MM genotypes, and 11.5% for LM genotypes, Table 15). Neither *PON1* polymorphisms significantly influenced in changes of PON3 activity towards *p*-nitophenyl butyrate (Table 15).

10. Influence of *PON2* polymorphism on baseline lipid profile and PON2 activity

Baseline lipid profile and PON2 activity towards *p*-nitrophenyl butyrate according to the *PON2* C311S polymorphism are shown in table 16. There were no significant differences in baseline lipid profile and PON2 activity between genotypes classified by *PON2* C311S polymorphism ($P > 0.05$).

11. Influence of the *PON2* polymorphism on its activity in response to atorvastatin treatment

There was no significant difference in the change of PON2 activity towards *p*-nitrophenyl butyrate after atorvastatin treatment between genotypes classified by *PON2* C311S polymorphism ($P > 0.05$, Table 17). However, CS and SS genotype changes the PON2 activity by 7.3% and 10.4%, respectively after 3 month of atorvastatin treatment.

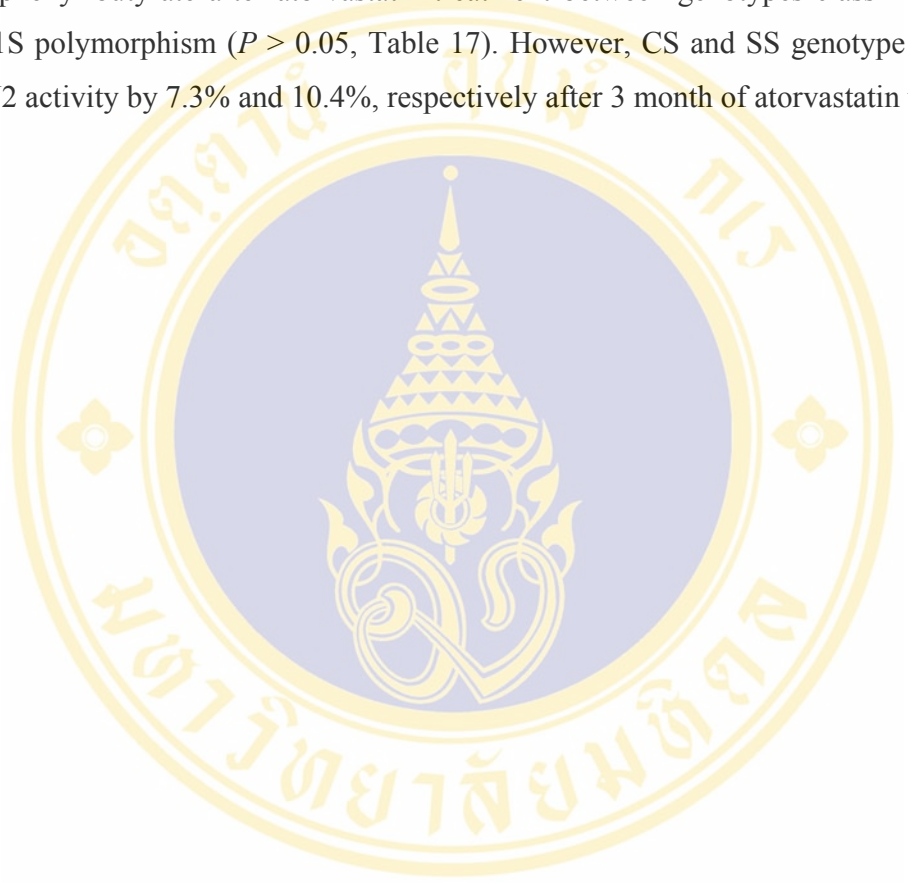


Table 13. Influence of *PON1* polymorphisms on lipid profile

Parameters	L55M	Q192R	T-107C
TC	LL : 251 (168-715)	QQ : 256 (219-297)	CC : 263 (219-715)
	LM : 263 (219-297)	QR : 305 (168-715)	CT : 264 (168-329)
	MM : 305	RR : 239 (191-264)	TT : 250 (239-305)
	$P = 0.54$	$P = 0.09$	$P = 0.94$
TG	LL : 164 (87-367)	QQ : 164 (106-316)	CC : 189 (106-367)
	LM : 194 (125-316)	QR : 229 (101-367)	CT : 133 (87-270)
	MM : 229	RR : 138 (87-247)	TT : 179 (144-247)
	$P = 0.75$	$P = 0.43$	$P = 0.25$
HDL	LL : 49 (37-86)	QQ : 48 (31-62)	CC : 50 (31-62)
	LM : 51 (31-62)	QR : 54 (41-86)	CT : 52 (45-86)
	MM : 41	RR : 42 (37-52)	TT : 41 (37-48)
	$P = 0.54$	$P = 0.11$	$P = 0.08$
LDL	LL : 166 (86-464)	QQ : 166 (113-188)	CC : 173 (113-464)
	LM : 155 (113-173)	QR : 194 (86-464)	CT : 167 (86-207)
	MM : 218	RR : 154 (137-185)	TT : 166 (137-218)
	$P = 0.24$	$P = 0.20$	$P = 0.93$

Values are median (range).

$P < 0.05$ is considered significant.

MM genotype was found only in 1 subject so no range value.

Table 14. Influence of *PON1* polymorphisms on PON1 activity

PON1 activity	L55M	Q192 R	T-107C
Paraoxon [a]	LL: 186 (56-324)	QQ: 171 (52-240)	CC: 195 (56-258)
	LM: 165 (52-204)	QR: 160 (22-324)	CT: 165 (52-324)
	MM: 20	RR: 231 (207-256)	TT: 167 (22-256)
	$P = 0.14$	$P = 0.12$	$P = 0.14$
Phenyl acetate	LL: 145 ± 22	QQ: 149 ± 15	CC: 155 ± 18
	LM: 148 ± 18	QR: 147 ± 32	CT: 136 ± 24
	MM: 82	RR: 118 ± 14	TT: 126 ± 29
	$P = 0.03$	$P = 0.09$	$P = 0.10$

Values are shown as mean ± SD. [a] values are median (range).

$P < 0.05$ is considered significant.

MM genotype was found only in 1 subject so no range value.

Table 15. Effect of attorvastatin on PON1 and PON3 activities according to the *PON1* polymorphisms

% Change of PON levels▼	L55M	Q192R	T-107C
PON1			
(Paraoxon)	LL: 13.3 ± 6.9	QQ: 14.8 ± 6.5	CC: 30.1 ± 8.7
	LM: 20.9 ± 12.1	QR: 17.3 ± 13.8	CT: 3.15 ± 8.54
	MM: -9	RR: 3.4 ± 7.5	TT: -2.09 ± 5.82
	P = 0.61	*P* = 0.69	*P* = 0.03
PON1			
(Phenyl acetate)	LL: 0.9 ± 2.6	QQ: 4.7 ± 4.5	CC: 4.13 ± 3.31
	LM: 11.5 ± 7.9	QR: -3.5 ± 4.3	CT: 2.23 ± 5.36
	MM: 1.3	RR: 3.2 ± 6.3	TT: -5.74 ± 7.29
	P = 0.03	*P* = 0.42	*P* = 0.43
PON3	LL: 12.2 ± 4.0	QQ: 14.0 ± 6.7	CC: 19.0 ± 5.9
(*p*-nitrophenyl butyrate)	LM: 22.7 ± 13.4	QR: 14.3 ± 7.1	CT: 13.6 ± 7.0
	MM: -1.2	RR: 9.3 ± 4.8	TT: 0.75 ± 3.35
	P = 0.43	*P* = 0.88	*P* = 0.21

Values are mean ± SEM.

MM genotype was found only in 1 subject so no range value.

$P < 0.05$ is considered significant.

Table 16. Influence of *PON2* polymorphism on lipid profiles and PON2 activity

	PON2 C311S			
Parameters	CC (n = 2)	CS (n = 6)	SS (n = 11)	*P*
TC [a]	291 (264-319)	263 (219-715)	250 (168-329)	0.4
TG [a]	250 (133-367)	147 (125-316)	183 (87-270)	0.67
LDL [a]	189 (185-194)	167 (113-464)	163 (86-218)	0.36
HDL [a]	51 (50-52)	53 (31-95)	48 (37-86)	0.73
PON2 activity	303 ± 256	389 ± 87	337 ± 91	0.53

Values are shown as mean ± SD. [a] values are in median (range).
$P < 0.05$ is considered significant.

Table 17. Effect of atorvastatin on PON2 activity according to *PON2* polymorphism

% change of PON2 level	*PON2* C311S		
CC (n = 2)	CS (n = 6)	SS (n = 11)	*P*
-45.4 (-149-59)	7.3 (-7.9-34.9)	10.4 (-130-75)	0.51

Values are shown as median (range).

$P < 0.05$ is considered significant.

CHAPTER V
DISCUSSION

One of the major hypotheses that is believed to be the underlying cause in pathogenesis of atherosclerosis is role of oxidative modification of lipid (see rev. Roland and Keaney, 2003). With this in mind, the paradox for prevention and regimen of atherosclerosis and its consequence diseases would be to reduce the lipid level and oxidative stress. Atorvastatin has been used as the drug of choice for reducing lipid in hypercholesterolaemic patients. Moreover, Liao and colleague reported that atorvastatin exerts beneficial cardiovascular effect independent of their lipid lowering property (Lio, 2002), and it might be due to its direct and indirect antioxidant properties (Sardo *et al*., 2005). In this study, 3 month atorvastatin treatment of hypercholesterolaemic subjects significantly lowered total cholesterol (TC), triglyceride (TG), and low density lipoprotein (LDL). The reduction of all these lipid parameters does not really come as a surprising fact. It can be explained on the basis that the major action of atorvastatin is to inhibit hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting step in *de novo* cholesterol synthesis. This pathway caused a decrease in the hepatocyte cholesterol synthesis and consequently cleared the LDL from the circulation by LDL receptor of hepatocyte cell surface. The effect of atorvastatin in lowering TC, TG, and LDL levels obtained in our study were similar to those described by Rosenblat *et al*. and Paragh *et al***.** (Paragh *et al*., 2004; Rosenblat *et al*., 2004). It has been reported that atorvastatin also has beneficial effect on increasing HDL with unknown mechanism(s) (Maron et al., 2000; Kural *et al*., 2004). However, we saw an insignificant increased in HDL by atorvastatin as previously reported by other (Paragh *et al*., 2004; Rosenblat *et al*., 2004). These discrepancies may partly be explained from the different factors associated with the subjects themselves who participate in the study that are beyond control such as the severity of dietary control, exercise, genetic effect, patient behavior, and disease state.

Atorvastatin exerts beneficial cardiovascular effect independent of their lipid lowering properties (see rev. Liao, 2002), and it might be due to its antioxidant effect (Sardo *et al*., 2005) which believed to come from HDL-associated enzymes, PON1 and PON3. There are considerable numbers of interests in the study of potential pharmacological effect of various lipid lowering drugs on PON activity. The increased in serum PON1 activity by statin is still unclear due to the disagreement of results from many studies. In this study, we found that atorvastatin significantly increased PON1 activity towards paraoxon ($P < 0.05$), however, there was a non significant trend for the increase in the PON1 activity towards phenyl acetate ($P = 0.24$). Similar result was seen in simvastatin treatment that was studied by Tomas and colleague (Tomas *et al*., 2000). PON1 activity has been shown to be substrate dependent and may show varied results for different substrates (Dragomir *et al*., 2005). Paraoxon is the most commonly used substrate for the determination of PON1 activity and this level of enzyme activity has been shown to reflect the antioxidant capacity of the enzyme (Cao *et al*., 1999). At the baseline, PON1 activity towards paraoxon was unaffected by the differences in sex but PON1 activity towards phenyl acetate was significantly higher in male than females ($P = 0.04$). After 3 month of atorvastatin treatment, PON1 activity towards phenyl acetate was significantly increased in male subjects ($P = 0.03$), but did not changed significantly in female subjects ($P = 0.76$). It is rather difficult to explain this finding about mechanism underlying the differences, since the numbers of subjects in this study were too small to make a meaningful statistical comparison. Moreover, most of our study subjects are menopause female and we need more clinical evidence to support this effect of the atorvastatin on sex differences. The significant increased of PON1 activity in our study may be due to increased PON1 concentration. Recently, Deakin and colleague had demonstrated that simvastatin increased the nuclear sterol regulating element binding protein-2. This protein binds to the paraoxonase promoter and cause an enhanced in promoter activity (Deakin et al., 2003). We found, after 3 month of atorvastatin treatment, percent change of PON1 activity towards paraoxon was positively correlated with percent change of TAS ($R^2 = 0.304$), which indicates PON1 contributes to decreased lipid

peroxidation and thereby reduce the risk of heart diseases as well. It has been suggested that PON1 inhibits lipid peroxidation by hydrolyzing lipid peroxide and hydrogen peroxide. On the other hand, it might possibly to reduce oxidative stress and cause a reduction in the inactivation of PON1 and thereby leading to the increase of PON1 activity after 3 month of treatment.

PON3 is a member of PON that is also associates with HDL and contributes to the antioxidant property of HDL. PON3 is still new in the research field, more work has to be done before any concrete conclusion can be made. In the present study, interestingly, high levels of serum PON3 activity towards *p*-nitrophenyl butyrate (mean = 2163.84 µmol/min/mL) was observed at the beginning of the study and significantly increased after 3 month of atorvastatin treatment. The effect of atorvastatin to increased PON3 activity is not influenced by sex. The significant increased of PON3 activity observed in our study by atorvastain treatment was similar to previous study by Rosenblat *et al*. (Rosenblat *et al*., 2004). Recently, it was reported that PON3 is more potent than PON1 to prevent LDL oxidation (Dragnov *et al*., 2000) and PON3 activity was decreased under oxidative stress (Rosenblat *et al*., 2003). This finding may explain the effect of atorvastatin to increase PON3 activity, since in our study oxidative stress was significantly reduced by atorvastatin treatment.

It is convincingly believed that PON2 is a cellular antioxidant and may play a role in atherosclerosis development at the subintima layer of artery wall. PON2 activity towards *p*-nitrophenyl butyrate, in monocytes was detected in this study before and after treatment. No significant changed on PON2 activity after atorvastatin treatment was observed. It was reported that atorvastatin reduced the PON2 activity in macrophage in hypercholesterolaemic subjects (Rosenblat *et al*., 2004). Rosenblat and colleague reported that macrophage PON2 activity was increased under oxidative stress (Rosenblat *et al*., 2003). We detected PON2 activity in monocytes of hypercholesterolaemic subjects whose oxidative stress was relatively high. Atorvastatin treatment significantly reduced oxidative stress, however, it did not reduce PON2 activity (-0.8%, $P < 0.76$). This finding was similar with the previous study which suggested that reduced PON2 in

hypercholesterolaemic subjects was a result of cellular cholesterol accumulation and not from the increased oxidative stress. They also demonstrated that atorvastatin reduced macrophage cholesterol content, up-regulated cellular PON2 expression, and leading to decreased macrophage oxidative stress (Rosenblat *et al*., 2004). Therefore, in view of these results, the insignificant change of PON2 activity observed in monocytes after atorvastatin therapy in our study should be viewed with caution.

In this study, we observed the distribution of all *PON1* genotype frequencies were in Hardy-Weinberg Equilibrium, indicating that a bias due to population sampling was ruled out. The L and M allele frequencies for the *PON1* coding region (L55M) polymorphism observed in our study, were similar to those of previously reported in Thai, Japanese, and Chinese population who display very low frequencies of the M allele (M = 0.16, L = 0.84) (Sanghera *et al*., 1998; Suehiro *et al*., 2000; Phuntuwate *et al*., 2005). The Q and R allele frequencies for the *PON1* Q192R polymorphism observed in our study were similar to previously reported in Thai and Caucasian populations (Q = 0.61, R = 0.39) (Suehiro *et al*., 2000; Phuntuwate *et al*., 2005) but differences with Japanese population (Zama *et al*., 1997). Interestingly, the C and T allele frequencies for *PON1* regulatory region (T-107C) polymorphism observed in our study were (C = 0.61, T = 0.39) similar to Italian, Japanese, and Caucasian population (Suehiro *et al*., 2000; Brophy et al., 2001; Sardo *et al*., 2005) but different from previously reported healthy Thai population by Phuntuwate *et al.* (Phuntuwate *et al*., 2005), which was surprising and we speculated that disease state may influence the differences in result. The C and S allele frequencies of *PON2* C311S (C = 0.26, S = 0.74) polymorphism in this study was the first study in Thai population and we found that the gene frequency was similar to those observed in English and Chinese population (Shi *et al*., 2004; Mackness B *et al*., 2005). Our data support the other previous studies that there are inter population differences in allele frequencies for the *PON* polymorphisms (Suehiro *et al*., 2000; Brophy et al., 2001). This may be a coincident or selection pressure may have acted on these polymorphisms to maintain specific allele frequencies across different ethnic groups. We observed no linkage disequilibrium between *PON1* and *PON2* polymorphisms.

In this study, significant variation in baseline PON1 activity towards phenyl acetate among genotypes classified by *PON1* L55M polymorphism was observed. This result was similar with the previous study that the L allele produced more *PON1* mRNA and that LL genotype had significantly more PON1 concentration than MM genotype (Mackness B *et al*., 1998). This can be speculated that *PON1* containing L allele mRNA may have greater stability than *PON1* containing M allele mRNA. Surprisingly, *PON1* Q192R and *PON1* T-107C did not significantly affect the variation in baseline PON1 activity towards both paraoxon and phenyl acetate substrates. These findings are in conflicts with the result reported that individuals with TT genotype had the lowest PON1 activity as compared to that of CC genotype (Sanghera *et al*., 1997). These differences could be based on ethnical consideration. However, it may be due a too small sample size of each genotypes group in this study. In addition to the correlation study of *PON1* polymorphisms and CHD, there are few studies suggested that *PON2* S311C polymorphism was associated with CHD (Leus *et al*., 2002; Pan *et al*., 2002) but another report suggested that *PON2* polymorphism was not associated with CHD (Pinizzotto *et al*., 2001; Chen et al., 2003). The data presented in this study showed that the *PON2* C311S polymorphism did not significantly affect the variation in baseline TC, TG, HDL, LDL, and PON2 activity in monocytes.

The number of previous intervention studies on the relationship between *PON1* genotypes and PON1 activities were very limited. Moreover, there was no report on the influence of *PON2* genotype on PON2 activity. Tomas and colleague reported that therapeutic response of PON1 activity to simvastatin therapy was independent from the *PON1* L55M and *PON1* Q192R polymorphisms (Tomas *et al*., 2000), however, Sardo and colleague reported that antioxidant effect of atorvastatin was significantly different among *PON1* T-107C polymorphism (Sardo *et al*., 2005). In addition, *PON1* polymorphisms have been found to modify the effect of pitavastatin on HDL (Malin *et al*., 2001). Since there were ethnical variations of *PON* genotypes, we detected the relationship between *PON* genotypes and PON activities, and therapeutic response of *PON* genotypes to atorvastatin treatment, among Thai hypercholesterolaemic subjects.

We found no significant differences in the therapeutic response of PON1 activity towards paraoxon after atorvastatin treatment between genotype groups classified by *PON1* Q192R and L55M polymorphisms. However, the therapeutic response of PON1 activity towards paraoxon was dependent of *PON1* T-107C polymorphism. In addition, the therapeutic response of PON1 activity towards phenyl acetate was dependent of *PON1* L55M polymorphism. Since PON2 activity was not significantly changed after treatment and also non significant differences in the therapeutic response of PON2 activity after atorvastatin treatment between genotype groups classified by the *PON2* C311S polymorphism was observed. These findings suggest that only *PON1* L55M and T-107C polymorphisms modify the therapeutic response of PON1 activities to atorvastatin treatment in hypercholesterolaemic subjects.

In the present study, we found that atorvastatin significantly reduced conjugated diene (CD) (4.4%), total peroxide (13.0%), and malondialdehyde (MDA) (15.2%), whereas, total antioxidant status (TAS) was significantly increased (27.3%). These parameters caused an overall significant reduction of oxidative stress index (OSI) (24.0%). The effect of atorvastatin in lowering CD, total peroxide, and MDA and increasing TAS levels obtained from our study were similar to some previously reported studies (Tomas *et al*., 2000; Kural *et al*., 2004; Sardo *et al*., 2005). The degree of decreased in lipid peroxidtion markers imply that PON may be really function by hydrolyze lipid peroxides since the level of the downstream lipid peroxidation markers, total peroxide and MDA, decreased more than CD. However, our result did not provide enough clear cut evidence to show the precise mechanism since a reduction of lipid peroxidation could partly be due to an effect from the decreased in lipid levels. In this study, our results demonstrated clearly that atorvastatin caused a significant reduction of OSI in hypercholesterolaemic subjects. It seems therefore reasonable to postulate that atrovastatin exerts beneficial effect on CHD, a mechanism that is beyond its lipid modifying properties. This beneficial effect may have an impact on the prevention of CHD.

In conclusion, our studies shows that atorvastatin cause an increased in TAS, PON1, and PON3 activities and a decrease in lipid peroxidation and oxidative stress. This might be due to antioxidant or pleiotropic properties of atorvastatin. A clear understanding of the effects of lipid lowering drugs on non-lipid risk factors of atherosclerosis will be helpful for selection of optimal treatment according to risk profile of individual patient. The knowledge obtain from this study can be applicable to pharmacogenetics and also essential for the development of more effective therapy. However, strong evidence need to be further investigate

CHAPTER VI
CONCLUSION

Atorvastatin treatment was effective in lowering total cholesterol, triglyceride, low density lipoprotein, conjugated diene, total peroxide, malondialdehyde, and oxidative stress index in hypercholesterolaemic subjects. Meanwhile, atorvastatin was significantly effective to increasing total antioxidant status. The mean value of high density lipoprotein did not significantly change after treatment. Atorvastatin treatment was not able to exert beneficial effect on anthropometric parameters including blood pressure. Atorvastatin exerted favorable effect on increasing PON1 activity towards paraoxon but not phenyl acetate, however, the effect of atorvastatin on the increasing of PON1 activity towards phenyl acetate was seem to be sex dependent in which males had significantly higher levels than in females. Atorvastatin also significantly increased PON3 activity to the same degree as PON1, however, no significant change of PON2 activity in monocyte was observed in this study. Decreasing oxidative stress and increasing antioxidant status with PON1 and PON3 activities by atorvastatin may be regarded as an additional favorable action, which might be due to pleiotropic properties of atorvastatin. The mechanism(s) of action for atorvastatin increased PON1 and PON3 activities could be due to an increase PON1 and PON3 concentrations or activities. Atorvastatin may play a role in inducing *PON1* gene expression. The antioxidant property of atorvastatin itself that is capable in reducing oxidative stress may lead to less inactive form or a decrease in the utilization of PON. Atorvastatin also may directly alter PON activity via a change in the reduction/oxidization pathways of PON. These speculations need to be scrutinized in the future studies.

In hypercholesterolaemic subjects, the genotype and allele frequencies for the *PON1* L55M, Q192R, and T-107C polymorphisms were 73.68% LL, 21.05% LM, 5.26% MM (L = 0.84, M = 0.16), 42.11% QQ, 36.84% QR, 21.05% RR (Q = 0.61, R = 0.39), 42.11% CC, 36.84% CT, and 21.05% TT (C = 0.61, T = 0.39), respectively.

The genotype and allele frequencies for the *PON2* C311S were 10.53% CC, 31.58% CS, and 57.89% SS (C = 0.26, S = 0.74). Chi-square test revealed no significant linkage disequilibrium between *PON1* and *PON2* polymorphisms ($P > 0.05$). The baseline PON1 activity towards phenyl acetate was significantly influenced by the *PON1* L55M polymorphism. The *PON1* L55M and T-107C polymorphisms influenced on the therapeutic response of PON1 activities to atorvastatin treatment.

In addition, atorvastatin was well tolerated and there was no adverse effects observed during our study periods in hypercholesterolaemic subjects. Therefore, atorvastatin showed the safety profile in Thai hypercholesterolaemic subjects. In conclusion, atorvastatin may contribute important antioxidant properties through increasing PON1 and PON3 activities, as a consequence of reducing oxidative stress and, leads to beneficial cardiovascular events. Moreover, the finding of PON1 polymorphism will be helpful in pharmacogenomics of atorvastain regimen in the future.

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