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Hydrolysis of Organophosphate and Model Substrates in African American and Caucasian Southerners by Serum Paraoxonase-1 (pon1) and its Relationship to Atherosclerosis

Ryan Hunter Coombes

HYDROLYSIS OF ORGANOPHOSPHATE AND MODEL SUBSTRATES IN AFRICAN AMERICAN AND CAUCASIAN SOUTHERNERS BY SERUM PARAOXONASE-1 (PON1) AND ITS RELATIONSHIP TO ATHEROSCLEROSIS

By

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Paraoxonase-1 (PON1) is a high density lipoprotein (HDL)-associated enzyme displaying esterase and lactonase activity. PON1 hydrolyzes the oxons of several organophosphorous insecticides (e.g. paraoxon, diazoxon and chlorpyrifos-oxon) and metabolizes lipid peroxides of low density lipoproteins (LDL) and HDL. As such, PON1 plays a relevant role in determining susceptibility of organophosphate toxicity and cardiovascular disease.

The objective of this study was to determine associations of PON1 status (i.e. genotype and activity levels) with atherosclerosis (ATH) in individuals from the Southeastern United States. An additional objective was to determine whether PON1 genotype and/or PON1 activity levels influence the capacity of PON1 to metabolize chlorpyrifos-oxon (CPO) at a relatively low concentration.

Data indicated increasing PON1 activity assessed by hydrolysis of phenyl acetate is associated with decreased odds of ATH. Furthermore, neither PON1 genotype nor

PON1 activity levels influence capacity of PON1 to metabolize CPO at a relatively low concentration.

Keywords: paraoxonase, atherosclerosis, coronary artery disease, cardiovascular disease, health disparities, chlorpyrifos-oxon, organophosphates, PON1

DEDICATION

To the casual observer, a project of this level may appear to be solitary work. However, to complete a project of this magnitude requires a network of support, and I am indebted to many people. I am most especially grateful to my friends and family for their guidance, continuous support, and extraordinary courage throughout the program.

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CHAPTER I

INTRODUCTION

In every year since 1900, cardiovascular disease (CVD) accounted for more deaths than any other major cause of death in the United States (Roger et al. 2011). States in the South (except for Florida) have higher annual age-adjusted mortality rates from CVD and stroke than states from other regions of the country. The cause for this disparity is unknown. Low socioeconomic status, exposure to environmental toxicants, and the increasing prevalence of hypertension are some theories that have developed to explain the higher stroke mortality rate in the South (Perry and Roccella 1998). CVD is also more prevalent in African Americans than in Caucasians (Roger et al. 2011).

Paraoxonase-1 (PON1; EC 3.1.8.1) is a calcium dependent enzyme which functions as an esterase and lactonase. PON1 is a member of a family of proteins that also includes PON2 and PON3 (Primo-Parmo et al. 1996). PON1 is synthesized primarily in the liver and is secreted into the plasma, where it is associated with high density lipoprotein (HDL) particles (Sorenson et al. 1999). Initially characterized for its ability to hydrolyze organophosphates (OP), the name, PON1, reflects its ability to hydrolyze paraoxon, the toxic metabolite of the OP insecticide parathion, which is its first and most studied substrate (Aldridge 1953a; Mazur 1946).

PON1 performs multiple functions. One physiological function of PON1 is the prevention of low density lipoprotein (LDL) and HDL oxidation by hydrolyzing lipid peroxides in the lipoprotein; therefore protecting against the development of

atherosclerosis (ATH) (Mackness et al. 1991; Shih et al. 1998). The PON1 Q192R polymorphism involves an arginine (R) to glutamine (Q) amino acid substitution (Atkins et al. 1993; Humbert et al. 1993). The relationship, if any, of the PON1 Q192R polymorphism with the development of ATH is unclear. Initial studies suggested that the $PON1_{192Q}$ alloform was more efficient at metabolizing lipid peroxides in human atherosclerotic plaque than the $PON1_{192R}$ alloform (Aviram et al. 2000), and thus was believed to be more effective at preventing ATH. However, recent research has reported that the $PON1_{192R}$ alloform binds to HDL with greater affinity than the $PON1_{192Q}$ alloform and therefore demonstrates increased stability and greater lipolactonase activity (Gaidukov et al. 2006). Thus the effect of the PON1 Q192R polymorphism in atherosclerotic (ATH) vascular disease is still unknown (Bhattacharyya et al. 2008).

African Americans are reported to have a higher weighted $PON1_{192R}$ allele frequency, while Caucasians have a higher weighted $PON1_{192Q}$ allele frequency (Costa et al. 2003b; Scacchi et al. 2003). The population distribution of the $PON1_{192}$ functional genotypes (QQ, QR, RR) can be inferred by plotting the rate of diazoxon hydrolysis (diazoxonase) by the rate of paraoxon hydrolysis (paraoxonase) (Richter and Furlong 1999). Data on the frequency distribution of the $PON1_{192}$ genotypes has been reported on numerous populations, but not specifically the Southern populations. The current consensus is that the PON1 phenotype (i.e. enzyme activity level) is more important than genotype in estimating an individual's risk of disease with individuals having higher PON1 enzyme activities being more protected from disease than those with lower enzyme activities (Mackness et al. 2001).

Another key function of PON1 is its ability to hydrolyze the toxic metabolites of several OP insecticides (e.g. chlorpyrifos-oxon, diazoxon and paraoxon). The PON1

Q192R polymorphism significantly affects the catalytic efficiency of hydrolysis of specific OP substrates (Costa et al. 2003b; Furlong et al. 2000). Transgenic mouse models of the human hPON1 Q192R polymorphism have demonstrated that the $PON1_{192R}$ alloform is significantly more efficient in hydrolyzing chlorpyrifos-oxon, the toxic metabolite of the OP anticholinesterase insecticide chlorpyrifos, than $PON1_{192Q}$ alloform and provides better protection against chlorpyrifos-oxon exposure (Richter et al. 2009). The catalytic efficiency with which PON1 degrades toxic OPs determines the degree of protection provided by PON1 (Costa et al. 2003b; Furlong et al. 2000). In addition, higher concentrations of PON1 in the serum provide better protection (Cole et al. 2010; Jansen et al. 2009). When given the wide range of use of OPs and their high toxicity, research on the role of PON1 in modulating their toxic effects remains of utmost importance.

Most epidemiological studies concerned with CVD have not focused on southern populations where cardiovascular health is the worst, nor have African Americans been an important proportion of the subjects in these studies. Additionally, little research has been published regarding the frequency distribution of $PON1_{192}$ genotypes of southern populations, which have the worst American health statistics. In this study, PON1 status was determined for African American and Caucasian male and female Southerners for whom demographic and clinical information was available. A model was developed to test the association of PON1 status with ATH while accounting for a core group of clinical and demographic variables. Also in this study, the hydrolysis of chlorpyrifos-oxon was measured at a relatively high concentration (320 µM) and a relatively low concentration (178 nM) by PON1 in the sera of the study population with a high and a low activity phenotype towards diazoxon and paraoxon.

Subjects were enrolled in the study from the clinical practice of Cardiology Associates of North Mississippi, LLC, a cardiology group located in Tupelo, Mississippi, which draws the vast majority of its patients from the 26 counties comprising northeast Mississippi and northwest Alabama. The research division of this clinic, Cardiology Associates Research, LLC (CARe), collected a total of 200 serum samples from Caucasians (60 male, 60 female) and African Americans (40 male, 40 female), ages 45 and older. A 60/40 split was chosen to reflect the racial composition of Mississippi. Subjects were excluded if they were being evaluated for acute coronary syndrome (recent myocardial infarction or unstable angina), were known or suspected to be infected with human immunodeficiency virus or hepatitis B, or did not self-declare as either Caucasian or African American. Participants underwent the diagnostic tests recommended by their cardiologist. Study records that identified the subject were kept confidential as required by Federal Privacy Regulations. The protocol was approved by both the Institutional Review Board of Mississippi State University and the IRB of North Mississippi Medical Center; the latter provides oversight for studies performed at CARe.

CHAPTER II

LITERATURE REVIEW

Introduction to Paraoxonase-1 (PON1)

Paraoxonase-1 (PON1; EC 3.1.8.1) is a calcium dependent enzyme which functions as an esterase and lactonase. In 1946, Mazur was the first to describe the enzymatic hydrolysis of organophosphorous (OP) compounds by enzymes in animal tissues (Mazur 1946). This led to the initial identification of the PON1 enzyme during the 1950s. PON1 was named after its ability to hydrolyze the OP substrate paraoxon, which is the toxic metabolite of the OP insecticide parathion. Because PON1 can also hydrolyze aromatic esters, such as phenylacetate (arylesterase; EC 3.1.1.2), the term *A-esterase* was coined for the enzyme hydrolyzing both compounds (Aldridge 1953a; Aldridge and Davison 1953b). Another group, *B-esterases* (e.g., cholinesterases and carboxylesterases), is inhibited by OPs in the progressive reaction which is time and temperature dependent (Aldridge 1953a; Aldridge and Davison 1953b). Studies in the late 1970s and early 1980s indicated a large variability of PON1 activity among individuals and that the gene frequency for the low activity varied considerably among different human populations (Diepgen and Geldmacher-von Mallinckrodt 1986; Playfer et al. 1976; Eckerson et al. 1983; Mueller et al. 1983), suggesting genetically based differential susceptibility to OP toxicity. It was not until the 1990s that the molecular basis of the PON1 activity polymorphisms (Humbert et al. 1993; Adkins et al. 1993) and its role in the toxicity of OP compounds (Lotti 1991a, b) have been determined. More

recently, the molecular structure of a recombinant PON1 protein has also been elucidated (Harel et al. 2004).

Currently, PON1 has been shown to have multiple functions. One physiological function of PON1 is the prevention of low density lipoprotein (LDL) and high density lipoprotein (HDL) oxidation by hydrolyzing lipid peroxides in the lipoprotein; therefore, it may protect against the development of atherosclerosis (ATH) (Aviram and Rosenblat 2004; Mackness et al. 1991). PON1 also functions to hydrolyze OP anticholinesterases (e.g. chlorpyrifos-oxon and diazoxon, the active metabolites of two OP insecticides, chlorpyrifos and diazinon, respectively), as well as nerve agents such as sarin, soman and VX thereby contributing to protection against the toxicity of OP insecticides and nerve agents (Davies et al. 1996; Li et al. 2000; Shih et al. 1998). This became of great importance in the field of toxicology, and investigations were conducted focusing on protection against organophosphate poisoning and prevention of atherosclerotic development.

The Paraoxonase Family

PON1 is a member of a family of proteins that also includes PON2 and PON3. The genes encoding for the various PONs are >60% identical and all three are mapped to nearby loci on the long arm of chromosome 7 (q21.22) in humans (Primo-Parmo et al. 1996). From a molecular evolutionary standpoint, PON2 appears to be the oldest member, followed by PON3 and PON1 (Draganov and La Du 2004). At the molecular level, all of the PON gene family members share the ability to hydrolyze aromatic and long-chain aliphatic lactones and thus the name PON is in fact a misnomer since PON2 and PON3 lack paraoxonase activity (Draganov et al. 2005). For that reason, the PON

gene family is most likely evolutionarily linked to lactonases (Kobayashi et al. 1998). Of the three members of the PON family, the most studied and hence best known is PON1 (van Himbergen et al. 2006).

The PON gene family is expressed in a variety of human tissues (Primo-Parmo et al. 1996). In humans, PON1 and PON3 mRNA is synthesized and expressed within the liver (Reddy et al. 2001), while PON2 is expressed in multiple tissues including the heart, kidney, liver, lung, and testis (Ng et al. 2005; Ng et al. 2001). In human serum, PON1 activity predominates; however, both PON1 and PON3 are secreted into the blood from the liver where it is tightly bound to HDL particles ($K_D \leq 1$ nM) (Gaidukov et al. 2010; Deakin et al. 2002). In contrast, the human PON2 protein is not found in HDL, LDL, or the media of cultured cells, but appears to remain intracellular with its active site protruding to the outside of the cell (Ng et al. 2001). Human PON1 can be found in a similar orientation in the liver before it is released by a docking process (i.e., HDL particles temporarily associate with the cell membrane and remove PON1 from the membrane) (Deakin et al. 2002; Moren et al. 2008).

The PON1 Polymorphisms

Hasset (Hassett et al. 1991) and Adkins (Adkins et al. 1993) are both credited for determining the human liver PON1 cDNA sequence and molecular characterization of its polymorphisms. The cloning of the gene resulted in the identification of over 200 single-nucleotide polymorphisms (SNPs) in different regions of the PON1 gene. An early study indicated that PON activity in human populations exhibited a polymorphic distribution, and individuals with high, intermediate, and low PON activity could be identified (Mueller et al. 1983). The molecular basis of PON activity polymorphisms is a missense

mutation in the coding region of PON1 resulting in leucine(L)/methionine(M) substitution at position 55 and glutamine(Q)/arginine(R) substitution at position 192 (La Du et al. 1993; Li et al. 1995). *PON1*$_{192}$ and *PON1*$_{55}$ genotypes have been established in several populations utilizing PCR-based methods. The polymorphism at position 192 has been the most studied, with a weighted *PON1*$_{192Q}$ allele frequency of 0.75 for Caucasians and a weighted *PON1*$_{192R}$ allele frequency of 0.63 for African Americans (Chen et al. 2003; Scacchi et al. 2003).

In addition to the PON1 Q192R and L55M polymorphisms, sequencing of the non-coding region of PON1 gene has led to the discovery of 13 additional polymorphisms, of which 5 have been characterized and show varying degrees of influence over its expression (Brophy et al. 2001; Leviev and James 2000; Suehiro et al. 2000). The most significant of the promoter region polymorphisms is that at position -108, with -108C allele providing levels of PON1 about twice as high as those seen with the -108T allele (Brophy et al. 2001). The other polymorphisms have not been characterized and need more investigation.

The catalytic efficiencies of both PON1 Q192R and L55M polymorphisms have been investigated for effects on the catalytic efficiencies towards specific substrates. The PON1 L55M polymorphism does not affect catalytic efficiency, but has been associated with plasma PON1 protein levels, with PON1$_{55M}$ alloform being associated with low plasma PON1 (Mackness et al. 1998). It is suggested that linkage disequilibrium with the low efficiency of the -108T allele of the -108 promoter region polymorphism plays a role (Brophy et al. 2001). Research has implied that the PON1$_{55M}$ alloform may be less stable than PON1$_{55L}$ alloform (Leviev et al. 2001; Roest et al. 2007).

The PON1 Q192R polymorphism significantly affects the catalytic efficiency of PON1. Early studies indicated that the $PON1_{192R}$ allozyme hydrolyzed paraoxon and chlorpyrifos-oxon more readily than the $PON1_{192Q}$ (Humbert et al. 1993; Adkins et al. 1993). Current research has indicated that this polymorphism was substrate dependent, as the $PON1_{192Q}$ alloform was found to hydrolyze diazoxon, sarin, and soman more rapidly than $PON1_{192R}$ *in vitro* (Davies et al. 1996). However, it has also been shown that both PON1 alloforms hydrolyze diazoxon with the same efficiency, under physiological conditions (Li et al. 2000). $PON1_{192Q}$ allozyme also has a higher efficiency in protecting against LDL oxidation than the $PON1_{192R}$ allozyme (Mackness et al. 1999).

The Structure of Paraoxonase

Human serum PON1 is a glycoprotein of 354 amino acids and its molecular weight is 43-47kDa (Furlong et al. 1991). PON is a six-bladed β-propeller with two calcium ions (i.e. Ca1 and Ca2), 7.4 Å apart, in a central tunnel of the PON propeller (Harel et al. 2004; Kuo and La Du 1998). The calcium in the central section (Ca1) is referred to as structural calcium, because its dissociation leads to irreversible denaturation of the protein (Harel et al. 2004; Kuo and La Du 1998). The second calcium (Ca2) is assigned as the catalytic calcium, because its removal from PON1 or the addition of a metal ion chelator (EDTA) results in inactivation of the Ca^{2+}-dependent PON1 activities towards paraoxon and phenylacetate, but does not affect its ability to protect LDL-cholesterol from oxidation (Kuo and La Du 1998). Although the exact mechanism of PON1's protective activities is unknown, results have illustrated that there are separate active sites on PON1 for paraoxonase and phenylacetate activities (Aviram 1999; Aviram et al. 1998b).

PON1 has three cysteine (Cys) residues at positions Cys^{42}, Cys^{284}, and Cys^{353}. Cysteine residues at positions Cys^{42} and Cys^{353} form a disulfide linkage; however, the Cys^{284} residue is free (Kuo and La Du 1998). Cys^{284} is essential for the enzyme's activity and the ability of PON1 to protect from LDL oxidation. Mutating C^{284} to alanine (Ala) or serine (Ser) in recombinant PON1 mutants decreases its paraoxonase and phenylacetate activities (Sorenson et al. 1995), and complete inhibition of its ability to protect against oxidation of LDL (Aviram et al. 1998b).

The active site of PON1 contains a histidine dyad formed by His^{115} and His^{134}. His^{115} acts as a general base to activate a water molecule attacking the carbonyl oxygen of the substrate, whereas His^{134} is a proton relay and the calcium is stabilizing the negatively charged intermediate (Harel et al. 2004; Khersonsky and Tawfik 2006).

PON1 and Cardiovascular Disease

The concept of PON1 binding to HDL cholesterol in human serum led to hypotheses by Mackness that the enzyme might play a role in lipid metabolism and protection against the development of ATH (Mackness et al. 1991). ATH is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. Risk factors have been implicated in the development of ATH which included obesity, hypertension, and dyslipidaemia (i.e. elevated total cholesterol, LDL cholesterol, triglyceride concentrations, and decreased HDL cholesterol) (McKenney 2003). The antioxidant activity of HDL cholesterol is largely due to its enzyme PON1, located on it (Mackness et al. 1991).

The most convincing link between PON1 activity and atherosclerosis comes from animal studies. *In vivo* experiments with knockout mice (Shih et al. 1998; Shih et al.

2000) provide convincing data for the involvement of PON1 in protection from atherosclerosis (Mackness et al. 1998). From these studies, PON1 knockout mice developed ATH when fed an atherogenic diet, and their HDL cholesterol failed to prevent LDL cholesterol oxidation in cultured artery wall cells, in contrast to wild-type HDL cholesterol. Conversely, transgenic mice over-expressing PON1 had a significant decrease in ATH lesion development (Tward et al. 2002).

Although animal studies have shown the inverse relationship between PON1 levels and ATH, PON1's antioxidant properties in humans are still being investigated. A large number of studies have tested the association of Q192R polymorphism and the development of ATH. The $PON1_{192Q}$ alloform is believed to be more protective of cardiovascular health than the $PON1_{192R}$ alloform because it has a greater capacity to metabolize oxidized lipids (Costa et al. 2005). However, another study suggests the $PON1_{192R}$ alloform binds to HDL cholesterol with better affinity than the $PON1_{192Q}$ alloform therefore demonstrating increased stability and greater lipolactonase activity (Gaidukov et al. 2006). A recent meta-analysis of 43 published studies revealed a weak association of the Q192R polymorphism with ATH (Wheeler et al. 2004). Any association of Q192R polymorphism with ATH remains unclear. Conflicting results could be due to small sample size from different populations, different genotyping methods, different sampling strategies, and different end points (Roest et al. 2007).

The catalytic efficiency with which PON1 metabolizes oxidized lipids determines the degree of protection provided by PON1. In addition, higher concentrations of PON1 provide better protection; therefore, when determining adequate risk assessment, it is important to know PON1 levels and activity. In a given population, plasma PON1 activity can vary up to 40-fold (Eckerson et al. 1983; Mueller et al. 1983; Davies et al.

1996; Richter and Furlong 1999), and differences in PON1 protein levels up to 13-fold are also present within a single *PON1*$_{192}$ genotype in adults (Davies et al. 1996). Studies that examine either plasma PON1 activity levels or PON1 status (i.e. activity levels and *PON1*$_{192}$ genotype) is a much better predictor of disease than *PON1*$_{192}$ genotype alone (Jarvik et al. 2000; Mackness et al. 2001; Graner et al. 2006; Gur et al. 2006). There are many factors that can influence PON1 activity such as diet, environmental factors, lifestyle, and disease (Costa et al. 2003a). In epidemiological studies, enzyme activity as well as genotype needs to be considered when determining associations of PON1 with disease.

PON1 and the Toxicity of Organophosphate (OP) Insecticides

While the ability of PON1 in hydrolyzing a number of OP substrates *in vitro* is well established, *in vivo* studies of the same OPs have emerged more slowly. Early experiments demonstrated that by injecting of partially purified PON1 from rabbit serum to rats would protect them against paraoxon and much better protection against chlorpyrifos-oxon exposures (Li et al. 1995; Furlong et al. 1991; Main 1956; Costa et al. 1990; Li et al. 1993). These studies indicate that increasing serum levels of PON1 by injection of exogenous source of PON1 (purified rabbit PON1) decreased the acute toxicity of specific OPs and that PON1 exerted a protective effect when given after OP exposure.

Recently, PON1 knockout and transgenic animals generated by Shih et al. (Shih et al. 1998) have provided important new tools to investigate the role of PON1 in modulating OP toxicity. In the absence of PON1, PON1 null mice showed a dramatic increase in sensitivity to chlorpyrifos-oxon and diazoxon (Li et al. 2000) when high doses

of chlorpyrifos or diazinon was used. It was also demonstrated that PON1 null mice did not show an increased sensitivity to paraoxon, in spite of having no paraoxonase activity in plasma and liver (Li et al. 2000). Experiments began to examine the catalytic efficiency of human PON1s in an attempt to explain why PON1 protected against some OP compounds and not others.

PON1 protects against OPs that are hydrolyzed with high catalytic efficiency. Transgenic mice (mice expressing either human $PON1_{192Q}$ allele or human $PON1_{192R}$ allele) or administration of exogenous PON1 to PON1 null mice experiments by Li et al. (Li et al. 2000) and Cole et al. (Cole et al. 2005) demonstrated that diazoxon was hydrolyzed by both PON1-192 alloforms with equal catalytic efficiency and increasing PON1 levels by injection of either purified human PON1-192 alloform restored resistance to diazoxon exposure. They also elucidated that the $PON1_{192R}$ alloform had better catalytic efficiency for chlorpyrifos-oxon and paraoxon hydrolysis than the $PON1_{192Q}$ alloform. Chambers (Chambers et al. 1994) and Pond (Pond et al. 1995) demonstrated that PON1 does not hydrolyze paraoxon efficiently at low concentrations *in vivo,* and that other pathways must be involved (e.g. cytochromes P450, carboxylesterase).

CHAPTER III

RELATIONSHIP OF PARAOXONASE-1 (PON1) SERUM ACTIVITY AND GENOTYPE WITH ATHEROSCLEROSIS IN INDIVIDUALS FROM THE DEEP SOUTH

The paraoxonase (PON) gene family has been extensively investigated recently. One member of the PON family, paraoxonase-1 (PON1) has been implicated in a wide variety of human illnesses including cardiovascular disease (CVD), diabetes mellitus, and obesity (Abbott et al. 1995; Ferretti et al. 2005; Shih et al. 1998). PON1 performs multiple functions. In humans, PON1 is mainly expressed in the liver, then transported to the blood where it is tightly bound to high density lipoprotein (HDL) particles (K_D <1 nM) (Gaidukov and Tawfik 2005). One physiological function is the prevention of low density lipoprotein (LDL) and HDL oxidation by hydrolyzing lipid peroxides in lipoprotein particles; therefore it may protect against the development of atherosclerosis (ATH) (Shih et al. 1998; Mackness et al. 1991). PON1 also protects against the acute toxicity of organophosphorus anticholinesterases including nerve agents and insecticides through hydrolysis of the compounds or their active metabolites (Aldridge and Davison 1953). In fact, paraoxonase received its name for its ability to hydrolyze paraoxon, the active metabolite of the insecticide parathion.

Studies using mouse models of ATH (ApoE$^{-/-}$ and LDL-R$^{-/-}$) have consistently shown that the over expression of PON1 reduces ATH by reducing oxidative stress, hydrolyzing lipid peroxides, promoting cholesterol efflux from macrophages, and

increasing reverse cholesterol transport (Aviram and Rosenblat 2004; Rozenberg et al. 2005; Tward et al. 2002). Conversely, studies have shown that PON1-deficient mice have increased susceptibility to LDL oxidation, increased measures of macrophage oxidative stress, and larger atherosclerotic lesions than controls (Rozenberg et al. 2003; Shih et al. 2000).

Differences in PON1 activity have been linked to common polymorphisms in both coding and promoter regions (Davies et al. 1996; Leviev and James 2000). Two common polymorphisms in the coding region of PON1 are PON1 L55M and PON1 Q192R. The PON1 Q192R polymorphism has the greater influence on enzyme activity measured *in vitro* (Davies et al. 1996; Adkins et al. 1993). This polymorphism involves a glutamine (Q) to arginine (R) substitution at position 192 (Adkins et al. 1993; Humbert et al. 1993). Several promoter region polymorphisms have been described with the C-108T polymorphism influencing expression the most (Leviev and James 2000). The recognition that PON1 prevents lipid peroxide accumulation and that the PON1 Q192R polymorphism affects the rate of hydrolysis of several pesticide metabolites led to investigation of the possible association of the PON1 Q192R polymorphism with atherosclerosis.

The relationship, if any, of the PON1 Q192R polymorphism with the development of atherosclerosis is unclear. Early research suggested that the $PON1_{192Q}$ alloform was more efficient at metabolizing lipid peroxides in human atherosclerotic plaques than the $PON1_{192R}$ alloform (Aviram et al. 2000), and thus was believed to be more effective at preventing ATH. In addition several early studies suggested an association of the $PON1_{192R}$ allele with atherosclerosis (reviewed by Costa et al. (Costa et al. 2003)). However, recent research indicated that the $PON1_{192R}$ alloform binds to HDL

with greater affinity than the $PON1_{192Q}$ alloform and therefore demonstrates increased stability and greater lipolactonase activity (Gaidukov et al. 2006). Also the prevalence of ATH was greatest in subjects with the *PON1*$_{192QQ}$ genotype (Bhattacharyya et al. 2008). A recent meta-analysis found only a weak association of *PON1*$_{192R}$ with vascular disease and pointed out the need for more definitive studies (Wang et al. 2011). Richter and Furlong (Richter and Furlong 1999) have argued that to accurately assess PON1's role in atherosclerosis that both PON1 genotype and phenotype (i.e. activity level), together known as the PON1 status, must be determined.

An individual's functional *PON1*$_{192}$ genotype can be determined using a 2-substrate assay to generate a 2-dimensional enzyme activity plot (Richter and Furlong 1999). Without actual genotyping, the plot of the rates of diazoxon hydrolysis in the presence of 2 M NaCl (diazoxonase; DZOaseNa) vs. paraoxon hydrolysis in the presence of 2 M NaCl (paraoxonase; POaseNa) separates individuals into 3 functional genotypes, $PON1_{192QQ}$, $PON1_{192QR}$ and $PON1_{192RR}$. PON1 activity determined using PhAc as a substrate in the absence of NaCl does not distinguish between the $PON1_{192Q}$ and the $PON1_{192R}$ alloforms and thus serves as a measure of PON1 activity independent of genotype (Richter et al. 2008).

Numerous epidemiological studies have identified factors that increase the risk of ATH. Important risk factors include: dyslipidemias (e.g. low HDL cholesterol and high LDL cholesterol), smoking, diabetes mellitus, and hypertension. States in the Southeast (except Florida) have higher age-adjusted annual mortality rates from CVD than any other geographic region in the US (Roger et al. 2011). The cause for this disparity is unknown. Low socioeconomic status, exposure to environmental toxicants, and the increasing prevalence of hypertension have been proposed to explain the higher stroke

mortality rate in the South (Perry and Roccella 1998). African American men and women have higher age-adjusted mortality rates from CVD than Caucasian men and women (Roger et al. 2011). Little information is available on the frequency distribution of $PON1_{192}$ genotypes of US Southern populations, which have among the worst health statistics in America. Early studies reported that African Americans have a weighted $PON1_{192Q}$ allele frequency of 0.37 and a weighted $PON1_{192R}$ allele frequency of 0.63, while Caucasians have a weighted $PON1_{192Q}$ allele frequency of 0.73 and a weighted $PON1_{192R}$ allele frequency of 0.27 (Chen et al. 2003). A study from our laboratory (Davis et al. 2009) showed a similar distribution with African Americans having a higher $PON1_{192R}$ allele frequency, 0.66, and Caucasians having a higher $PON1_{192Q}$ allele frequency, 0.77.

In the present study, the $PON1_{192}$ genotype and the PON1 enzymatic activity (phenotype) were determined for African American and Caucasian male and female Southerners for whom demographic and clinical information was available. The enzymatic activity was assessed with three substrates, paraoxon, diazoxon and PhAc. Statistically significant associations of clinical characteristics and $PON1_{192}$ genotype and phenotype with ATH were determined using logistic regression. A model was developed to test the association of PON1 status with ATH while accounting for a core group of clinical and demographic variables.

Materials and methods

Study Population

Subjects were enrolled in the study from the clinical practice of Cardiology Associates of North Mississippi, LLC, a cardiology group located in Tupelo, Mississippi,

which draws the vast majority of its patients from the 26 counties comprising northeast Mississippi and northwest Alabama. The research division of this clinic, Cardiology Associates Research, LLC (CARe), collected a total of 200 serum samples from Caucasians (60 male, 60 female) and African Americans (40 male, 40 female), ages 45 and older. A 60/40 split was chosen to reflect the racial composition of Mississippi.

Subjects were excluded if they were being evaluated for acute coronary syndrome (recent myocardial infarction or unstable angina), were known or suspected to be infected with human immunodeficiency virus or hepatitis B, or did not self-declare as either Caucasian or African American. Participants underwent the diagnostic tests recommended by their cardiologist. Study records that identified the subject were kept confidential as required by Federal Privacy Regulations. The protocol was approved by both the Institutional Review Board (IRB) of Mississippi State University and the IRB of North Mississippi Medical Center; the latter provides oversight for studies performed at CARe.

After obtaining informed written consent, blood samples were collected when vascular access was established for clinical testing. Serum was isolated by allowing blood to clot, then centrifuging at 2500-3000 x g at 4°C for 10 to 20 minutes. The serum was frozen on dry ice and stored at -70°C until analysis. An aliquot of whole blood anti-coagulated with K_2EDTA was stored at -70°C for *PON1* genotypic analysis.

Age, gender, race, diabetes, history of tobacco use, family history of coronary artery disease or stroke, presence of hypertension, the use of statins, height, weight, waist circumference in inches, blood pressure, and pulse were also obtained for each individual. A fasting lipid panel was obtained on the serum collected. All clinical information was de-identified before release from the clinic. Subjects were classified as

having ATH if they had either a history of a clinical event resulting from or a mechanical revascularization procedure for treating coronary artery disease, cerebrovascular disease, abdomino-aortic disease, or peripheral vascular disease. Subjects were also considered to have ATH if they had reversible perfusion defects interpreted as myocardial ischemia on a nuclear stress test. Subjects who did not meet the criteria for ATH were considered not to have ATH. All diagnostic tests were interpreted by a CARe physician who had no knowledge of the results of the PON1 assays.

Statin usage of a subject was designated as 'yes' if the subject was currently on a statin drug or designated as 'no' if the subject was not taking a statin. Smoking status of an individual was assigned an 'ever' if the subject was still smoking or had ever smoked or 'never' if the subject had never smoked.

Because of the large number of participants on statins, LDL cholesterol levels and statin usage were used to place subjects into one of two categories. Subjects were considered normal for LDL cholesterol if their levels were ≤ 160 mg/dL, they did not meet criteria for pharmacologic treatment (McKenney 2003), and they were not currently taking a statin. Subjects' LDL cholesterol levels were considered elevated if their levels were >160 mg/dL, they were currently taking a statin, or they met the criteria for pharmacological treatment of elevated LDL cholesterol (McKenney 2003). Subjects were considered normal for HDL cholesterol if their levels were ≥ 40 mg/dL and low if their levels were < 40 mg/dL.

Subjects were considered normal for triglycerides if their levels were <200 mg/dL and they were not taking a fibrate. Subjects were considered abnormal for triglycerides if their levels were ≥200 mg/dL or they were taking a fibrate. Subjects who were on statin therapy and had triglycerides <200 mg/dL were considered indeterminate as it was

assumed statin therapy was prescribed for elevated LDL cholesterol but would influence triglyceride levels and prevent an accurate designation of baseline triglyceride status (i.e. off statin therapy).

Subjects were considered normal for total cholesterol if their levels were <200 mg/dL and they were not taking statins. Subjects were considered abnormal for total cholesterol if their levels were ≥200 mg/dL. Subjects who were on statin therapy and had a total cholesterol <200 mg/dL were considered indeterminate as it was assumed statin therapy was prescribed for elevated LDL cholesterol but would influence total cholesterol levels and prevent an accurate designation of baseline total cholesterol status (i.e. off statin therapy).

Chemicals and Samples

Paraoxon was synthesized as described previously (Chambers and Chambers 1990). Diazoxon was purchased from ChemService (West Chester, PA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Paraoxonase and Diazoxonase Assays

Paraoxonase and diazoxonase activities were determined by the hydrolysis of paraoxon and diazoxon, respectively, as described by Richter and Furlong (Richter and Furlong 1999) with minor modifications (Davis et al. 2009). The paraoxonase assay was conducted in the presence of 2 M NaCl (POaseNa) and the absence of NaCl (POase). The diazoxonase assay was conducted in the presence of 2 M NaCl (DZOaseNa) and the absence of NaCl (DZOase). Samples were run in triplicate. Data were expressed as units per liter of serum (U/L) where 1 unit = 1 micromole of paraoxon or diazoxon hydrolyzed per minute.

The functional genotype was determined by plotting the DZOaseNa activity versus the POaseNa activity for each sample (Richter and Furlong 1999).

Arylesterase Assay

Arylesterase activity was determined using PhAc as a substrate. The phenol released from PhAc hydrolysis was measured spectrophotometrically as described previously (Davis et al. 2009) and the activity was expressed as U/L where 1 unit = 1 micromole of PhAc hydrolyzed per minute. Samples were run in triplicate.

***PON1* Genotyping**

Genomic DNA was isolated from whole blood using a Sigma-Aldrich® GenElute™ Blood Genomic DNA Kit and concentrated using a Qiagen QIAEX®II Gel Extraction Kit. Following the method received from the Medical Genetics Department of the University of Washington (Jane Ranchalis, personal communication), a region containing the $PON1_{192}$ codon was amplified by PCR to yield a 99 bp product using the primer pair F 5' TATTGTTGCTGTGGGACCTGAG 3' and R 5' TGAAAGACTTAAACT 3'. Each PCR reaction contained ~200 ng of purified human DNA at ~100 ng/µl, 2 µl of 10X AmpliTaq Gold® 360 PCR Buffer (ABI), 2 µl of 25 mM $MgCl_2$, 0.16 µl 25 mM dNTPs (Promega), 0.2 µl of each primer at 25 µM, 0.2 µl AmpliTaq Gold polymerase (ABI) at 5U/µl, 1 µl of dimethylsulfoxide and water sufficient to bring the final volume to 20 µl. Since there is a native *Alw* I restriction site at codon 192, 10 µl of the PCR product solution was digested with *Alw* I (NEB) overnight and visualized by ethidium bromide staining on a 3% agarose gel next to 10 µl of undigested product solution. The digestion resulted in 63- and 36-bp fragments for individuals with the $PON1_{192RR}$ genotype; 99-, 63-, and 36-bp fragments for individuals

with the *PON1*$_{192QR}$ genotype; and a single, non-digested 99-bp fragment for individuals with the *PON1*$_{192QQ}$ genotype. Sequence data confirmed the results from the restriction digest analyses for all 50 of the 200 participants sequenced**.**

Statistical Analysis and Model Development

A chi-square (χ^2) test (PROC FREQ, SAS for Windows 9.1.3, SAS Institute Inc., Cary, NC, USA) with Bonferroni correction for multiple comparisons when a significant χ^2 was found ($p \leq 0.05$), was used to determine differences among race-gender groups within PON1 genotypes. One-way analysis of variance was used (PROC GLM, SAS for Windows 9.1.3) to determine if significant differences among race-gender groups ($p \leq 0.05$) for PON1 enzymatic activities. If the model indicated significant differences among groups, mean separation tests were conducted using the Tukey adjustment.

The strength of association, expressed as odds ratios (OR), between the occurrence of ATH and each of the explanatory variables listed in Table 3 was assessed by logistic regression (PROC LOGISTIC, SAS for Windows v. 9.1.3). Clinical variables that were found to be associated with ATH with a p-value ≤ 0.25 were used as candidates in a multivariable model (Hosmer and Lemeshow 1989). After the model was fit, the variable with the highest p-value was removed manually and the remaining explanatory variables reassessed. This process was continued until only clinical variables with p-value ≤ 0.05 remained in the model. This multivariable model was designated the core factor model. The experimental variables for PON1 activity (i.e. activities for hydrolysis of paraoxon, diazoxon, and PhAc) were then added individually to the core factor model to test the association between ATH and the experimental variables for PON1 activity while accounting for the core clinical variables.

Results

Characterization of the Study Population

Clinical variables were expressed as mean ± SD for continuous data and ratios for categorical data for each race-gender group (Table 1). Height and weight were used to determine body mass index (BMI). The categorical variables LDL group, triglyceride (trig) group, and TotalChol group were determined using the appropriate values from the lipid panel and information of statin use as described in detail in the Methods section.

PON1 Genotypes

Separation of the 200 individuals of this study into the three functional $PON1_{192}$ genotypes through the 2-dimensional plot of POaseNa vs. DZOaseNa was not as clear as previously reported (Richter and Furlong 1999; Davis et al. 2009) (Table 4Figure 1a). In fact, the DZOaseNa vs POaseNa plot alone was unable to unambiguously assign a functional genotype to a number of individuals. Therefore, the $PON1_{192}$ genotype was determined using a restriction fragment length polymorphism assay. Assignment of genotypes to the data points on the DZOaseNa vs POaseNa plot showed that individuals with each of the three $PON1_{192}$ genotypes were located in the expected region of the plot (Figure 2). A greater portion of Caucasians had the $PON1_{192QQ}$ genotype ($p \leq 0.0001$) than did the African Americans, while the reverse was true for the $PON1_{192RR}$ genotype ($p \leq 0.0001$) (Figure 1b, c and Table 2). No significant differences were noted in the population distribution of the $PON1_{192QR}$ genotype (Table 2).

Differences in Serum Enzyme Activities

Serum enzyme activities were expressed as mean ± SD for each race-gender group (Table 2). African American females had significantly higher POaseNa activities

than Caucasian females (p= 0.0005) and Caucasian males (p= 0.0002) but no differences between the other groups were observed (p> 0.1171). Caucasian females had significantly higher DZOaseNa activities than African American males (p= 0.0321) but no differences between the other groups were observed (p> 0.1470). African American females had significantly higher POase activities than Caucasian females (p= 0.0004) and Caucasian males (p= 0.0003) but no differences between the other groups were observed (p> 0.1382). No significant differences (p> 0.1054) were detected with DZOase activity levels among the race-gender groups. African American females had significantly higher PhAc activities than Caucasian females (p= 0.0216) and Caucasian males (p= 0.0049) but no difference between the other groups were observed (p> 0.2898).

Associations of ATH with Clinical and Serum Factors

Statistically significant associations ($p \leq 0.05$) between the presence of ATH and each of the clinical and serum factors individually were determined by logistic regression (Table 3). In our study population, increasing age, gender, smoking status, and classification in the elevated LDL, the low HDL, and the abnormal triglyceride groups were all associated with ATH. Classification in the elevated LDL cholesterol group had the strongest association with ATH of all the explanatory variables. Surprisingly, increasing body mass index (BMI) was associated with decreased odds of ATH. Increased levels of PON1 activity (expressed as POaseNa, DZOaseNa, POase, DZOase or PON1 activity determined by the hydrolysis of PhAc) were associated with decreased odds of ATH.

Factors that were included in the analysis but did not have statistically significant associations with ATH included race (p= 0.3264), hypertension (p= 0.0898), diabetes

(p=0.6778), family history of ATH (p= 0.1242), waist circumference (p=0.8021), total cholesterol group (p= 0.8427), ratio LDL/HDL (p= 0.3466), and *PON1*$_{192}$ genotype (p=0.9792).

Multivariable Logistic Regression Model

A multivariable logistic regression model was developed that showed the association of individual clinical and serum factors with the occurrence of ATH (Table 4). Triglyceride and total cholesterol groups were not included as variables in the model because over half the sample population was indeterminate for the variables due to statin therapy. Statin usage was not included as a variable in the model because it was used to define the LDL cholesterol groups. The factors age, gender, smoking status, hypertension, and LDL cholesterol group remained in the model to constitute the core model. Age showed a direct association with ATH (OR=1.04, p= 0.0251). Gender was associated with ATH (OR=3.13, p= 0.0011), with males being more likely than females to have ATH. Subjects who had a history of smoking (OR=1.98, p= 0.0560) or had hypertension (OR=2.05, p= 0.0888) were more likely to have ATH. History of smoking and hypertension were both left in the core model, even though their p-values were >0.05, because they are well-established risk factors for ATH.

Following construction of the core factor model, the various PON1 activity measurements were added individually to the model to test the association of PON1 activity and ATH. PON1 activity as measured by PhAc hydrolysis was the only activity measurement that was significantly associated with ATH (p= 0.0317). The presence of hydrolysis of PhAc in the model increased the maximum-rescaled generalized r^2 value from 0.35 to 0.37.

Discussion

PON1 is now known to hydrolyze many substrates including organophosphorus insecticide metabolites and pharmaceuticals (Aldridge and Davison 1953; Draganov et al. 2005). PON1 has also been found to play an important role in the prevention of ATH by the metabolism of pro-atherogenic oxidized lipids (Shih et al. 1998). To accurately assess the association of PON1 with ATH an individual's PON1 status (genotype and phenotype) must be determined. Even though individuals from the Deep South (except Floridians) have higher age-adjusted mortality rates of CVD than people in other regions of the United States, little is known about the PON1 status in the Southern population.

In the present study, each individual's PON1 status was determined. This analysis included determination of PON1 activity using the three substrates diazoxon, paraoxon, and PhAc and determination of each individual's $PON1_{192}$ genotype. A core model consisting of risk factors known to be associated with ATH including age, gender, history of smoking, presence of hypertension, and LDL cholesterol group was developed. Family history was not associated with ATH in our study population possibly because many subjects were unable to provide enough information to accurately assign their family history. Diabetes also was not associated with ATH in our study population. The lack of association of diabetes with ATH may be the result of the over representation of diabetics in the group of individuals without ATH (35 of 104, more than would be expected in a random sampling of the population, 12.3 % prevalence of diabetes in MS). Most of the study subjects had been referred for diagnostic testing for coronary disease and, not surprisingly, had a much higher prevalence of diabetes than the general population. Increasing BMI was associated with decreased odds of ATH in our study population. The reason for this is not known though individuals with ATH may have undergone more

intensive dietary counseling and been more motivated to follow the dietary recommendations than individuals without ATH.

The association of the various components of PON1 status with ATH was determined. Interestingly, all of the PON1 enzymatic measurements had an association with ATH when assessed individually. However, when assessed in the presence of the clinical factors comprising the core model, only PON1 activity determined by the hydrolysis of PhAc was associated with ATH with a $p \leq 0.05$. The maximum-rescaled generalized r^2 value of the core model was 0.35. The addition of PON1 activity (assessed by the hydrolysis of PhAc) to the core model resulted in an increase of the maximum-rescaled generalized r^2 value to 0.37. While this increase is not large, this small increase is of interest because only 5-10% of the risk of ATH is not accounted for by known risk factors in studies using very large numbers of patients (Yusuf et al. 2004).

The results of this study must be interpreted with a degree of caution. First, the study population was not randomly selected but consisted of individuals being treated or evaluated in a cardiology clinic. This selection bias resulted in an over representation of diabetes in individuals without ATH in the study population and prevented an adequate evaluation of diabetes in this study. Second, most patients with ATH in this study had a previous history of ATH and were already being treated (as evidenced by the extensive use of statins in the study population) thus allowing for the possible alteration of PON1 enzymatic activities. However, use of statins and fibrates has in general been associated with an increase in PON1 serum activity measures as (reviewed by Paragh et al. (Paragh et al. 2008)). If such an increase had occurred in the study population, it would obscure a sparing effect of PON1 activity on ATH occurrence. The ongoing statin treatment also resulted in the inability to include triglyceride levels and total cholesterol levels in the

model. In addition, the only screening test for ATH was cardiac nuclear perfusion imaging for hemodynamically significant coronary artery disease. This may not have detected subclinical ATH as sensitively as some other imaging modalities used to diagnose atherosclerosis (e.g. carotid ultrasound) used in previously published studies (Jarvik et al. 2003; Jarvik et al. 2000). This would result in individuals with subclinical coronary artery disease being classified as normal (i.e. no atherosclerosis). Despite these limitations, this study did demonstrate that increased PON1 activity, as measured by PhAc hydrolysis, is associated with decreased odds of ATH in this Southern population. Interestingly, in this population the $PON1_{192}$ genotype was not significantly associated with ATH.

Conclusions

This study documents a sparing effect of serum PON1 activity as assessed by the hydrolysis of PhAc on ATH occurrence adjusted for the effect of age, gender, history of smoking, hypertension, and elevated LDL-cholesterol. Diabetes, family history of ATH, total cholesterol levels and triglyceride levels are not well investigated in this model. The possibility of PON1 activity as an independent risk marker of ATH warrants further investigation to clarify these issues. This is the first study to document a relationship of PON1 activity with ATH in a population from the Deep South. In addition, the study has produced a model that will have utility for characterizing ATH risk factors utilizing larger data sets in the future.

Table 1 Characterization of study population by race-gender group.

Variables[1]	**Units**	**TotalPop**	**AAF**[2]	**AAM**[2]	**CF**[2]	**CM**[2]
		n=200	n=40	n=40	n=60	n=60
Age	Years	63 ± 9	60 ± 11	60 ± 7	65 ± 9	64 ± 9
Smoker (Ever/Never)		113/87	13/27	27/13	31/29	42/18
Diabetes (Yes/No)		70/130	18/22	15/25	19/41	18/42
Hypertension (Yes/No)		154/46	35/5	34/6	44/16	41/19
Height	Inches	67 ± 4	65 ± 3	69 ± 3	65 ± 3	70 ± 3
Weight	pounds	198 ± 44	197 ± 54	201 ± 36	183 ± 43	214 ± 38
BMI	kg/m^2	31 ± 6	33 ± 8	30 ± 5	31 ± 7	30 ± 5
HDL	mg/dL	48 ± 16	56 ±13	43 ±12	51 ± 16	40 ± 13
LDL	mg/dL	107 ± 36	123 ± 37	98 ± 30	116 ± 40	95 ± 29
Triglycerides	mg/dL	149 ± 97	119 ± 53	126 ± 113	162 ± 73	171 ± 122
TotalChol	mg/dL	185 ± 45	203 ± 43	165 ± 34	202 ± 48	168 ± 39
Statin Use (Yes/No)		121/79	19/21	29/11	30/30	43/17
HDL group (Low/Normal)		76/120	5/34	18/22	14/44	39/20
LDL group (Elevated/Normal)		156/41	25/14	35/5	43/15	53/7
Trig group (Abnormal/Normal)[3]		43/63	3/19	5/9	19/20	16/15
TotalChol group (Abnormal/Normal)[3]		69/37	21/9	7/8	29/10	12/10
Atherosclerosis (Yes/No)		96/104	10/30	25/15	21/39	40/20

[1] Variables: Age, Height, Weight, Body Mass Index (BMI), Total Cholesterol, HDL cholesterol, and LDL cholesterol are expressed as means ± SD. Variables: Smoker, Diabetes, Hypertension, Statin Use, HDL group, LDL group, Trig group, and TotalChol group were expressed as ratios.

[2] AAF=African American Female, AAM=African American Male, CF=Caucasian Female, and CM=Caucasian Male.

[3] Subjects receiving statin therapy were not included in the summary statistics for these variables.

Table 2 Serum PON1 parameters of Caucasian and African American males and females.

Variables	Units	TotalPop	AAF	AAM	CF	CM
		n = 200	n = 40	n = 40	n = 60	n = 60
POaseNa[1]	U/L	401 ± 188	509 ± 183^{A}	421 ± 189^{AB}	363 ± 173^{B}	354 ± 178^{B}
DZOaseNa[1]	U/L	8460 ± 2757	8649 ± 2566^{AB}	7699 ± 2231^{B}	9191 ± 3110^{A}	8130 ± 2741^{AB}
POase[1]	U/L	98 ± 54	129 ± 56^{A}	104 ± 53^{AB}	85 ± 50^{B}	85 ± 50^{B}
DZOase[1]	U/L	6437 ± 2249	6141 ± 1814^{A}	6033 ± 1858^{A}	7052 ± 2472^{A}	6300 ± 2445^{A}
PhAc[1]	U/L	15404 ± 3351	16919 ± 3165^{A}	15685 ± 3542^{AB}	14984 ± 3386^{B}	14656 ± 3041^{B}
Genotype[2]						
QQ		68	5^{A}	9^{A}	29^{B}	25^{B}
QR		87	17^{A}	18^{A}	25^{A}	27^{A}
RR		40	16^{A}	13^{A}	5^{B}	6^{B}

[1] Activities of POaseNa, DZOaseNa, POase, DZOase, and hydrolysis of PhAc are expressed as micromoles substrate hydrolyzed per minute per liter of serum (U/L), means ± SD.

[2] Number of individuals in each race-gender group with the indicated genotype.

[A, B] Values within a row with the same superscript letter are not significantly different ($p \leq 0.05$) as determined by analysis of variance with Tukey adjustment or by analysis of chi-square (χ^2) with Bonferroni adjustment.

Table 3 Odds ratios of atherosclerosis associated with clinical and serum factors.

Factor[1]	Comparison	Units	n	Odds Ratio	95% CI	P-Value
Age		5 years	200	1.22	(1.04,1.43)	0.0138
Gender	Male vs Female		200	4.13	(2.29,7.46)	<0.0001
Smoker	Ever vs Never		200	2.45	(1.38,4.31)	0.0023
LDLGroup	Elevated vs Normal		197	10.08	(3.75,27.08)	<0.0001
HDLGroup	Low vs Normal		196	2.66	(1.47,4.82)	0.0012
Triglyceride	Abnormal vs Normal		97	6.57	(2.70,15.97)	0.0267
BMI		5 kg/m^2	198	0.75	(0.59,0.95)	0.018
POaseNa[2]		150 U/L	199	0.78	(0.62,0.98)	0.0326
DZOaseNa[2]		1000 U/L	199	0.89	(0.80,0.99)	0.0346
POase[2]		15 U/L	199	0.92	(0.85,0.99)	0.0334
DZOase[2]		1000 U/L	199	0.85	(0.75,0.97)	0.0169
PhAc[2]		1000 U/L	199	0.89	(0.82,0.98)	0.0128

[1] Factors that were included in study but not found significant at $p \leq 0.05$ included race, hypertension, diabetes, family history, waist circumference, total cholesterol group, LDL/HDL ratio, genotype, DZOaseNa/POaseNa ratios, and DZOase/POase ratios

[2] Activities of POaseNa, DZOaseNa, POase, DZOase, and hydrolysis of PhAc are expressed as micromoles substrate hydrolyzed per minute per liter of serum (U/L), means ± SD.

Table 4 Multivariable logistic regression model of clinical and serum factors associated with atherosclerosis occurrence

Factor	Comparison	Units	Odds Ratio	95% CI	P-Value
Age		5 years	1.04	(1.01,1.08)	0.0251
Gender	Male vs. Female		3.13	(1.58,6.23)	0.0011
Smoker[1]	Ever vs. Never		1.98	(0.98,3.99)	0.0560
Hypertension[1]	Yes vs. No		2.05	(0.90,4.70)	0.0888
LDLGroup	High vs. Normal		9.56	(3.17, 28.79)	<0.0001
PhAc		1000 U/L	0.89	(0.80,0.99)	0.0317

[1] Although the p-values for the association of smoking status and hypertension were greater than 0.05, the variables were retained in the model because each is an established risk factor for atherosclerosis

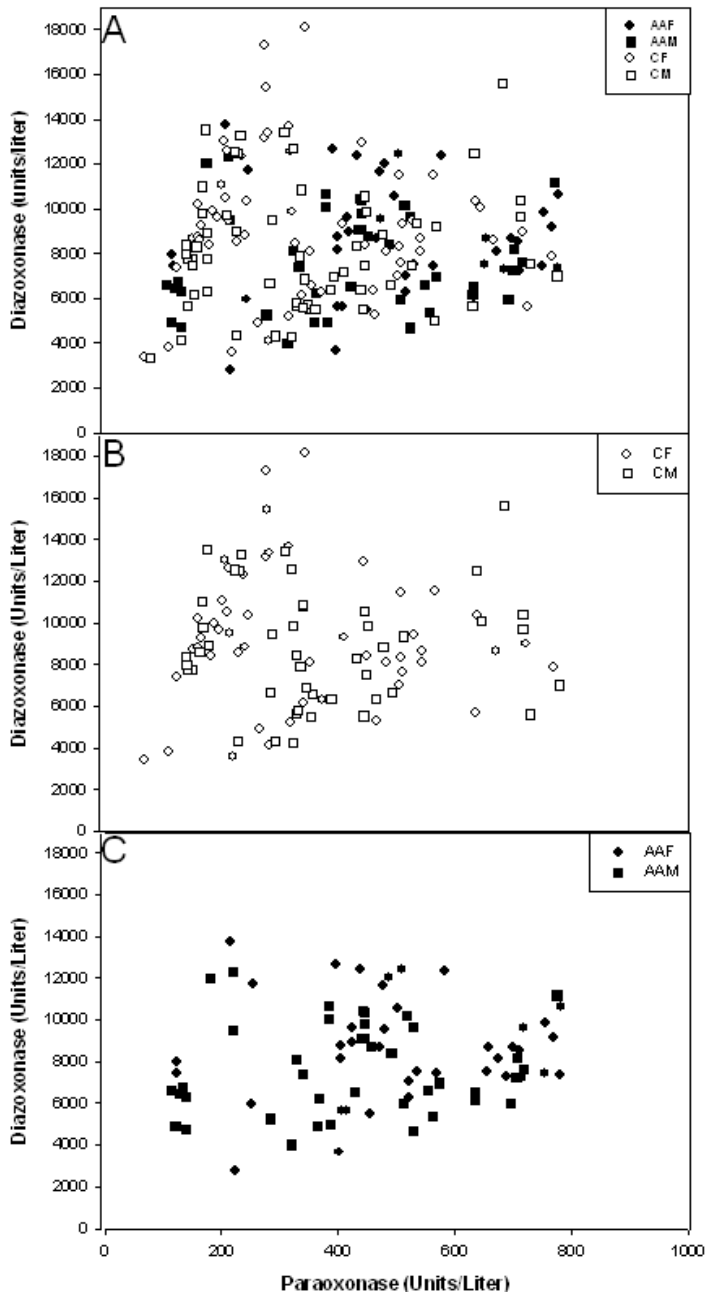

Figure 1 Plot of serum DZOaseNa vs. POaseNa enzymatic activities.

The sample population (n=200) consisted of 120 Caucasians (60 male, 60 female) and 80 African Americans (40 male, 40 female). (A) Plot of DZOaseNa activities vs. POaseNa activities in the sera of male and female African American and Caucasian Southerners. (B) Plot of DZOaseNa activities vs. POaseNa activities in the sera of male and female Caucasian Southerners. (C) Plot of DZOaseNa activities vs. POaseNa activities in the sera of male and female African American Southerners
Abbreviations: AAF, African American Female (●); AAM, African American Male (■); CF, Caucasian Female (○); CM, Caucasian Male (□)

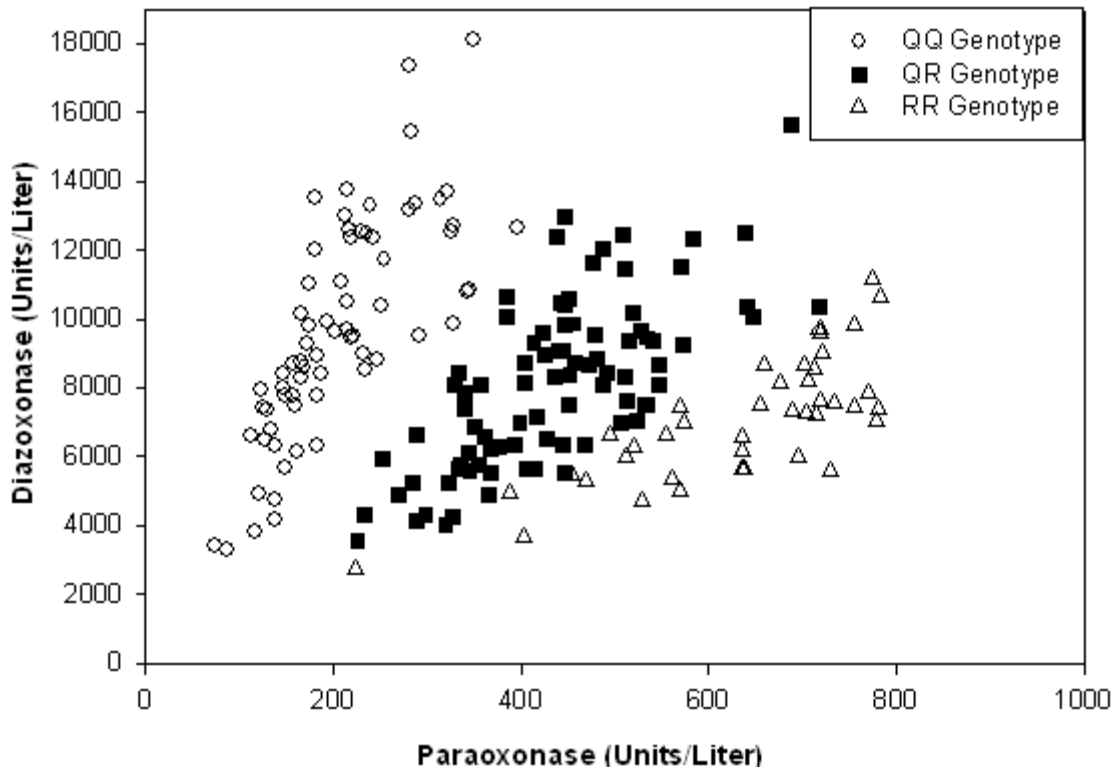

Figure 2 Plot of DZOaseNa vs. POaseNa enzymatic activities in the sera of male and female African American and Caucasian Southerners.

Genotypes are $PON1_{192QQ}$ (○); $PON1_{192QR}$ (■); and $PON1_{192RR}$ (Δ). The sample population (n=200) consisted of 120 Caucasians (60 male, 60 female) and 80 African Americans (40 male, 40 female)

CHAPTER IV

PARAOXONASE-1 (PON1) STATUS AND HYDROLYSIS OF CHLORPYRIFOS-OXON AT HIGH AND LOW CONCENTRATIONS

Since the removal of organochlorine insecticides (e.g. DDT) from use in the United States in the 1970's, organophosphorous (OP) insecticides have become the most widely used insecticides available today. With the increasing demand of insecticide products, as well as insecticide-contaminated food and different formulas, the question of their safety has become a serious world public health issue. Chlorpyrifos has been used globally as an insecticide to control crop pests in agriculture, specifically on corn. Over the past 40 years, research has focused on assessing human exposure to chlorpyrifos (rat oral LD_{50}, 122-163 mg/kg; (Gaines 1969; McCollister et al. 1974). Our lab has developed an interest in chlorpyrifos-oxon (CPO), the active metabolite of the phosphorothionate insecticide chlorpyrifos. This cytochrome P450-generated metabolite exerts acute toxicity by persistent inhibition of acetylcholinesterase (AChE; E.C. 3.1.1.7) in the peripheral and central cholinergic nervous systems causing cholinergic overstimulation (Aldridge and Davison 1953b).

Paraoxonase-1 (PON1; E.C. 3.1.8.1) is synthesized in the liver and circulates in the plasma associated with both apolipoprotein A1 and high density lipoproteins (Gaidukov and Tawfik 2005). PON1 protects against the acute toxic effects of OP anticholinesterases including nerve agents and OP insecticides through hydrolysis of the compounds or their active metabolite (i.e. the oxons of some OP insecticides) (Aldridge

and Davison 1953b). In fact, paraoxonase takes its name from its capability to hydrolyze paraoxon, the active metabolite of the OP insecticide parathion.

The involvement of PON1 in the *in vivo* detoxication of CPO and diazoxon, the active metabolite of the OP insecticide diazinon, has been demonstrated with transgenic animals. Although mice with a deleted PON1 gene are more susceptible to the toxic effect of CPO and diazoxon than wild-type mice, surprisingly they are not more susceptible to paraoxon than wild-type animals (Cole et al. 2005; Li et al. 2000; Shih et al. 1998). Other studies also indicate that the administration of purified plasma PON1 protein or, more recently, recombinant PON1 (Stevens et al. 2008), confers additional resistance to chlorpyrifos and CPO (Li et al. 2000; Li et al. 1995; Costa et al. 1990). The PON1 Q192R polymorphism significantly affects the catalytic efficiency of PON1 to modulate the acute toxicity of OPs, which varies among compounds (Li et al. 2000; Adkins et al. 1993; Davies et al. 1996; Humbert et al. 1993). For CPO, the $PON1_{R192}$ alloform provided higher catalytic efficiency of hydrolysis than the $PON1_{Q192}$ alloform in both *in vitro* and *in vivo* testing (Li et al. 2000). Studies using humanized transgenic mice (i.e. mice with a deleted PON1 gene and human PON1 transgenes inserted that express either the $PON1_{Q192}$ or $PON1_{R192}$ alleles) indicated that the mice expressing the $PON1_{R192}$ allele were significantly less sensitive to the toxicity of CPO than the mice expressing the $PON1_{Q192}$ allele, even with the same level of PON1 protein in the liver and plasma (Cole et al. 2005; Cole et al. 2003). Therefore, at high dose concentrations of CPO, both the level of expression and the $PON1_{192}$ genotype are important determinants of susceptibility.

In the present study, the $PON1_{192}$ genotype and the PON1 phenotype (i.e. activity levels) were determined for African American and Caucasian male and female

Southerners as described in Chapter 3. The *PON1*$_{192}$ genotype was determined using a restriction fragment length polymorphism assay and the PON1 phenotype was assessed with two substrates, paraoxon and diazoxon. Forty subjects were chosen that were designated as a *PON1*$_{192QQ}$ or *PON1*$_{192RR}$ genotype and that had either high or low activities towards diazoxon and paraoxon. The hydrolysis of a high and a relatively low concentration of CPO by PON1 was assessed using either a direct spectrophotometric method or indirect method, respectively. Current research using humanized transgenic mice use high doses of CPO to demonstrate that *PON1*$_{192}$ genotype and level of activity are significant determinants of susceptibility (Cole et al. 2005). However, studies fail to demonstrate this principle in human serum at concentrations that are more relevant to everyday exposure. The indirect method allows quantitation of hydrolysis of CPO at a relatively low concentration that is closer to toxicologically relevant concentrations than are the high concentrations assayed in traditional direct assay methods. The indirect assay assesses the ability of residual CPO not hydrolyzed by PON1 to inhibit an exogenous source of acetylcholinesterase. Acetylcholinesterase activity can be measured at levels well below the detection limits of direct spectrophotometric methods and therefore, the indirect method can measure CPO concentration levels that would be more reflective of realistic exposure scenarios.

Materials and methods

Study Population

Subjects were enrolled in the study from the clinical practice of Cardiology Associates of North Mississippi, LLC, a cardiology group located in Tupelo, Mississippi, which draws the vast majority of its patients from the 26 counties comprising northeast

Mississippi and northwest Alabama. The research division of this clinic, Cardiology Associates Research, LLC (CARe), collected a total of 200 serum samples from Caucasians (60 male, 60 female) and African Americans (40 male, 40 female), ages 45 and older. A 60/40 split was chosen to reflect the racial composition of Mississippi.

After obtaining written informed consent, blood samples were collected when vascular access was established for clinical testing. Serum was isolated by allowing blood to clot, then centrifuging at 2500-3000 x g at 4°C for 10 to 20 minutes. The serum was frozen on dry ice and stored at -70°C until analysis of PON1 activity. An aliquot of whole blood anti-coagulated with K_2EDTA was stored at -70°C for PON1 genotypic analysis.

The study protocol was approved by both the Institutional Review Board of Mississippi State University and the IRB of North Mississippi Medical Center; the latter provides oversight for studies performed at CARe.

PON1 phenotype of a subject was determined using diazoxon and paraoxon as substrates (see paraoxonase and diazoxonase assay in methods) (Figure 3a). A subject's PON1 phenotype was designated as "high" if he/she had high enzyme activity towards diazoxon or paraoxon or designated as "low" if he/she had low enzyme activity towards diazoxon or paraoxon (Figure 3c).

The *PON1*$_{192}$ genotype for each individual was determined using a restriction fragment length polymorphism assay (see PON1 genotyping in methods) (Figure 3a). Subjects used in this study were determined to have either the PON1$_{Q192}$ alloform or the PON1$_{R192}$ alloform (Figure 3b). Subjects that were determined to be heterozygous (i.e. QR192) were not used in this study (Figure 3b).

Chemicals and samples

Paraoxon and chlorpyrifos-oxon were synthesized by Dr. Howard Chambers (Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University. Diazoxon was purchased from ChemService (West Chester, PA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Paraoxonase and Diazoxonase Assays

Paraoxonase activity was determined by the hydrolysis of paraoxon as described in detail by (Richter and Furlong 1999)with minor modifications (Davis et al. 2009). Diazoxonase activity was determined by the hydrolysis of diazoxon as described in detail by (Richter and Furlong 1999) with minor modifications (Davis et al. 2009). The paraoxonase and diazoxonase assays were conducted in the presence of 2 M NaCl. Samples were run in triplicate. Data were expressed as micromoles of paraoxon hydrolyzed per minute per liter of serum for the paraoxonase assay and micromoles of diazoxon hydrolyzed per minute per liter of serum for the diazoxonase assay.

Direct A-esterase assay for activity for chlorpyrifos-oxon activity

The direct assay of chlorpyrifos-oxonase activity was determined by the hydrolysis of chlorpyrifos-oxon by PON1 as described in detail by Furlong et al. (1989) with minor modifications by Pond et al. (1998).

A 5 µl aliquot of human serum was added to each tube in a set of 4 tubes in which 1 tube contained 1 ml EDTA buffer [Tris-HCl (0.1 M, pH 8.0), 1.0 mM EDTA] to inhibit PON1 activity and the remaining 3 tubes containing 1 ml calcium buffer [Tris-HCl (0.1 M, pH 8.0), 2.0 mM $CaCl_2$] to stimulate PON1 activity. A 10 µl aliquot of stock 32 mM

CPO in ethanol was added to each tube (320 µM final concentration), and the tubes were vortexed and incubated in a shaking water bath at 37ºC for 15 min. After incubation, 500 µl of 2% sodium dodecyl sulfate (SDS) was added to each tube. The solution was vortexed and immediately transferred into a 1.5 ml disposable cuvette. The liberation of 3,5,6-trichloropyridinol (TCP) by chlorpyrifos-oxon hydrolysis was calculated using a molar extinction coefficient of 7.704×10^3 M^{-1} cm^{-1}. Absorbances were read at 315 nm using the Thermo Biomate 3 spectrophotometer. Samples were analyzed in triplicate and the values averaged. Data were expressed as micromoles of chlorpyrifos-oxon hydrolyzed per minute per liter of serum.

Calculation of Data

The change in absorbance units (Δ AU) was calculated from the difference between the Ca^{2+}-fortified sample (AU_{Ca2+}) and the EDTA-containing sample (AU_{EDTA}) for each tube:

$$\Delta \text{ AU} = (\text{AVG } AU_{Ca2+}) - (\text{AVG } AU_{EDTA}) \qquad \text{Equation 1}$$

Micromoles of chlorpyrifos-oxon hydrolyzed per min per liter of serum was calculated using a molar extinction coefficient of 7.704×10^3 M^{-1} cm^{-1} from Pond et al. (1998):

$$\text{Activity} = \Delta \text{ AU} \times \frac{7.704 \times 10^3 \text{ µmol}}{(\text{AU})(\text{L})(\text{min})} \qquad \text{Equation 2}$$

Here:

$$\frac{7.704 \times 10^3 \text{ µmol}}{(\text{AU})(\text{L})(\text{min})} = \text{conversion factor} \qquad \text{Equation 3}$$

Indirect A-esterase assay for activity for chlorpyrifos-oxon activity

A 10 µl aliquot of human serum was added to 4 sets of 4 tubes in which 2 tubes contained 1 ml EDTA buffer [Tris-HCl (0.1 M, pH 8.0), 1.0 mM EDTA] to inhibit PON1

activity and 2 tubes containing 1 ml calcium buffer [Tris-HCl (0.1 M, pH 8.0), 2.0 mM $CaCl_2$] to stimulate PON1 activity. A 10 µl aliquot of iso-OMPA (tetraisopropylpyrophosphoramide; 0.01 mM stock concentration in ethanol), to inhibit serum butyrylcholinesterase activity, was added to each tube (0.0001 mM final concentration), and all the tubes were vortexed and incubated in a shaker bath at 37ºC for 5 min. After the incubation, 10 µl of ethanol, to serve as a control, or chlorpyrifos-oxon (178 nM in ethanol final concentration) was added to 2 tubes of each set, and all the tubes were vortexed and incubated in a shaker bath at 37ºC for 15 min. The 178 nM CPO concentration was determined from a log-logit standard concentration curve that produced ~90% electric eel acetylcholinesterase (AChE) inhibition with EDTA buffer and ~20% electric eel AChE inhibition with calcium buffer. After the incubation, 1 ml of 0.15 unit/ml electric eel AChE was added to each tube (0.075 unit/ml final concentration), vortexed, and incubated in a shaker bath at 37ºC for 15 min. Eight mls of Tris-HCl buffer (0.05 M, pH 7.7, 25ºC) was added to sixteen 15-ml centrifuge tubes. After incubation, the entire reaction m A 10 µl aliquot of human serum was added to 4 sets of 4 tubes in which 2 tubes contained 1 ml EDTA buffer [Tris-HCl (0.1 M, pH 8.0), 1.0 mM EDTA] to inhibit PON1 activity and 2 tubes containing 1 ml calcium buffer [Tris-HCl (0.1 M, pH 8.0), 2.0 mM $CaCl_2$] to stimulate PON1 activity. A 10 µl aliquot of iso-OMPA (tetraisopropylpyrophosphoramide; 0.01 mM stock concentration in ethanol), to inhibit serum butyrylcholinesterase activity, was added to each tube (0.0001 mM final concentration), and all the tubes were vortexed and incubated in a shaker bath at 37ºC for 5 min. After the incubation, 10 µl of ethanol, to serve as a control, or chlorpyrifos-oxon (178 nM in ethanol final concentration) was added to 2 tubes of each set, and all the tubes were vortexed and incubated in a shaker bath at 37ºC for 15 min. The 178 nM CPO

concentration was determined from a log-logit standard concentration curve that produced ~90% electric eel acetylcholinesterase (AChE) inhibition with EDTA buffer and ~20% electric eel AChE inhibition with calcium buffer. After the incubation, 1 ml of 0.15 unit/ml electric eel AChE was added to each tube (0.075 unit/ml final concentration), vortexed, and incubated in a shaker bath at 37ºC for 15 min. Eight mls of Tris-HCl buffer (0.05 M, pH 7.7, 25ºC) was added to sixteen 15-ml centrifuge tubes. After incubation, the entire reaction mixture of 1 tube was transferred into one of the sixteen 15-ml centrifuge tubes and vortexed. ixture of 1 tube was transferred into one of the sixteen 15-ml centrifuge tubes and vortexed.

Acetylcholinesterase Assay

Each of the sixteen 15-ml centrifuge tubes were aliquoted into 2 ml assay volume (0.015 unit/ml electric eel AChE final concentration) into 16 sets of 4 tubes (one 15-ml centrifuge tube per set). To inhibit electric eel AChE activity, 20 µl of eserine sulfate (1.0 mM stock concentration in 0.05 M Tris-HCl buffer) was added to the first tube of each set (0.01 mM final concentration) and vortexed. These tubes were designated as blanks. All 16 sets of 4 tubes were then incubated in a shaker bath at 37ºC for 15 min. After incubation, 20 µl of acetylthiocholine (ATCh; 100 mM stock concentration in ethanol) was added to all tubes (1 mM final concentration), vortexed, and incubated in a shaker bath at 37ºC for an additional 15 min. In a 50-ml beaker, 20 mls of 5% SDS (sodium dodecyl sulfate) and 5 mls of 2.6 mM DTNB [5,5'-dithio-bis (2-nitrobenzoic acid)] were added together to form a 5% SDS/DTNB mixture. After the incubation, 250 µl of the 5% SDS/DTNB mixture were added to all tubes. The solutions were vortexed and immediately transferred into a 1.5 ml disposable cuvettes. The electric eel AChE activity

was measured using a Thermo Biomate 3 spectrophotometer at 412 nm. Samples were analyzed in triplicate and the values averaged. Data were expressed in nanomoles CPO hydrolyzed per min per liter of serum.

Calculation of Data

The indirect method monitored the inhibition of electric eel AChE to quantify the residual oxon not hydrolyzed by PON1. The percent inhibition (%I) for EDTA and calcium assay tubes were converted to logits by taking the log of [(%I) ÷ (100-%I)] for both EDTA and calcium. A log-logit standard curve plot for chlorpyrifos-oxon inhibition of electric eel AChE vs. CPO concentration was then used to determine the nanomolar concentration of CPO not hydrolyzed by PON1. The concentration of CPO which produced about 20% electric eel AChE inhibition with calcium was subtracted from the concentration of CPO which produced about 90% electric eel AChE inhibition with EDTA (reflecting non-PON-mediated hydrolysis of CPO). Data were expressed in nanomoles CPO hydrolyzed per minute per liter of serum.

PON1 Genotyping

Genomic DNA was isolated from whole blood using a Sigma-Aldrich® GenElute™ Blood Genomic DNA Kit and concentrated using a Qiagen QIAEX®II Gel Extraction Kit. Following the method received from the Medical Genetics Department of the University of Washington (Jane Ranchalis, personal communication), a region containing the $PON1_{192}$ codon was amplified by PCR to yield a 99 bp product using the primer pair F 5' TATTGTTGCTGTGGGACCTGAG 3' and R 5' TGAAAGACTTAAACT 3'. Each PCR reaction contained ~200 ng of purified human DNA at ~100 ng/µl, 2 µl of 10X AmpliTaq Gold® 360 PCR Buffer (ABI), 2 µl of 25

mM $MgCl_2$, 0.16 µl 25 mM dNTPs (Promega), 0.2 µl of each primer at 25 µM, 0.2 µl AmpliTaq Gold polymerase (ABI) at 5U/µl, 1 µl of dimethylsulfoxide and water sufficient to bring the final volume to 20 µl. Since there is a native *Alw* I restriction site at codon 192, 10 µl of the PCR product solution was digested with *Alw* I (NEB) overnight and visualized by ethidium bromide staining on a 3% agarose gel next to 10 µl of undigested product solution. The digestion resulted in 63- and 36-bp fragments for individuals with the $PON1_{192RR}$ genotype; 99-, 63-, and 36-bp fragments for individuals with the $PON1_{192QR}$ genotype; and a single, non-digested 99-bp fragment for individuals with the $PON1_{192QQ}$ genotype. Sequence data confirmed the results from the restriction digest analyses for all 50 of the 200 participants sequenced**.**

Statistical Analysis

A two way analysis of variance using PROC GLM (SAS for Windows 9.1.3, SAS Institute Inc., Cary, NC, USA) was used to determine if there were statistically significant differences among $PON1_{192}$ genotypes, PON1 phenotypes, and $PON1_{192}$ genotype-PON1 phenotype interactions. If interaction term was found to be significant, mean separation tests were conducted using Tukey adjustment of the least square means. All statistical comparisons used a p-value ≤ 0.05 to demonstrate statistical significance.

Results

PON1 Genotypes

Separation of the 200 individuals of this study into the three functional $PON1_{192}$ genotypes through the 2-dimensional plot of diazoxonase vs. paraoxonase in the presence of 2 M NaCl was not as clear as previously reported (Richter and Furlong 1999; Davis et al. 2009). In fact, the diazoxonase vs. paraoxonase in the presence of 2 M NaCl plot alone

was unable to unambiguously assign a functional genotype to a number of individuals. Therefore, the *PON1*$_{192}$ genotype was determined using a restriction fragment length polymorphism assay. Assignment of genotypes to the data points on the diazoxonase vs. paraoxonase in the presence of 2 M NaCl plot showed that individuals with each of the three PON1$_{192}$ genotypes were located in the expected region of the plot (Figure 3a).

Chlorpyrifos-oxon Hydrolysis using Direct Assay

Statistically significant differences ($p \leq 0.05$) between *PON1*$_{192}$ genotypes, PON1 phenotypes, *PON1*$_{192}$ genotype-PON1 phenotype interactions were determined by two-way ANOVA. Subjects who expressed the PON1$_{R192}$ alloform hydrolyzed significantly more CPO than subjects who expressed the PON1$_{Q192}$ alloform ($p < 0.0001$). Subjects who expressed the PON1$_{Q192}$ alloform and had a high activity phenotype (i.e. high QQ) hydrolyzed significantly more CPO than subjects who expressed the PON1$_{Q192}$ alloform and had a low activity phenotype (i.e. low QQ) ($p < 0.0001$) (Table 5). Similarly, subjects who expressed the PON1$_{R192}$ alloform and had a high activity phenotype (i.e. high RR) hydrolyzed significantly more CPO than subjects who expressed the PON1$_{R192}$ alloform and had a low activity phenotype (i.e. low RR) ($p = 0.0002$) (Table 5).

Subjects who expressed the PON1$_{R192}$ alloform with a high activity phenotype hydrolyzed significantly more CPO than subjects who expressed the PON1$_{Q192}$ alloform with a high activity phenotype ($p < 0.0001$) (Table 5). Additionally, subjects who expressed the PON1$_{R192}$ alloform with a low activity phenotype hydrolyzed significantly more CPO than subjects who expressed the PON1$_{Q192}$ alloform with a low activity phenotype ($p < 0.0001$) (Table 5).

Chlorpyrifos-oxon Hydrolysis using Indirect Assay

No significant associations ($p \leq 0.05$) between *PON1*$_{192}$ genotype, PON1 phenotype, *PON1*$_{192}$ genotype-PON1 phenotype interactions were determined by two-way ANOVA. There were no significant differences in the hydrolysis of CPO between subjects who expressed the $PON1_{R192}$ or $PON1_{Q192}$ alloforms ($p= 0.0749$). There were no significant differences in the hydrolysis of CPO between subjects who expressed the $PON1_{Q192}$ alloform with a high or a low activity phenotype ($p= 0.7416$) (Table 2). Similarly, there were no significant differences in the hydrolysis of CPO between subjects who expressed the $PON1_{R192}$ alloform with a high or a low activity phenotype ($p= 0.5278$) (Table 6).

Subjects who expressed the $PON1_{R192}$ alloform with a high activity phenotype did not hydrolyze CPO more efficiently than subjects who expressed the $PON1_{Q192}$ alloform with a high activity phenotype ($p= 0.0769$) (Table 6). Additionally, subjects who expressed the $PON1_{R192}$ alloform with a low activity phenotype did not hydrolyze CPO more efficiently than subjects who expressed the $PON1_{Q192}$ alloform with a low activity phenotype ($p= 0.1237$) (Table 6).

Discussion

Plasma PON1 phenotypes are highly variable among human populations with individuals exhibiting high, intermediate, or low paraoxonase activity (Diepgen and Geldmacher-von Mallinckrodt 1986; Eckerson et al. 1983; Mueller et al. 1983). The coding region polymorphisms of PON1 have also been investigated for effects of catalytic efficiencies of certain OPs. Studies have elucidated that the PON1 Q192R polymorphism significantly affects the catalytic efficiency of PON1 but is substrate dependent (Li et al. 2000; Adkins et al. 1993; Davies et al. 1996; Humbert et al. 1993).

In this study, PON1 hydrolysis of CPO at a relatively high concentration (320 µM) measured by direct assay using human serum concur with previous reports that both serum PON1 phenotype and *PON1*$_{192}$ genotype are important determinants of CPO sensitivity at a high dose *in vivo* in transgenic animal models (Cole et al. 2005; Li et al. 2000; Shih et al. 1998). Our data indicate that differences in serum PON1 phenotypes are important for predicting sensitivity towards CPO at high concentrations with subjects having high PON1 phenotypes hydrolyzing significantly more CPO than subjects with low PON1 phenotypes. Additionally, subjects who expressed the PON1$_{R192}$ alloform hydrolyzed significantly more CPO than subjects expressing the PON1$_{Q192}$ alloform at the high CPO concentration.

However, PON1 hydrolysis of CPO at a relatively low concentration (178 nM) measured by our indirect method using human serum contradicted studies using direct methods of measurement and high concentrations of CPO (Cole et al. 2005; Li et al. 2000; Shih et al. 1998; Jansen et al. 2009). At a low CPO concentration that is closer to a toxicologically relevant concentration, the data indicated that neither serum PON1 phenotypes nor *PON1*$_{192}$ genotype influence PON1's ability to hydrolyze CPO. This is likely due to the amount of CPO present in the assay not being able to saturate the PON1 enzyme, and therefore PON1 was not functioning at maximal velocity (Cole et al. 2005; Li et al. 2000; Li et al. 1995; Jansen et al. 2009).

Conclusion

This is the first study to demonstrate hydrolysis of CPO by PON1 at a low and more toxicologically relevant concentration (178 nM) in human serum. Similar to humanized mouse models, significant differences were shown between the *PON1*$_{192}$

genotypes and between high and low serum PON1 phenotypes with the $PON1_{R192}$ alloform hydrolyzing significantly more CPO than the $PON1_{Q192}$ alloform at a relatively high concentration (320 μM). However, no significant differences were demonstrated between the *PON1*$_{192}$ genotypes and between high and low serum PON1 phenotypes at a low CPO concentration (178 nM). Therefore, at a low concentration more reflective of levels that would occur under realistic exposure scenarios, neither *PON1*$_{192}$ genotype nor serum PON1 phenotypes influences the capacity of PON1 to metabolize CPO presumably because PON1 is not saturated at these low CPO concentrations.

Table 5 Direct assay measurement of chlorpyrifos-oxon hydrolysis

High RR	Low RR	High QQ	Low QQ
11023 ± 722[A]	9467 ± 798[B]	8809 ± 672[B]	6030 ± 1015[C]

[a] Activities are expressed as micromoles of chlorpyrifos-oxon hydrolyzed per minute per L of serum, means ± SD
[A] Values with the same superscript letter are not significantly different ($p \leq 0.05$) as determined by analysis of variance with Tukey adjustment

Table 6 Indirect assay measurement of chlorpyrifos-oxon hydrolysis

High RR	Low RR	High QQ	Low QQ
231 ± 27[A]	219 ± 52[A]	193 ± 59[A]	185 ± 43[A]

[a] Activities are expressed as nanomoles of chlorpyrifos-oxon hydrolyzed per minute per L of serum, means ± SD
[A] Values with the same superscript letter are not significantly different ($p \leq 0.05$) as determined by analysis of variance with Tukey adjustment

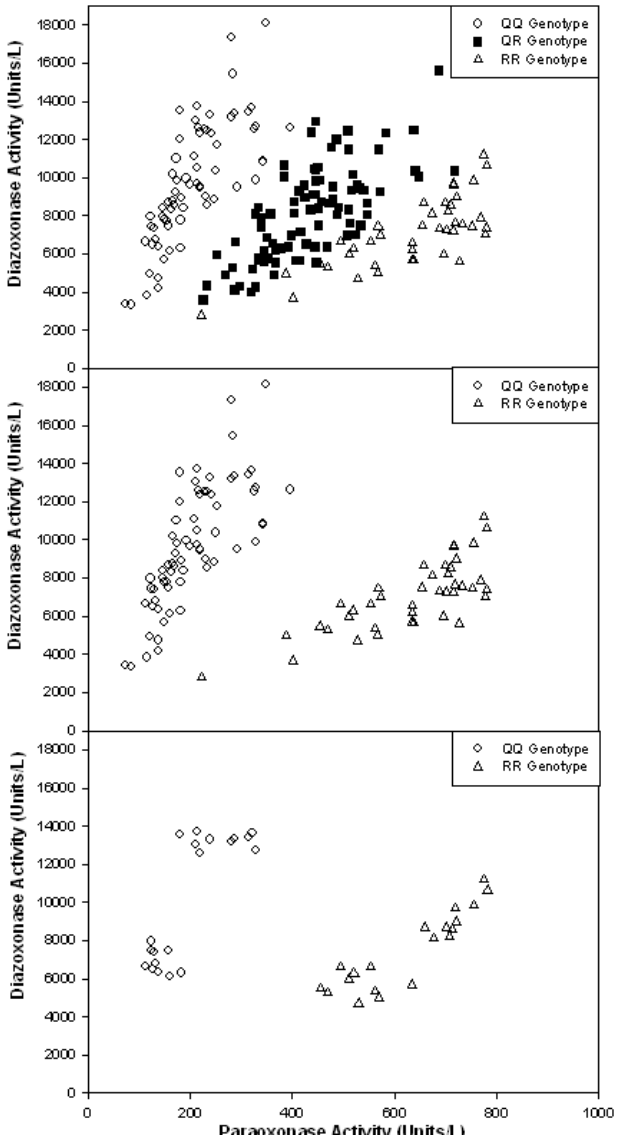

Figure 3 Plot of serum DZOaseNa vs. POaseNa enzymatic activities.

The sample population (n=200) consisted of 120 Caucasians (60 male, 60 female) and 80 African Americans (40 male, 40 female). (A) Plot of diazoxonase activities vs. paraoxonase activities in 2 M NaCl in the sera of male and female African American and Caucasian Southerners with the $PON1_{192QQ}$ (○); $PON1_{192QR}$ (■); $PON1_{192RR}$ (Δ) genotypes for each individual. (B) Plot of diazoxonase activities vs. paraoxonase activities in 2 M NaCl in the sera of male and female African American and Caucasian Southerners with the $PON1_{192QQ}$ (○) and $PON1_{192RR}$ (Δ) genotypes only. (C) Plot of diazoxonase activities vs. paraoxonase activities in 2 M NaCl in the sera of male and female African American and Caucasian Southerners with the $PON1_{192QQ}$ (○) and $PON1_{192RR}$ (Δ) genotypes used in this study.

CHAPTER V

CONCLUSIONS

Overwhelming health disparities occur in the southeastern United States, specifically Mississippi, and among those prominent health disparities is the prevalence of cardiovascular disease. Within these Southern states the occurrence of cardiovascular disease is more prevalent in the African American population than in the Caucasian population. Epidemiological studies concerned with cardiovascular disease have not focused on southern populations, nor have African Americans been an important proportion of these studies where cardiovascular health is the worst.

This study is one of the first to report the frequency distribution of the $PON1_{192}$ genotypes (QQ, QR, RR) in African American and Caucasian Southerners and the possible differences between $PON1_{192}$ enzymatic activities (diazoxonase, paraoxonase and phenylacetate) within race, gender, age and presence of atherosclerotic disease. PON1 genotyping documented that the African American population displayed a greater proportion of the $PON1_{192RR}$ genotype than did Caucasians, while the reverse was true with the $PON1_{192QQ}$ genotype. In addition, this study developed a model that documented a protective effect of serum PON1 enzyme activity, as assessed by the hydrolysis of phenylacetate, on atherosclerosis occurrence while accounting for age, gender, history of smoking, hypertension and elevated low density lipoprotein cholesterol levels.

This study is also the first to document the hydrolysis of chlorpyrifos-oxon by PON1 at a low more toxicologically relevant concentration (178 nM) in the sera of the

study population using an indirect spectrophotometric method. The indirect method reported that neither *PON1*$_{192}$ genotype nor serum PON1$_{192}$ activity levels had a significant effect on the hydrolysis of chlorpyrifos-oxon by PON1 at a low concentration (178 nM) which is more reflective of levels that would occur under realistic exposure scenarios.

These data suggest that a greater subject population of African American and Caucasian Southerners are warranted where cardiovascular health status, demographic and clinical information are known. This study focused on the PON1 Q192R polymorphism and correlations with atherosclerosis; however, other polymorphisms which may demonstrate different sensitivity to organophosphate toxicity or correlations with atherosclerosis were not observed. Additionally, the subject population, whether participating in farming or not, were likely exposed to agricultural chemicals including pesticides since the area is rural and highly agricultural. Our lab is currently engaged in determining the PON1 L55M polymorphism of the subject population and determining any associations to atherosclerosis. In addition, our lab is determining organochlorine pesticide levels in the sera of the subject population and determining any association with the PON1 Q192R polymorphism and atherosclerosis.

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