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An investigation of paraoxonase-1 activities in the serum of southerners as related to gender and race

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AN INVESTIGATION OF PARAOXONASE-1 ($PON1_{192}$) ACTIVITIES IN THE SERUM OF SOUTHERNERS AS RELATED TO GENDER AND RACE

By

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Paraoxonase-1 (PON1) has an anti-oxidative function in preventing the formation of oxidized lipoproteins (LDL and HDL) and hydrolyzing the active metabolites of some organophosphate insecticides (e.g., paraoxon and diazoxon) and other non-physiological substrates. $PON1_{Q192R}$ affects PON1 hydrolytic activity and its protective role against oxidative stress, thereby influencing susceptibility to cardiovascular disease among individuals.

The objectives of this study were to determine the effect of race, gender, and age on PON1 activities and $PON1_{192}$ genotypes in Caucasian and African American Southerners. Serum samples from 200 individuals (equally distributed race and gender classes, ages 25-55) were assayed spectrophotometrically for paraoxon and diazoxon hydrolysis.

Data indicate a positive correspondence between $PON1_{192}$ genotypes and race and PON1 activity and race. Data do not indicate an influence of gender and age on

PON1 activities or $PON1_{192}$ genotypes. These results are useful in explaining the increased risk of cardiovascular disease in African Americans compared to Caucasians.

Key words: PON1, polymorphism Q192R, cardiovascular disease, diazoxonase, paraoxonase

DEDICATION

This research is dedicated to my parents, Lee and Rosa Davis, for their continuous support and encouragement.

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CHAPTER I

INTRODUCTION

Paraoxonase-1 (PON1) is an enzyme that has an anti-oxidative function in preventing the formation of oxidized lipoproteins (LDL and HDL) and hydrolyzing the active metabolites of some organophosphate insecticides (e.g., paraoxon and diazoxon) and other non- physiological substrates (van Himbergen et al., 2006). $PON1_{Q192R}$ polymorphism affects PON1 hydrolytic activity and its protective role against oxidative stress, thereby influencing susceptibility to cardiovascular disease among individuals. The R allele is less protective for cardiovascular disease due to its lesser capacity to metabolize oxidized lipoproteins and its greater efficiency for hydrolyzing paraoxon (Jarvik et al., 2002; Costa et al., 2005). The Q allele is more protective for cardiovascular disease due to its efficient metabolism of oxidized lipoproteins and its greater efficiency for hydrolyzing diazoxon than does the R allele. Recent studies have suggested that diazoxon hydrolytic activity indicates the antioxidative ability of PON1 more than other non-physiological substrates because of its association with HDL and LDL particle size, and its dominance in the protective Q allele (Vekic et al., 2007).

Cardiovascular disease is more prevalent in the African American population than the Caucasian population and causes higher annual age-adjusted mortality in the South (except Florida) than in other regions of the country. The onset of CVD is caused by environmental, dietary, and genetic influences. Knowledge of $PON1_{192}$ enzymatic

activities and genotypes as related to race, gender, and age within a population may provide a useful explanation for a portion of the cardiovascular health disparity.

The measurement of $PON1_{192}$ diazoxonase and paraoxonase activities in the serum of African American and Caucasian Southerners may be used to indicate the distribution of $PON1_{192}$ genotypes (QQ, QR, and RR) and possible differences within race, gender, and age associated with $PON1_{192}$ activities. Data on the frequency distribution of $PON1_{192}$ genotypes have been reported on numerous populations, but not specifically the Southern population. These data appear in journals such as: *Environmental Health Perspectives*, *European Journal of Clinical Investigation*, *Experimental and Molecular Pathology*, *Human Biology,* among others.

However, further studies are needed to understand the relationship between PON1 activities and genotypes and their role in cardiovascular disease, the distribution of PON1 phenotypes and genotypes within human populations, the possible differences of PON1 activities within race, gender, and age groups, and why PON1 activities are a better predictor of cardiovascular disease than genotype. Also, studies of PON1 have not been conducted on Southern populations or many African American populations, so neither the activities of PON1 nor the proportion of the two genotypic forms (Q and R) of PON1 are known among the individuals with the highest mortality rate from cardiovascular disease.

Our study samples came from Integrated Laboratory Services-Biotech (ILSbio), which had obtained serum samples from blood banks in Alabama and Tennessee. The Institutional Review Board approved the study protocol. A total of two hundred serum samples were obtained from men and women, equally distributed race and gender classes, who were self-identified as being Caucasian or of African American origin. The

following individuals were excluded from the study: subjects under the age of twenty-five or over sixty-five, subjects known or suspected to be infected with human immunodeficiency virus or hepatitis, and subjects who were not Caucasian or African-American. The cardiovascular health status, dietary and lifestyle habits, and environmental toxicant exposure of subjects are unknown.

Our study will be the first to report the distribution of $PON1_{192}$ genotypes in the serum of African American and Caucasian Southerners and the possible differences in PON1 (paraoxonase and diazoxonase) activities within race, gender, and age groups. With the data obtained, we statistically determined that African Americans in the South have lower DZOase/POase ratios than Caucasians and the frequency distribution of QQ, QR, and RR genotypes differ between the two races. Data from our study strongly suggest a positive correlation between mean DZOase/POase ratios and genotypes and their role in increased susceptibility to cardiovascular disease in African Americans. Also, these results can be used to compare with future studies in which more samples may be obtained and/or more parameters are studied.

CHAPTER II

LITERATURE REVIEW

Paraoxonase-1 (PON1) is a mammalian enzyme that is synthesized primarily in the liver and is secreted into the blood, where it is associated with high- density lipoproteins (Costa et al., 2004). The structure of PON1 is a six-bladed β- propeller with two calcium ions (Ca1, Ca2) located in its central tunnel (Harel et al., 2004). The catalytic calcium (Ca1) has higher solvent accessibility and the structural calcium (Ca2) stabilizes the structure of PON1 (Harel et al., 2004; van Himbergen et al., 2006). PON1 belongs to a family of calcium-dependent hydrolases that also includes PON2 and PON3 (Gaidukov et al., 2006). The three classes hydrolyze aromatic and long-chain aliphatic lactones, but PON2 and PON3 lack paraoxonase (phosphotriesterase) and arylesterase activities (Costa et al., 2004).

PON1 hydrolyzes paraoxon (the active metabolite of the organophosphate insecticide parathion), diazoxon (the active metabolite of the organophosphate insecticide diazinon), chlorpyrifos- oxon (the active metabolite of the organophosphate insecticide chlorpyrifos), organophosphate chemical warfare agents (e.g., soman and sarin), and aromatic esters (e.g., phenyl acetate) as non-physiological substrates (van Himbergen et al., 2006). Recent structure-activity model studies show that lactones are the preferred substrate of PON1 and the physiological activity of PON1 is to act as an interfacially

activated lipolactonase that associates with HDL carrying apolipoprotein A-I (apoA-I) and apolipoprotein J (apoJ), with the prevention of atherosclerosis (Gaidukov et al., 2006). PON1 has a physiological function for the prevention of the formation of oxidized low-density lipoproteins (LDL), inactivation of LDL-derived phospholipids once they are oxidized, and protection of phospholipids in high-density lipoproteins (HDL) from oxidation (Costa et al., 2004).

Formation of Oxidized Lipoproteins (HDL and LDL)

Oxidized LDL originates from oxidation in the arterial wall by lipoxygenases and myeloperoxidases. It has been proposed that biologically active lipids in LDL are formed in three phases (Mertens and Holvoet, 2001; Navab et al., 2000). First, LDL interacts with metabolic products of linoleic acid, metabolic products of arachidonic acid, and hydrogen peroxides. Linoleic acid and arachidonic acid are polyunsaturated fatty acids (PUFAs) that are protected from free radical attack and oxidation by several antioxidants. PUFAs and antioxidant levels indicate LDL oxidation susceptibility (Ramos et al., 1995). Second, LDL is trapped in the subendothelial space and continuous generation of additional reactive oxygen species in LDL occurs. 15- Lipoxygenase converts polyunsaturated fatty acids into lipid hydroperoxides causing oxidation of LDL; also 12-hydroxyeicosatetraenoic acid is a lipoxygenase metabolite of arachidonic acid that can cause greater adhesion of monocytes to endothelium (Mertens and Holvoet, 2001). Third, the nonenzymatic oxidation of LDL phospholipids occurs, causing formation of specific oxidized phospholipids that induce monocyte binding, chemotaxis, and differentiation into macrophages (Mertens and Holvoet, 2001; Navab et al., 2000).

Myeloperoxidases generate reactive species that oxidize antioxidants, lipids, and protein of LDL. Nitric oxide (reactive nitrogen species) can be converted to secondary oxidizing species that increase membrane and lipoprotein lipid oxidation. Oxidized LDL leads to atherosclerosis (Mertens and Holvoet, 2001).

Cardiovascular Disease

Approximately every year since 1900, cardiovascular disease (CVD) has been the leading cause of death in the United States. In general, states in the South (except Florida) have higher annual age- adjusted mortality rates from CVD and stroke than states from other regions of the country (American Heart Association, Heart Disease, and Stroke Statistics-2007 update). Some of the critical risk factors for CVD include: hypertension, high cholesterol (particularly LDL cholesterol), smoking, and obesity. The contributions of these risk factors to the state- specific age- adjusted mortality rates from CVD are unknown. Several theories have been developed to explain the higher stroke mortality rate (a subset of CVD mortality) in the South including: low socio-economic status, an increased prevalence and severity of hypertension, and the presence of environmental toxicants (Perry and Rocella, 1998). Soft water has been reported to be associated with increased cardiovascular mortality because the southeastern region of the United States has the largest soft water area in the U.S. (Perry and Rocella, 1998). The CVD health disparity in the South suggests that an environmental factor(s) more prominent in the South than in other regions of the country might synergize the known risk factors, perhaps in conjunction with a genetic pre-disposition prominent in the South.

PON1 Protective Function

Earlier studies have suggested that the PON1 *N*-terminus anchors it to HDL, thereby contributing to the antioxidative properties of HDL (Harel et al., 2004; Rozek et al., 2004). *In vivo* studies in laboratory animals show that PON1 reduces the generation of lipid peroxides by hydrolyzing biologically active LDL and preventing the formation of fatty streak lesions (Gamboa et al., 2006; Watson et al., 1995). This function also inactivates PON1 arylesterase activity by the interaction of oxidized LDL phospholipids and cholesteryl esters with the PON1-free sulfhydryl group at cysteine-284 (Aviram et al., 2000). PON1 has three cysteine (Cys) residues in positions 42, 284, and 353. Cys-42 and Cys-353 are essential for PON1 hydrolase activity while Cys-284 is close to the active site and is essential for PON1 to be protective against LDL oxidation (Bartels et al., 2002; Sorenson et al., 1995; Aviram et al., 1998; Costa et al., 2004). Paraoxonase also causes HDL to become resistant to oxidation therefore maintaining the capacity of HDL to participate in reverse cholesterol transport.

It has recently been shown that HDL particles carrying apolipoprotein A-I bind PON1 with high affinity and greatly stabilize the enzyme and stimulate its lipolactonase activity (Gaidukov et al., 2006). HDL and apolipoprotein A-I (apoA-I) inhibit the oxidation of biologically active lipids. ApoAI-HDL causes LDL to become resistant to oxidation by removing 13(S)-hydroxyperoxyoctadecadienoic acid [(13(S) - HPODE)] and 15(S)-hydroxyperoxyeicosatetraenoic acid [(15(S) - HPETE)] from LDL. Both enhance phospholipid auto- oxidation products such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-

phosphocholine (PGPC), as well as cholesterol linoleate (Mertens and Holvoet, 2001; Navab et al., 2000).

Apolipoprotein J (apoJ) is a glycoprotein present in HDL with PON1 and apoA-I that has been reported to function in complement regulation and prevention of cytolysis, reverse cholesterol transport, apoptosis, and membrane protection at fluid-tissue interfaces (Navab et al., 1997). Evidence has suggested that apoJ stimulates cholesterol and phospholipid efflux from the human macrophage cell lines THP-1 and U937 therefore contributing to reverse cholesterol transport (Gelissen et al., 1998). Reverse cholesterol transport involves removing excess cholesterol from peripheral cells and then returning it to the liver for excretion as bile acids. Navab and colleagues (1997) reported that pre- incubation of LDL or artery wall cells in co- culture with purified apoJ resulted in a dose-dependent inhibition of LDL- induced lipid hydroperoxide formation, MCP-1 production, and monocyte transmigration. Their data suggested that apoJ reduces the oxidative potential of LDL and artery wall cells.

The strongest connection of PON1 to the hydrolysis of lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxide comes from a study using transgenic mice. The transgenic mice that lacked PON1 were more susceptible to atherosclerosis, had HDLs and LDLs that were more susceptible to *in vitro* oxidation, and inefficient HDL for hydrolysis of LDL *in vitro* (Shih et al., 1998). HDLs isolated from the transgenic mice with PON1 had an enhanced ability to protect LDLs from oxidation (Tward et al., 2002). The ability of PON1 and its polymorphisms to metabolize various lipid peroxides and prevent their formation is related to susceptibility to CVD (Costa et al., 2003).

Mechanism of PON1 Activity

The mechanism of PON1 protection against oxidation and atherosclerosis is still not defined (Rozek et al., 2004). It has been suggested that the mechanism of PON1 antioxidative activity is due to the presence of detergents or unidentified proteins (Teiber et al., 2004). Another mechanism suggests that when PON1 is released into the blood a HDL receptor, scavenger receptor class B type 1 (SR-BI), allows the brief association of HDL with the hepatocyte membrane without altering HDL (Thomas- Maya et al., 2006). In the blood, PON1 function with HDL is stabilized by apoA-I and apolipoprotein J (apoJ). Studies have also suggested that PON1's protective mechanism against lipid oxidation comes from having peroxidase, phospholipase A_2 (PLA_2), and hydrolytic activity toward hydroperoxides and hydroxides (Teiber et al., 2004; Costa et. al., 2003). PON1 hydrolyzes phospholipid and cholesteryl ester hydroperoxides and hydroxides from arachidonic acid and reduces linoleic acid hydroperoxides to linoleic acid hydroxides (Costa et al., 2003; Watson et al., 1995; Aviram et al., 1998). Other studies have shown that copper and free radical generator 2,2'-azobis-2-amidinopropane hydrochloride (AAPH), initiate lipid peroxidation and that possibly PON1 inhibits this activity (Teiber et al., 2004).

PON1 Polymorphisms

The PON1 cDNA encodes a protein of 355 amino acids with a molecular mass of 43kDa, which is located on human chromosome 7q21-22 (Costa et al, 2003). The PON1 gene contains seven single nucleotide polymorphisms (SNPs), five in the promoter sequence (G-126C, C-108T, A-162G, G-832A, G-909C) and two in the coding sequence

(L55M, Q192R) with linkage disequilibrium (Chen et al., 2005). Polymorphisms that significantly affect PON1 include: L55M, Q192R, and C-108T. The L55M polymorphism affects plasma enzymatic stability and lipid peroxidation, the Q192R polymorphism affects the catalytic efficiency of the enzyme for various substrates, and the C-108T polymorphism has the most significant effect on PON1 expression (Davies et al., 1996; Chen et al., 2003). In the promoter region, the C-108T polymorphism has the most significant effect on PON1 expression with the –108C allele resulting in PON1 levels twice that of the –108T allele (Brophy et al., 2001; Costa et al., 2005; Chen et al., 2003). Approximately two hundred polymorphisms of PON1 have been identified, but their function(s) have not yet been described. It has been suggested, that they are involved in splicing efficiency and the efficiency of polyadenylation (Costa et al., 2005). Paraoxonase activity polymorphisms result from a missense mutation in the coding sequence of PON1. In a missense mutation, the new base alters a codon resulting in a different amino acid being incorporated into the protein chain.

Linkage Disequilibrium

Recent work suggests the effect of polymorphism L55M on plasma enzymatic activity is due to linkage disequilibrium with the low efficiency –108T allele of the C-108T promoter region polymorphism (Chen et al., 2005). Linkage disequilibrium is the non- random association of alleles at two or more loci. In linkage disequilibrium, some combinations of alleles or genetic markers occur in a population more or less frequently than expected due to random formation of haplotypes from alleles based on their frequencies. Factors that cause linkage disequilibrium are: interactions between genes,

genetic linkage and the recombination rate, random drift or non- random mating, and population structure.

Tests have shown significant linkage disequilibrium among the three promoter polymorphisms (-909, -162, and –108) and between the promoter polymorphisms and coding polymorphism L55M. Chen et al. (2003) used maternal blood samples (n = 402) and umbilical cord blood samples (n = 229) to analyze linkage disequilibrium in the Caucasian, African American, and Caribbean Hispanic population. Their results showed that promoter polymorphisms are in almost complete linkage disequilibrium with one another. For Caucasians, there was significant linkage disequilibrium between Q192R and L55M and between the promoter polymorphisms and L55M. In African Americans, the promoter polymorphisms C-108T and G-909C are in disequilibrium with Q192R, but not in the Caucasian sample population (Chen et al., 2003). Knowledge of linkage disequilibrium within populations may provide strategies for testing PON1 polymorphism effects in diseases, such as CVD (Jarvik et al., 2003).

$PON1_{Q192R}$ and CVD

The polymorphism at position 192 involves the amino acid substitution of glutamine (Q) for arginine (R). Q192R has been shown to have significant roles in substrate hydrolysis, drug metabolism, and susceptibility to CVD (Harel et al., 2004). Results from previous studies have shown that $PON1_{Q192R}$ accounts for much variation in PON1 hydrolysis activity of paraoxon (POase activity) and lesser variation in PON1 hydrolysis activity of diazoxon (DZOase activity) *in vitro*, so using diazoxon as a substrate provides a better indication of overall PON1 activities (Jarvik et al., 2002; Costa

et al., 2005). When measuring the substrate concentration for half maximal velocity (K_m) divided by maximal initial velocity (V_m), Li and colleagues (2000) observed that $PON1_{R192}$ allele (Km/Vm= 6.27) is more efficient at hydrolyzing paraoxon than $PON1_{Q192}$ (Km/Vm= 0.71). However, diazoxon and the nerve gases soman and sarin are hydrolyzed more efficiently by $PON1_{Q192}$ than $PON1_{R192}$ (Durrington et al., 2001).

The Q192R polymorphism also affects the protective role of PON1 against oxidative stress, where Q192 is more efficient at metabolizing oxidized HDL and LDL than R192. Earlier data reported that approximately 30% of Caucasians are homozygous for the R genotype, 40% are heterozygous for the QR genotype, and 30% are homozygous for the Q genotype (Navab et al., 1997). Recent data on the distribution of weighted PON1 allele frequencies in various populations showed that African Americans have a weighted $PON1_{Q192}$ allele frequency of 0.37 and a weighted $PON1_{R192}$ allele frequency of 0.63 (Schacchi et al., 2003). Data have shown that Caucasians have a weighted $PON1_{Q192}$ allele frequency of 0.75 (Costa et al., 2003). Allele frequencies were determined using the gene-counting method and the genotype distribution was estimated by the χ^2 test (Schacchi et al., 2003). These data suggest that individuals carrying the $PON1_{R192}$ allele could be at increased risk for coronary artery disease when they are exposed to environmental changes, changes in dietary habits, or contact with insecticides and other toxic substances (Schacchi et al., 2003).

Studies on PON1 genotype suggest that genotype is an important indication of susceptibility to cardiovascular disease. Mackness et al. (1998) found that HDL containing the $PON1_{55MM}$ genotype provided the most protection against LDL oxidation, followed by $PON1_{55LM}$, and lastly $PON1_{55LL}$. They also found that of the $PON1_{192}$

genotype $PON1_{192QQ}$ provided the most protection against LDL oxidation, followed by $PON1_{Q192R}$, and lastly $PON1_{192RR}$. However, PON1 polymorphisms do not determine the level of plasma PON1 activity. The variability in PON1 activity in each individual explains why just knowing the genotype does not provide an accurate prediction of the association of PON1 polymorphisms with disease (Costa et al., 2003).

Metabolism of PON1 Substrates

Paraoxon (diethyl 4- nitrophenyl phosphate), the active metabolite of parathion, is formed by microsomal oxidation of parathion through desulfuration and is degraded by hydrolysis resulting in 4-nitrophenol and diethyl phosphate via the cytochrome P450/PON1 pathway (Neal, 1967).

Diazoxon, the active metabolite of diazinon, is formed by the microsomal oxidation of diazinon through desulfuration and is degraded by hydrolysis resulting in isopropyl-4-methyl-6-hydroxypyrimidine (IMHP) and diethyl phosphate via the cytochrome P450/PON1 pathway (Shishido and Fukami, 1972).

Measuring PON1 Activity

Measuring the rate of diazoxon and paraoxon hydrolysis at high salt concentrations (2 M NaCl), provides an accurate functional assessment of the plasma $PON1_{192}$ genotype, as well as the plasma PON1 activity for individuals (Costa et al., 2003; Costa et al., 2005). Knowledge of genotype and phenotype is important for determining the relationship of PON1 polymorphisms with susceptibilities to disease, the tolerance of organophosphate insecticides, and pharmacokinetic status of drug

metabolism (Richter et al., 1999). A plot of the rates of diazoxon hydrolysis vs. paraoxon hydrolysis (DZOase/POase ratio), can be used to separate individuals into three groups, which corresponds to: $PON1_{192QQ}$, $PON1_{192QR}$, and $PON1_{192RR}$.

Factors that Influence PON1 Activity

Many factors have been reported to affect serum PON1 activity. Simvastatin, a drug used to reduce the amount of cholesterol and fatty substances in the blood, has been reported to increase PON1 activity (Deakin et al., 2003). Fibrates, fibric acid derivatives that are used to lower blood triglyceride levels, have been reported to increase PON1 activity (Gouedard et al., 2003). Aspirin has also been reported to increase PON1 activity (Blater-Garin et al., 2003). Dietary polyphenols have been reported to increase PON1 expression by an aryl hydrocarbon- receptor dependent mechanism (Gouedard et al., 2004). Increased dietary intake of Vitamins C and E has been reported to increase PON1 activity by scavenging free- oxygen radical products that inhibit PON1 activity (Jarvik et al., 2002). Other studies have not confirmed these results (Ferre et al., 2003; Kleemola et al., 2002). Also, light alcohol consumption has been shown to increase PON1 activity, while heavy alcohol consumption decreases PON1 activity (Rao et al., 2003).

Smoking has been reported to decrease PON1 activity due to the presence of reactive aldehydes and aromatic hydrocarbons in cigarettes (Nishio and Watanabe, 1997; James et al., 2000; Jarvik et al., 2002; Senti et al., 2003; Ferre et al., 2003; Costa et al., 2005). Heavy alcohol consumption (Costa et. al., 2005), diabetes mellitus (Boemi et al., 2001), fat- enriched diets (Costa et al., 2005), various HDL deficiencies (Costa et al.,

2003; Mackness et al., 1987), and liver cirrhosis (Costa et al., 2003; Ferre et al., 2001) all have been reported to lower PON1 activity. Studies have confirmed that low serum PON1 activity is a predictor of vascular disease (Jarvik et al., 2000; Mackness et al., 2001; Jarvik et al., 2003).

PON1 and Age, Gender, and Race

Some data have been reported concerning the effect of age, gender, and race on PON1 activity. Studies have shown that serum PON1 activity is lower during development and birth, but increases over time (Chen et al., 2003). Other studies have reported that PON1 activities remain constant within adults or decrease in elderly subjects (Jarvik et al., 2002; Miochevitch and Khalil, 2001; Seres et al., 2004; Costa et al., 2005). In relation to gender, there have been conflicting studies. One study reported a higher mean value of PON1 activity in females than males (Mueller et al., 1983). Data presented on race and PON1, indicate that Caucasians have the more protective genotype than African Americans (Schacchi et al., 2003; Costa et al., 2003).

Because of the limited nature of the existing data regarding the PON1 polymorphism distributions in African Americans, and the lack of this type of information on Southern populations, this study was designed to investigate age, gender, and race differences that are associated with PON1 in Caucasians and African Americans from the South.

CHAPTER II

MATERIALS AND METHODS

Substrates and Materials

Measuring the rate of diazoxon and paraoxon hydrolysis at high salt concentrations (2 M NaCl) provides an accurate functional assessment of $PON1_{192}$ genotypes, as well as the PON1 activities of individuals (Costa et al., 2003; Costa et al., 2005). The diazoxon used in this study was obtained from Chem Service (West Chester, PA). Paraoxon was synthesized and provided by Dr. Howard Chambers (Department of Entomology and Plant Pathology, Mississippi State University).

The serum used in this study was obtained from blood banks in Alabama and Tennessee through Integrated Laboratory Services-Biotech (ILSbio, LLC) in Chestertown, Maryland. A total of two hundred serum samples were obtained from men and women, equally distributed race and gender classes, who were self-identified as being Caucasian or of African American origin. The following individuals were excluded from the study: subjects under the age of twenty- five or over sixty-five, subjects known or suspected to be infected with human immunodeficiency virus or hepatitis, and subjects who were not Caucasian or African-American. The cardiovascular health status, dietary and lifestyle habits, and environmental toxicant exposure of subjects are unknown.

Paraoxon Hydrolysis

The discontinuous Paraoxonase assay monitors the calcium-dependent hydrolysis of paraoxon. In the presence of salt, paraoxon hydrolysis by PON1 is activated and the release of 4-nitrophenol is yellow in alkaline solution. The 4-nitrophenol released by paraoxon hydrolysis was measured spectrophotometrically according to a method described by Richter and Furlong (1999).

Paraoxonase Assay

Paraoxon, the traditional PON substrate (1.2 mM final concentration; stock solution in dry ethanol), was incubated in paired serum samples. A calcium containing buffer solution of Tris-HCl (0.1 M, pH 8.0), 2 mM $CaCl_2$, and 2 M NaCl was prepared to activate PON1 activity. An EDTA containing buffer solution of Tris-HCl (0.1 M, pH 8.0), 1 mM EDTA, and 2 M NaCl was prepared to eliminate PON1 activity. 175 µL of calcium buffer was added to each of six wells of a microtiter plate (96 well format) and 175 µL of EDTA buffer was added to each of three wells of a microtiter plate. Six dilutions of serum were then prepared: three calcium dilutions (60 µL calcium buffer and 15 µL of serum) and three EDTA dilutions (40 µL of EDTA buffer and 10 µL of serum). Using a positive displacement pipette, 25 µL of the calcium dilution was added to the six wells of the microtiter plate already containing 175 µL of calcium buffer and 25 µL of the EDTA dilution was added to each of three wells of the microtiter plate containing 175 µL of EDTA buffer. The solution in the microtiter plate was mixed and incubated at 37°C for five minutes in the Jitterbug® microtiter plate shaker. Following the incubation time, 2 µL of paraoxon (1.2 mM final concentration) was added to the solution in each well of

the microtiter plate. The solution in the microtiter plate was mixed and incubated at 37°C for twenty minutes in the Jitterbug® microtiter plate shaker. Following the incubation time, the enzyme reactions were terminated by the addition of a solution of 50 µL of 20 mM EDTA and 2% Tris base solution in distilled water. The 4- nitrophenol released by paraoxon hydrolysis was quantified spectrophotometrically at 405 nm using a TECAN microplate spectrophotometer.

Calculation of Units/Liter

The factor for determining µmol 4-nitrophenol produced per minute per liter serum (U/L) came from Richter and Furlong (1999), but not numbers used in the equation:

$$\frac{(73.963\ \underline{\text{nmol/mL}}\ \text{AU})\ (10^6\ \mu\text{L/L})}{(20\ \text{min})\ (25\ \mu\text{L serum/mL})\ (10^3\ \text{nmol/}\mu\text{mol})} \qquad (3\text{-}1)$$

$$= 147.93\ (\text{U/L})/\ \Delta\ \text{AU}$$

where:

73.963	=	$1/E_{\mu m}$
20 min.	=	reaction time
25	=	µL of serum per mL
10^3	=	conversion factor
10^6	=	conversion factor

The change in absorbance units (Δ AU) was calculated from the difference between Ca^{++} and EDTA containing samples:

$$\Delta\ \text{AU} = (\text{AVG. } Ca^{++}\ \text{ABS}) - (\text{AVG. EDTA ABS}) \qquad (3\text{-}2)$$

The paraoxonase activity (U/L) was then calculated:

$$\text{Activity} = \frac{\Delta \text{ AU}}{0.8333 \text{ cm}} \times 147.93 \qquad (3\text{-}3)$$

where:

0.8333 cm = pathlength in microplate spectrophotometer

Diazoxon Hydrolysis

To distinguish between the Q192 and R192, Richter and Furlong (1999) used an additional substrate, diazoxon that is also well hydrolyzed by PON1. The continuous diazoxonase assay monitors the calcium dependent production of 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP). In the presence of salt, diazoxon hydrolysis by PON1 is activated. The IMHP released was measured spectrophotometrically according to a method described by Richter and Furlong (1999).

Diazoxonase Assay

Diazoxon (2.0 mM final concentration; stock solution in dry ethanol) was incubated in a single sample. A calcium buffer solution of Tris-HCl (0.1 M, pH 8.0), 2 mM $CaCl_2$, and 2 M NaCl was prepared to activate diazoxonase activity. 20 µL of stored human serum was pipetted into four test tubes each containing 1950 µL of calcium buffer. The samples were warmed in a 37°C shaker bath for approximately 5 minutes. Following the five-minute incubation time, 20 µL of diazoxon (2.0 mM final concentration) was added to the calcium buffer and serum solution in each test tube. The solution was vortexed and immediately placed into a 1.5 mL ultra-violet (UV) cuvette.

The IMHP released by diazoxon hydrolysis was quantified continuously using a conventional spectrophotometer for two minutes at 270 nm.

Calculation of Units/Liter:

The factor for determining μmol IMHP hydrolyzed per minute per liter serum (U/L) came from Richter and Furlong (1999):

$$\frac{(330\ \underline{\text{nmol/mL}}\ )\ (10^6\ \mu\text{L/L})}{\text{AU}} \qquad (3\text{-}4)$$

$$(2\ \text{min})\ (10\ \mu\text{L serum/mL})\ (10^3\ \text{nmol}/\mu\text{mol})$$

$$= \ 16{,}500\ (\text{U/L})/\ \Delta\ \text{AU}$$

where:

330	=	$1/E_{\mu m}$
2 min.	=	reaction time
10	=	μL of serum per mL
10^3	=	conversion factor
10^6	=	conversion factor

The change in absorbance units (Δ AU) were calculated from the difference between final absorbance unit (124 seconds) and initial absorbance unit (4 seconds):

$$\Delta\ \text{AU} = (\text{ABS 124 sec.} - \text{ABS 4 sec.}) \qquad (3\text{-}5)$$

The diazoxonase activity (U/L) was then calculated:

$$\text{Activity} = \Delta\ \text{AU} \times 16{,}500 \qquad (3\text{-}6)$$

Calculation of DZOase/POase Ratio

According to Richter and Furlong (1999), plotting diazoxon hydrolysis vs. paraoxon hydrolysis (DZOase/POase ratio), separates individuals into three genotypes of PON1 activity: QQ, QR, and RR. This ratio can be determined by dividing diazoxonase activity by paraoxonase activity:

$$\text{Ratio} = \frac{\text{DZOase Activity}}{\text{POase Activity}} \qquad (3\text{-}7)$$

Statistical Analysis

The results obtained from this study were statistically analyzed using Sigma Plot 8.0, Microsoft Excel, and SAS® System for Windows, Version 9.1.

All calculations performed using the SAS® System for Windows, Version 9.1, was at the 0.05 level of significance. We used the Kolmogorov –Smirnov method to test if variables were normally distributed. To check differences in $PON1_{192}$ activities (diazoxonase and paraoxonase) and variables (race, gender, and age) the General Linear Model (GLM) with least square means (LSMEANS) procedure was performed. A Wilcoxon rank sum test was also used for comparison. To check the proportion of the genotype (QQ), we used the Binomial distribution test. Chi-Square analysis (Fisher's exact test) was used to test race and gender interactions ($p < 0.05$).

CHAPTER IV

RESULTS

Distribution of DZOase/POase Ratios and Genotype

The DZOase/POase ratio and the population distribution of $PON1_{192}$ genotypes can be determined by plotting diazoxon hydrolysis (y- axis) vs. paraoxon hydrolysis (x-axis). The diazoxonase rates vs. paraoxonase rates in the serum of African American and Caucasian Southerners (n = 200) can be seen in Figure 1 and Table 1. Similar to previous studies by Richter and Furlong (1999), plotting hydrolytic rates separated individuals into one of three $PON1_{192}$ phenotypes for determining PON1 status. $PON1_{192QQ}$ individuals possess higher diazoxonase activities than $PON1_{192QR}$ and $PON1_{192RR}$ individuals. In contrast, $PON1_{192QR}$ and $PON1_{192RR}$ individuals possess higher paraoxonase activities than PON_{192QQ} individuals. The distribution of data points for individuals possessing the QQ genotype are visibly separated from individuals possessing QR and RR genotypes; however, individuals possessing QR and RR genotypes are not clearly delineated. Diazoxonase and paraoxonase assays were repeated for fifteen data points to exclude human error. Nine data points (.045) were still indeterminate for QR and RR genotypes by plotting diazoxonase and paraoxonase activities and analyzing mean DZOase/POase ratios (Figure 2).

The frequency distribution of QQ (.375), QR (.325), and RR (.255) excluding indeterminate genotypic data points can be seen in Figure 3. Chi-square analysis

(Fisher's exact test) was used to determine if gender relates to race in QQ, QR, and RR genotype (including and excluding indeterminate data points). Results indicate that gender does not relate to race including and excluding the indeterminate data points within the QQ and QR genotypes. However, results do indicate that gender does relate to race including ($p = 0.0495$) and excluding ($p = 0.0344$) indeterminate data points within the RR genotype. Race and gender interactions were not expected and may be attributed to the samples obtained in this study.

Observed Differences in African Americans and Caucasians

Results from the General Linear Model (GLM) with least squares means (LSMEANS) procedure indicate significant race ($p = 6.39^{-14}$) differences in mean DZOase/POase ratios of African Americans and Caucasians (Figure 4). Results from the Wilcoxon rank sum test also indicate differences at $p<0.0001$.

$PON1_{192}$ genotype distribution and mean DZOase/POase ratios were determined for African American Southerners ($n = 100$). Plotted diazoxonase activities vs. paraoxonase activities in the serum of African American Southerners can be seen in Figure 5a. The frequency distribution of QQ (.15), QR (.34), and RR (.44) genotypes within the African American population were determined. The mean DZOase/POase ratio of African American females (31.19 ± 25.96) and males (25.87 ± 21.49) is shown in Table 2. The lower ratio indicates that the R allele is more prevalent in the sample population than the Q allele. For seven data points of African Americans (3 females; 4 males), the genotype (QR or RR) could not be determined. $PON1_{192}$ genotype distribution and mean DZOase/POase ratios were also determined for Caucasian

Southerners (n = 100). The frequency distribution of QQ (.60), QR (.31), and RR (.07) genotypes can be seen in Figure 5b. The mean DZOase/POase ratio of Caucasian females (65.36 ± 28.43) and males (54.18 ± 31.62) was also calculated (Table 2). The higher frequency distribution of QQ and their significantly higher mean DZOase/POase ratios than African Americans indicates that that the Q allele is more prevalent than the R allele. For two data points of Caucasians (1 female; 1male), the genotype (QR or RR) could not be determined.

A 95% confidence interval was calculated for the proportion (P) of the QQ data for African Americans (.15) and Caucasians (.60):

$$95\%\ CI = P \pm 1.96 \frac{\sqrt{P\,(1-P)}}{\sqrt{n}} \qquad (4\text{-}1)$$

The 95% CI for Caucasians (.60 ± .15) and African Americans (.15 ± .11) indicate a significant difference in the QQ genotype between the two populations. Results from the binomial distribution test also indicate a significant difference in the proportion of African Americans and Caucasians with the QQ genotype ($p < .0001$). Statistical analyses of $PON1_{192}$ genotype distribution and mean DZOase/POase ratios in the serum of African American and Caucasian Southerners indicate a positive correlation between $PON1_{192}$ genotype and mean DZOase/POase ratios.

Differences in Race and $PON1_{192}$

Differences in DZOase/POase ratios were further analyzed by calculating diazoxonase and paraoxonase activities independently to examine possible differences between the races. The mean diazoxonase activities of African Americans (5972.73 ±

2064.62) and Caucasians (7720.30 ± 2226.64) were compared (Table 3). Results from the GLM with LSMEANS procedure show that Caucasians exhibit significantly higher diazoxonase activities than African Americans, $p = 3.25^{-08}$ (Figure 6).

The mean paraoxonase activities of African Americans (297.36 ± 143.93) and Caucasians (183.36 ± 120.81) were also analyzed (Table 3). Results from the GLM with LSMEANS procedure show that African Americans exhibit higher paraoxonase activities than Caucasians, $p = 6.52^{-09}$ (Figure 7). The significant differences in diazoxonase activities and paraoxonase activities between the races suggest a positive association between PON1 activities and race.

Differences in Gender and $PON1_{192}$

Results from the GLM with LSMEANS procedure indicate no significant gender differences when comparing diazoxonase (Figure 8a) and paraoxonase (Figure 9a) activities. There were also no significant gender differences in diazoxonase activities within race (Figure 8b) or gender differences in paraoxonase activities within race (Figure 9b).

Statistical analysis did indicate a significant difference in DZOase/POase ratios when comparing females and males, p = .0328 (Figure 10). These results indicate that females (48.27 ± 32.07) have higher DZOase/POase ratios than males (40.03 ± 30.43). Differences in genotype distribution among females and males were not expected and may be attributed to the samples obtained in this study or to some as yet unidentified sex linkage.

Differences in Age and PON1$_{192}$

Conflicting results on age and PON1 activity led us to analyze age as a function that possibly affects PON1 enzymatic activities. Individuals were separated into three age groups: 20-30 years, 30-40 years, and 40^{+} years for statistical analysis. Results from the GLM with LSMEANS procedure showed a statistically significant difference in paraoxonase activities ($p = .0366$) and DZOase/POase ratios ($p = .0062$) between the youngest ($n = 32$) and oldest ($n = 32$) age groups in females (Figures 12 and 13).

The findings suggest differential changes may occur in PON1$_{192}$ activities with age. Results show that the youngest females have higher paraoxonase activities (278.23 ± 147.22) than the oldest females (179.92 ± 135.99). The opposite is true for diazoxonase activities in the youngest females (6527.68 ± 2226.01) compared to the oldest females (7503.27 ± 2577.52). This analysis indicates that with age, changes in paraoxonase activities may render a higher DZOase/POase ratio in females.

Results from the GLM with LSMEANS procedure also showed a statistical significant difference in paraoxonase activities ($p = .0209$) and a marginally significant difference in DZOase/POase ratios ($p = .0543$) between the youngest ($n = 28$) and the oldest ($n = 45$) age groups in males (Figures 15 and 16).

A different trend was found when comparing PON1$_{192}$ activities in males. Results showed that the oldest males (284.68 ± 138.64) have higher paraoxonase activities than the youngest males (210.48 ± 122.25). The opposite is true for diazoxonase activities in the youngest males (7064.31 ± 2263.73) compared to the oldest males (6519.52 ± 2222.66). This analysis indicates that with age, changes in paraoxonase activities may render a lower DZOase/POase ratio in males.

However, results did not show significant differences when analyzing the middle age group or differences in diazoxonase activities among the three age groups in females and males (Figures 11 and 14). Differences found in mean DZOase/POase ratios of both genders may also be due to the samples obtained.

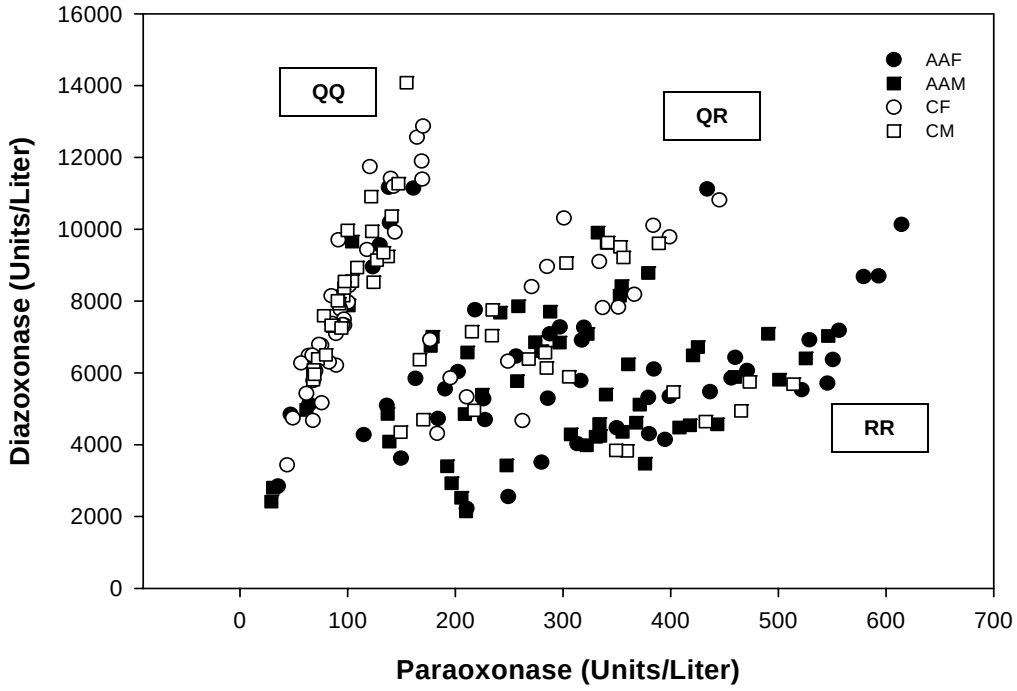

Figure 1. DZOase vs. POase Activities in the Serum of African American and Caucasian Southerners

Abbreviations are as follows: AAF, African American female (●); AAM, African American male (■); CF, Caucasian female (○); CM, Caucasian male (□). Sample population (n = 200) consisted of 50 AAF, 50 AAM, 50 CF, and 50 CM. The distribution of individuals into three $PON1_{192}$ genotypes (QQ, QR, and RR) is also shown.

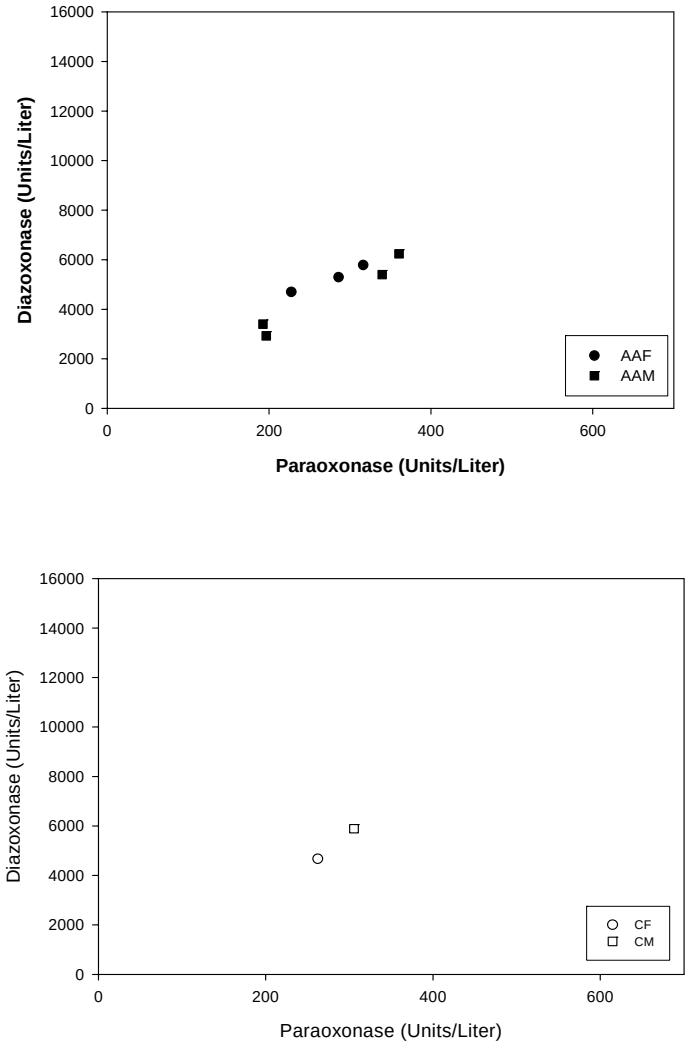

Figure 2. DZOase vs. POase Activities in the Serum of African American and Caucasian Southerners with Indeterminate Data Points for QR and RR

Distribution of data points for nine individuals where QR and RR genotypes were indeterminate using diazoxonase and paraoxonase activities and mean DZOase/POase ratios. Abbreviations and symbols are identical as in Figure 1.

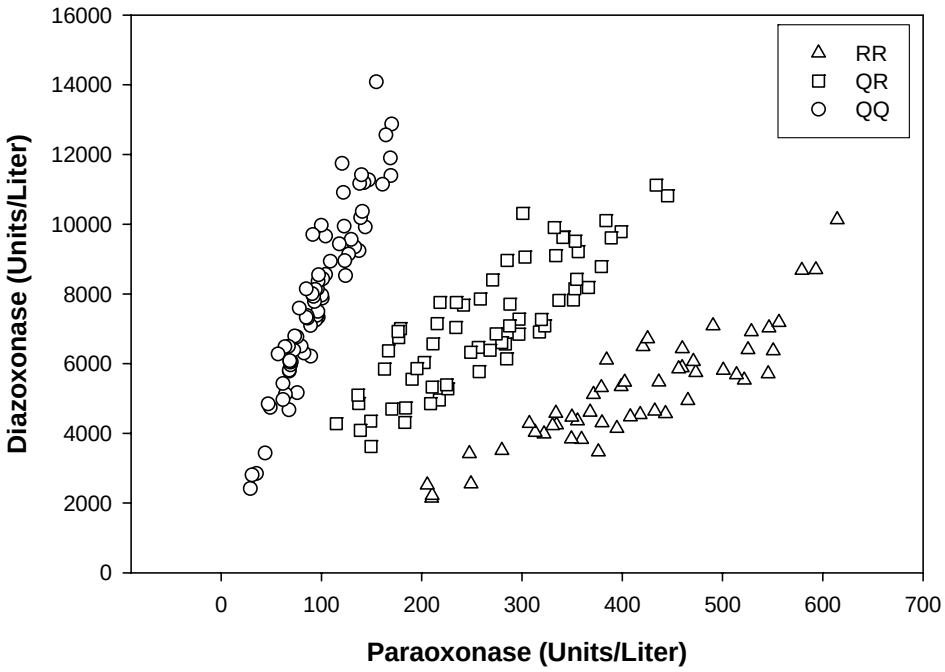

Figure 3. DZOase vs. POase Activities in the Serum of African American and Caucasian Southerners Excluding Indeterminate Data Points

Frequency distribution of QQ, QR, and RR in 47 AAF, 46 AAM, 49 CF, and 49 CM. Indeterminate data points were excluded (3 AAF; 4AAM; 1 CF; and 1 CM).

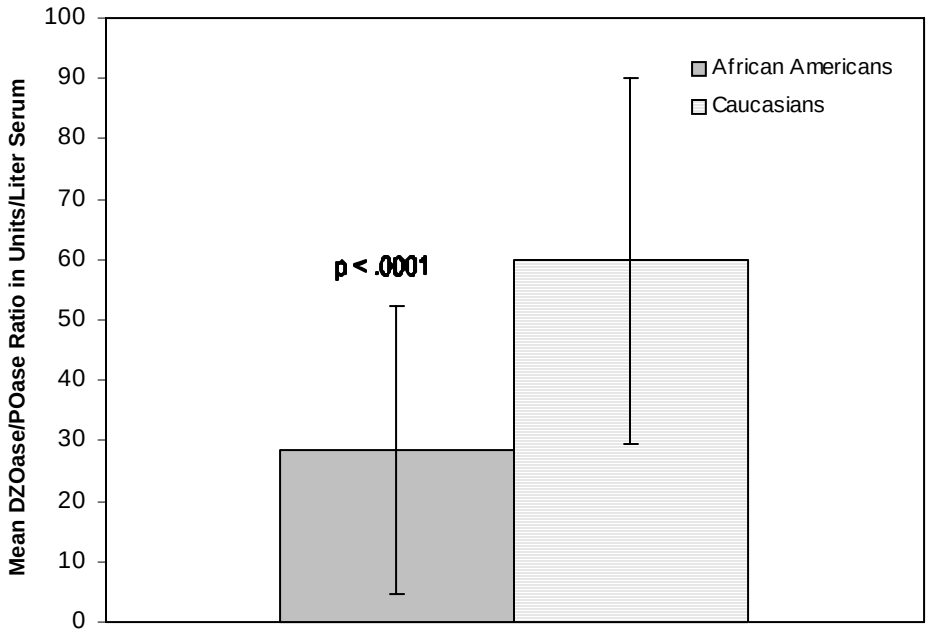

Figure 4. Mean DZOase/POase Ratios in the Serum of African American and Caucasian Southerners

Values are expressed as mean ± SD (n = 100) within each race. Significant differences were determined using the GLM with LSMEANS procedure ($p<0.05$). P –value represents significant difference found when comparing race.

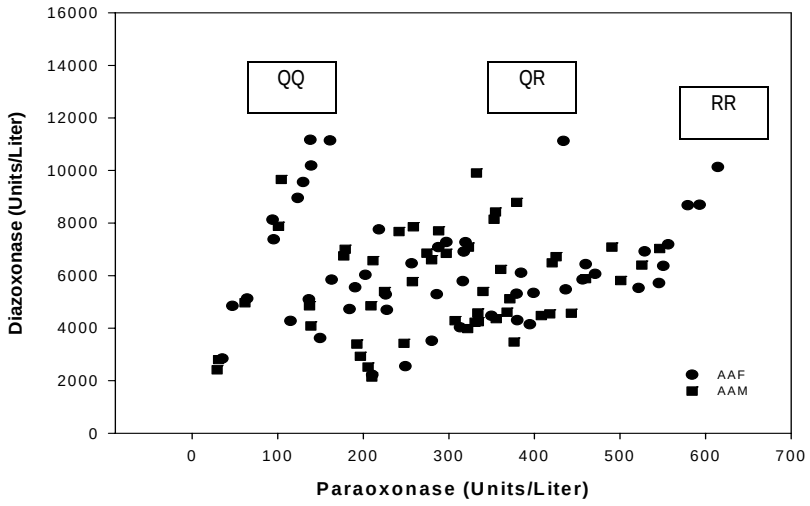

Figure 5a. DZOase vs. POase Activities in the Serum of African American Southerners

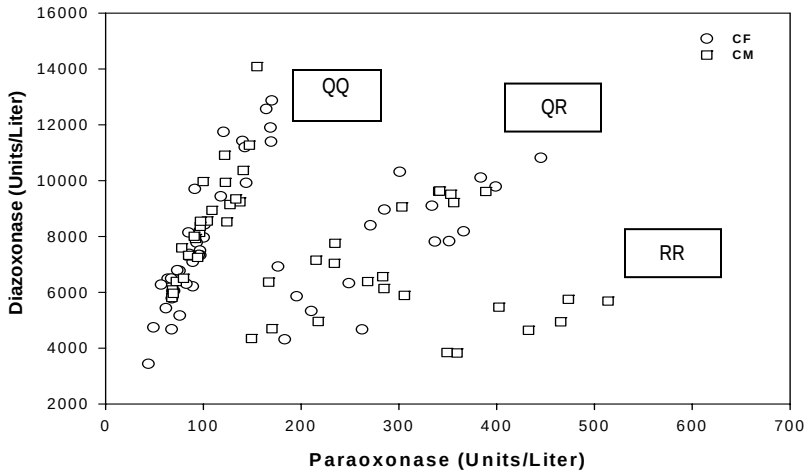

Figure 5b. DZOase vs. POase Activities in the Serum of Caucasian Southerners

Abbreviations and symbols are identical as in Figure 1. The frequency distribution QQ, QR, and RR can be determined. Indeterminate data points for QR and RR genotypes in African Americans (7) and Caucasians (2) can be seen in Figure 2.

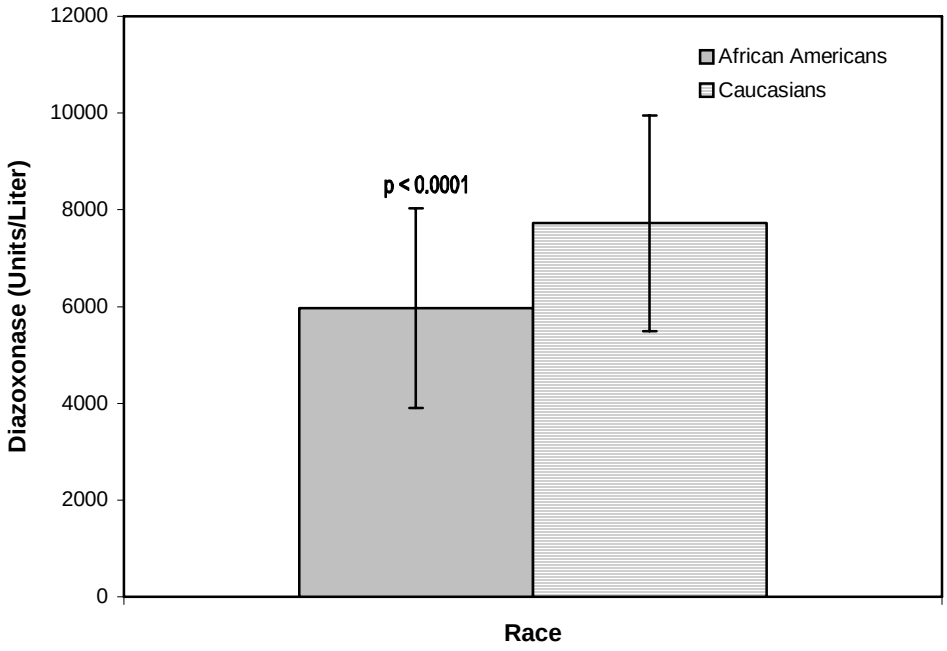

Figure 6. Mean DZOase Activities in the Serum of African American and Caucasian Southerners

Values are expressed as mean ± SD (n = 100) within each race. Significant differences were determined using the GLM with LSMEANS procedure ($p<0.05$). P –value represents significant difference found when comparing race.

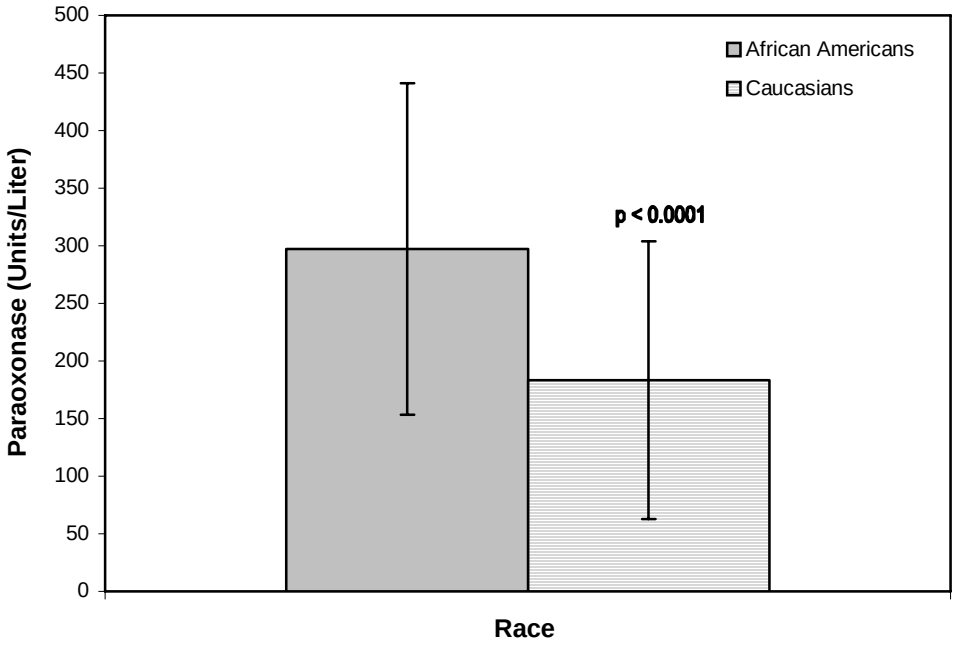

Figure 7. Mean POase Activities in the Serum of African American and Caucasian Southerners

Abbreviations are identical as in Figure 1. Values are expressed as mean ± SD (n = 200). Significant differences were determined using the GLM with LSMEANS procedure ($p<0.05$). P–value represents significant difference found when comparing race.

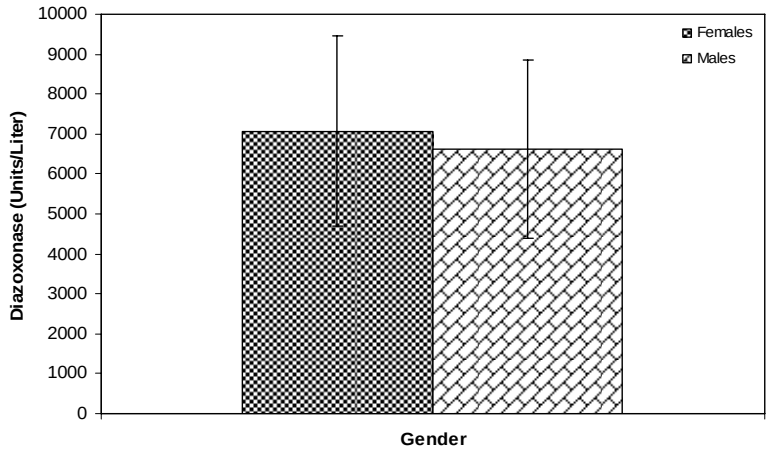

Figure 8a. Mean DZOase Activities in the Serum of Females and Males

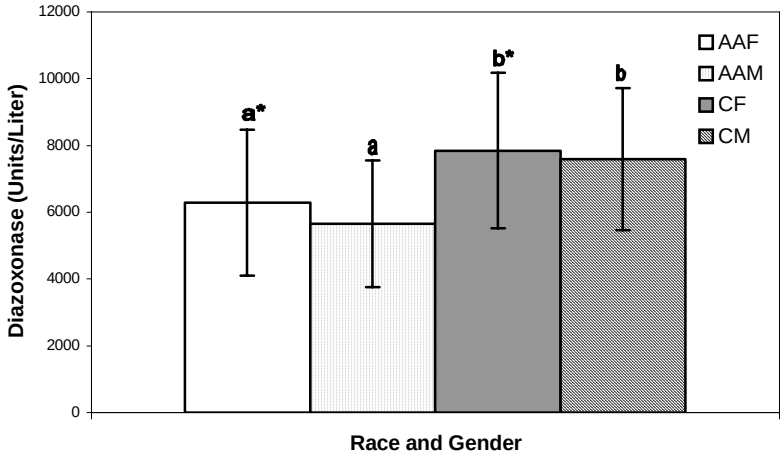

Figure 8b. Mean DZOase Activities in the Serum of African American and Caucasian Southerners

Abbreviations are identical as in Figure 1. (a) Values are expressed as mean ± SD (n = 100) within each gender. (b) Values are expressed as mean ± SD (n = 50) within each gender and race. Significant differences were determined using the GLM with LSMEANS procedure ($p<0.05$). Contrasting letters (a* & b* -females; a & b - males) represent significant differences found comparing race. No differences within groups having similar letters.

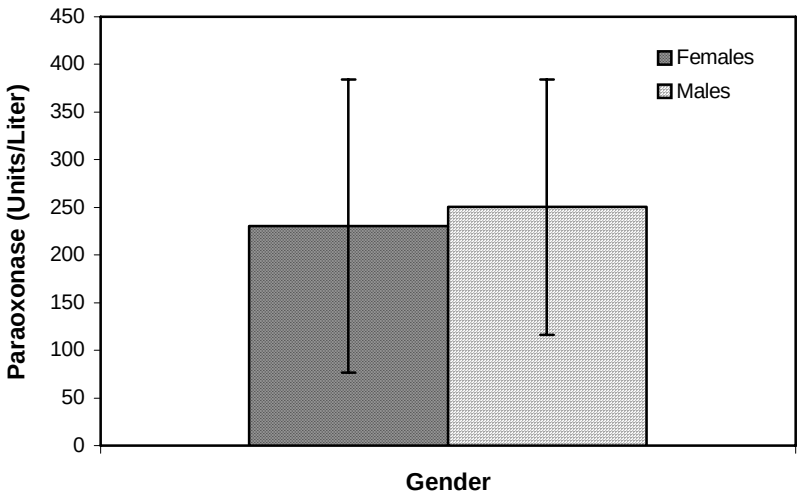

Figure 9a. Mean POase Activities in the Serum of Females and Males

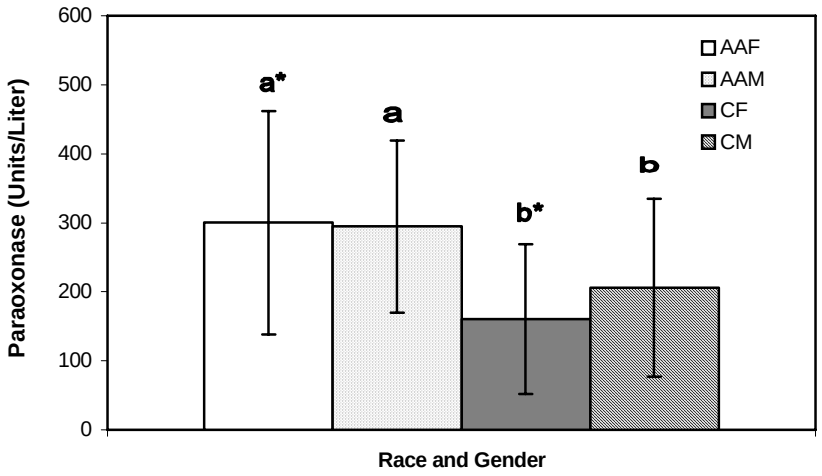

Figure 9b. Mean POase Activities in the Serum of African American and Caucasian Southerners

Abbreviations are identical as in Figure 1. (a) Values are expressed as mean ± SD (n = 100) within each gender. (b) Values are expressed as mean ± SD (n = 50) within each gender and race. Significant differences were determined using the GLM with LSMEANS procedure ($p<0.05$). Contrasting letters (a* & b* -females; a & b - males) represent significant differences found comparing race. No differences within groups having similar letters.

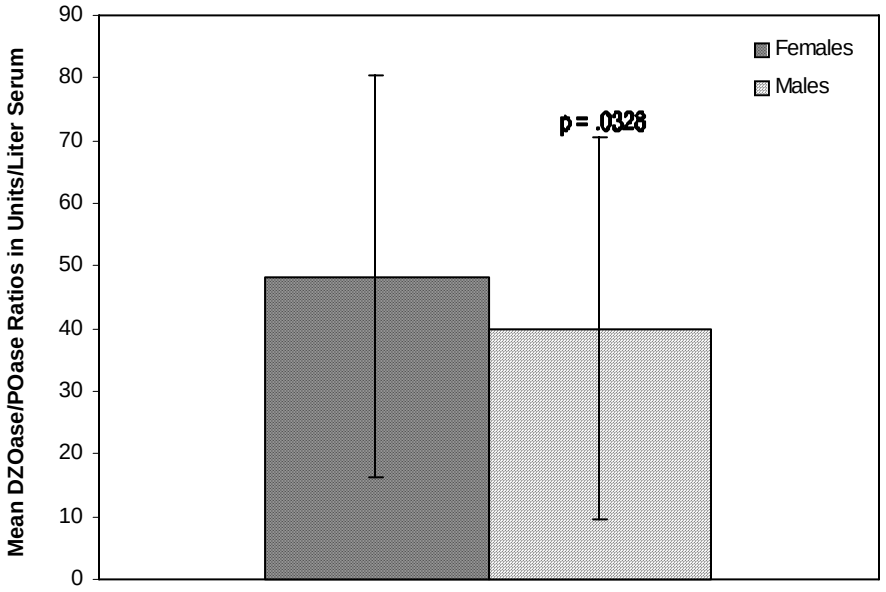

Figure 10. Mean DZOase/POase Ratios in the Serum of Females and Males

Values are expressed as mean ± SD (n = 100) within each gender. Significant differences were determined using the GLM with LSMEANS procedure ($p<0.05$). P –value represents significant difference found when comparing race.

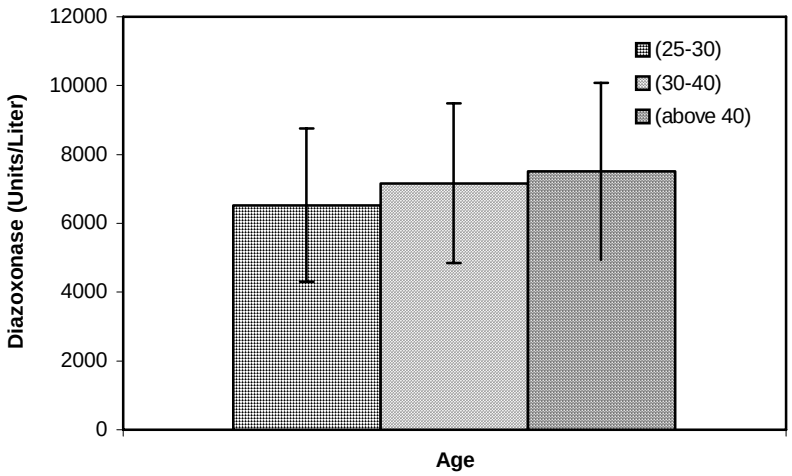

Figure 11. Mean DZOase Activities in the Serum of Three Age Groups African American and Caucasian Female Southerners

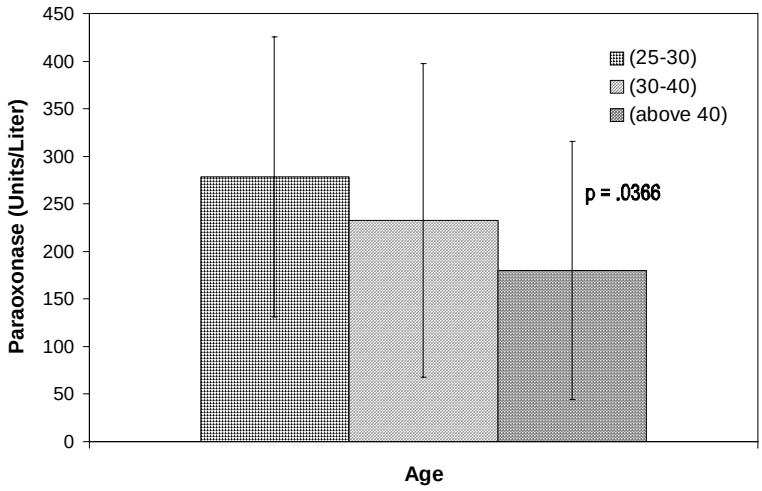

Figure 12. Mean POase Activities in the Serum of Three Age Groups African American and Caucasian Female Southerners

Above figures, values are expressed as mean ± SD (n = 100): females 25-30 (n = 32), 30-40 (n = 36) and above 40 (n = 32). Significant differences were determined using the binomial distribution test, $p < 0.05$. For paraoxonase, p- value represents the significant difference found among 25-30 (n = 32) and above 40 females.

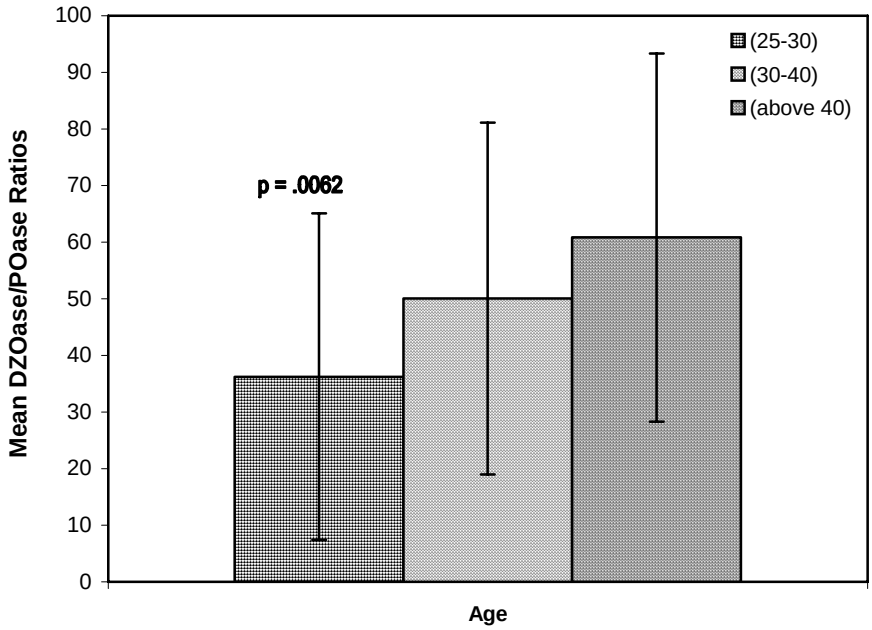

Figure 13. Mean DZOase/POase Ratios in the Serum of Three Age Groups of African American and Caucasian Female Southerners

Values are expressed as mean ± SD (n = 96). Significant differences were determined using the binomial distribution test, $p < 0.05$. P- value represents the significant difference found among 25-30 (n = 30) and above 40 (n = 30) females. Ratios of indeterminate data points were not included.

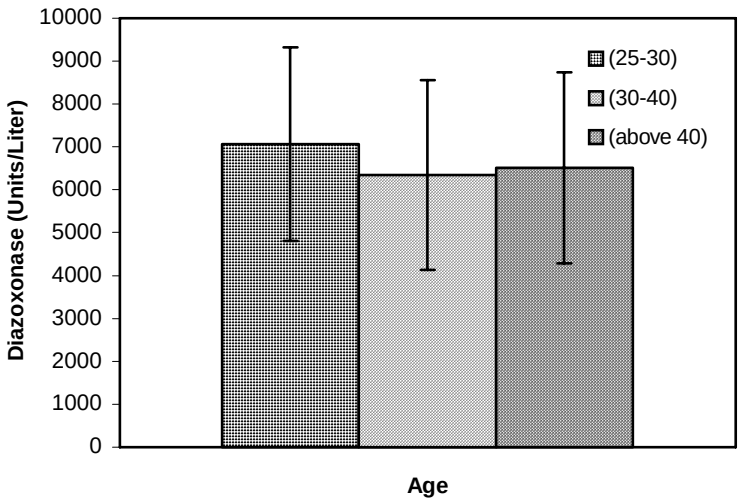

Figure 14. Mean DZOase Activities in the Serum of Three Age Groups of African American and Caucasian Male Southerners

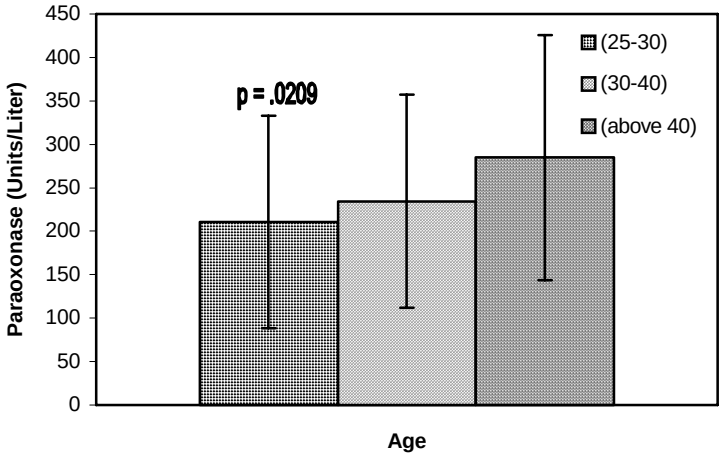

Figure 15. Mean POase Activities in the Serum of Three Age Groups of African American and Caucasian Male Southerners

Above figures, values are expressed as mean ± SD (n = 100); males 25-30 (n = 28), 30-40 (n = 25) and above 40 (n = 47). Significant differences were determined using the binomial distribution test, $p < 0.05$. For paraoxonase, p- value represents the significant difference found among 25-30 (n = 28) and above 40 (n = 47) males.

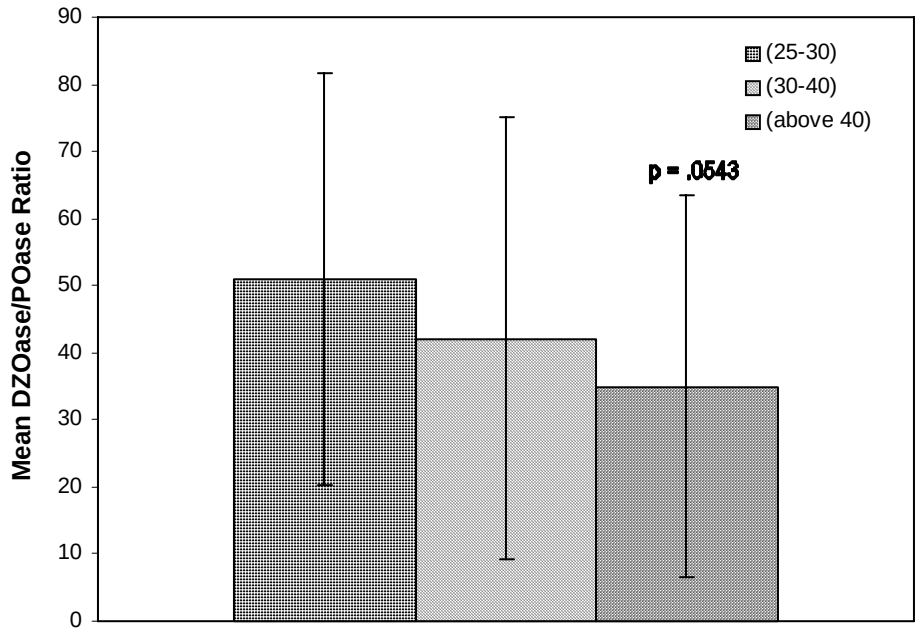

Figure 16. Mean DZOase/POase Ratios in the Serum of Three Age Groups of African American and Caucasian Male Southerners

Values are expressed as mean ± SD (n = 99). Significant differences were determined using the binomial distribution test, $p < 0.05$. P- value represents the significant difference found among 25-30 (n = 27) and above 40 (n = 45) males.

Table 1. PON1$_{192}$ Genotype Distribution in the Serum of African American and Caucasian Southerners

	POase (Units/Liter)		**DZOase (Units/Liter)**		
n = 200	Range	Mean ± SD	Range	Mean ± SD	Frequency
All	29 - 614	240 ± 144	2140 -14083	6847 ± 2314	
QQ	29 - 170	98 ± 34	2417 - 14083	7950 ± 2446	.375
QR	115 - 445	265 ± 80	3622 - 10312	7132 ± 1825	.325
RR	206 - 614	408 ± 104	2140 - 10131	5210 ± 1595	.255

Data from Sigma Plot 8.0 (Figure 1).

Table 2. Mean PON1$_{192}$ Activities and DZOase/POase Ratios in the Serum of African American and Caucasian Southerners by Gender

Race and Gender	Paraoxonase Activity	Diazoxonase Activity	DZOase/POase ratios
AA- female [a]	300.10 ± 161.95	6288.85 ± 2193.03	31.19 ± 25.96
AA- male [a]	294.61 ± 124.95	5656.61 ± 1897.09	25.87 ± 21.49
C- female [b]	160.63 ± 108.73	7849.22 ± 2334.96	65.36 ± 28.43
C- male [b]	206.08 ± 128.88	7591.38 ± 2128.62	54.18 ± 31.62

Values are represented as mean ± SD of fifty individuals of each race and gender.

[a,b] Significant differences in activities and DZOase/POase ratios

Table 3. Mean PON1$_{192}$ Activities and DZOase/POase Ratios in the Serum of African American and Caucasian Southerners

Race	Paraoxonase Activity	Diazoxonase Activity	DZOase/POase ratios
African Americans	297.36 ± 143.93[a]	5972.73 ± 2064.62	28.53 ± 23.86
Caucasians	183.36 ± 120.81	7720.30 ± 2226.64[a]	59.77 ± 30.44[a]

Values are represented as mean ± SD of two hundred individuals.

[a] Significant differences in activities and DZOase/POase ratios ($p < 0.05$).

CHAPTER V

DISCUSSION

PON1 plays an important role in hydrolyzing many substrates, including oxidized lipoproteins and active metabolites of organophosphate insecticides. The single nucleotide polymorphism at position 192 has a significant effect on PON1 hydrolytic activity, drug metabolism, and the onset of cardiovascular disease (Harel et al., 2004). Regarding the PON1Q192R polymorphism, the Q allele has been shown to be more efficient in metabolizing diazoxon and to have the more protective function in decreased susceptibility to cardiovascular disease. In contrast, the R allele has been shown to be more efficient in metabolizing paraoxon and less protective in preventing the onset of cardiovascular disease (CVD). Statistical analysis has indicated that CVD is more prevalent in the African American population and causes higher annual age-adjusted mortality rates in the South (excluding Florida) than any other region in the United States. Many environmental, dietary, lifestyle, and genetic factors have been reported to be associated with the onset of CVD; however, data on the distribution of PON_{Q192R} in the serum of Southerners and African Americans in the South remain unknown.

This study has determined the $PON1_{192}$ genotype distribution, $PON1_{192}$ enzymatic activities (paraoxonase and diazoxonase), and mean DZOase/POase ratios in the serum of two hundred African American and Caucasian Southerners. This examination also

revealed a significant association of $PON1_{192}$ activities with race, gender, and age. The $PON1_{192}$ genotype frequency distribution in the Southern population: QQ (.375), QR (.325), and RR (.255) excluding indeterminate (QR and RR) data points (.045) indicate that more data points from the population fall into the protective genotype. Results from this study show that Caucasians (60%) have an overwhelmingly higher distribution of the QQ genotype than African Americans (15%). Our study supports data reported by Costa (2003) and Schacchi (2003) on Q and R allele frequencies in African Americans and Caucasians. African Americans were reported to have a higher R allele frequency (0.63) and Caucasians were reported to have a higher Q allele frequency (0.75). Data strongly suggest that more African Americans in the South have the genotypes associated with increased susceptibility to CVD than Caucasians.

Differences seen among Caucasians and African Americans suggest that linkage disequilibrium may play a critical role in determining the distribution of $PON1_{192}$ genotypes. According to a study by Chen et al. (2003), in Caucasians there is linkage disequilibrium between polymorphisms Q192R and L55M and between the promoter polymorphisms and L55M. In African Americans, the promoter polymorphisms C-108T and G-909C are in disequilibrium with Q192R, but not in the Caucasian sample population. Factors that cause linkage disequilibrium are: interactions between genes, genetic linkage and the recombination rate, random drift or non- random mating, and population structure. The risk of CVD may be synergized with the less protective genotype and factors such as poor lifestyle habits (high alcohol consumption, smoking), fat- enriched dietary intake, and environmental toxicant exposure (insecticides). It would

be of interest to also study lifestyle, dietary, and environmental factors that affect $PON1_{192}$ activities.

The mean DZOase/POase ratios were significantly different when comparing African Americans to Caucasians using the GLM with LSMEANS procedure. The mean DZOase/POase ratios are indicative of the $PON1_{192}$ genotype distribution within each population. The higher mean DZOase/POase ratio of Caucasians compared to African Americans suggests a higher frequency of the more protective $PON1_{192}$ genotype (QQ) and higher diazoxonase activities within the Caucasian population. It has recently been revealed in a Serbian study that HDL and LDL particle size are associated with diazoxonase activity (Vekic et al., 2007). Small and dense LDL particles (sdLDL) undergo oxidation readily and the large size of HDL has been associated with hydrolyzing sdLDL particles. Results from this study found that sdLDL particles increase oxidative stress, which stimulates PON1 diazoxonase activity. The increased oxidative stress causes an accumulation of smaller, denser HDL particles decreasing HDL antioxidative activity (Vekic et al., 2007).

Examining the $PON1_{192}$ genotype distribution and mean DZOase/POase ratios, we hypothesized higher diazoxonase activities in Caucasians and higher paraoxonase activities in African Americans. Our results show significant differences in diazoxonase and paraoxonase activities between African American and Caucasian Southerners independently. Caucasians demonstrated higher mean diazoxonase activities than African Americans; in contrast, African Americans demonstrated higher mean paraoxonase activities than Caucasians. The higher paraoxonase activity in African Americans denotes the dominant presence of the R allele. The higher diazoxonase

activity in Caucasians denotes the dominant presence of the Q allele. Our study indicates a positive correspondence between $PON1_{192}$ activities and race.

Gender differences in PON1 activities have not been shown to be significant in many studies (Mueller et al., 1983). Results from our study do not indicate a significant difference when comparing diazoxonase and paraoxonase activities of females and males separately or within each race. Our results did indicate a significant difference when comparing DZOase/POase ratios of females and males. Differences in the higher mean DZOase/POase ratios of females than males may be attributed to one or two factors, the samples obtained in this study or to some as yet unidentified sex linkage. Our serum samples were randomly obtained from individuals in Alabama and Tennessee blood banks, so more females with QQ genotype may have been selected than males with the QQ genotype. If non- random sampling was not involved, our data suggest that regardless of $PON1_{192}$ activities, females are more likely to be born with the more protective genotype than males. Further studies are needed to determine if direct correlation between gender and $PON1_{192}$ DZOase/POase ratios exists.

Our study reports that with age, paraoxonase activity decreases in females and DZOase/POase ratios shift to become higher. In males, we found that paraoxonase activity increases with age and DZOase/POase ratios shift to become lower. These results indicate that with age, changes in paraoxonase activities may render a lower DZOase/POase ratio in the oldest males, but higher DZOase/POase ratios in the oldest females. A higher DZOase/POase ratio is indicative of having the more protective $PON1_{192}$ genotype associated with decreased susceptibility to CVD. A lower

DZOase/POase ratio is indicative of having the less protective $PON1_{192}$ genotype associated with increased susceptibility to CVD.

Studies have shown that serum PON1 activity is lower during development and birth, but increases over time (Chen et al., 2003). Other studies have reported that PON1 activities remain constant within adults or decrease in elderly subjects (Jarvik et al., 2002; Miochevitch and Khalil, 2001; Seres et al., 2004; Costa et al., 2005). From the data presented here, and previous studies, it is clear that further studies are needed to determine the relationship between $PON1_{192}$ and age.

In summary, our results report the $PON1_{192}$ genotype distribution, $PON1_{192}$ enzymatic activities (paraoxonase and diazoxonase), and mean DZOase/POase ratios in the serum of African American and Caucasian Southerners. Most importantly, our study reports significant race, gender, and age differences associated with $PON1_{192}$ activities. A higher frequency of African Americans possess the less protective genotype and a higher frequency of Caucasians possess the more protective genotype linked with CVD. This is due to the higher paraoxonase activities of African American Southerners and higher diazoxonase activities of Caucasian Southerners.

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