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INVESTIGATION OF A POSSIBLE ASSOCIATION BETWEEN PON1 POLYMORPHISMS L55M AND Q192R WITH CORONARY ARTERY DISEASE AND TYPE 2 DIABETES PATIENTS WITHIN A SOUTHERN POPULATION

By

Chiquita Yvette McDaniel

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Sciences
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2012

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POLYMORPHISMS L55M AND Q192R WITH CORONARY

ARTERY DISEASE AND TYPE 2 DIABETES

PATIENTS WITHIN A SOUTHERN

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Pages in Study: 34

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Mississippi has a very high prevalence of coronary artery disease (CAD) and type 2 diabetes (T2D), especially among African Americans compared to Caucasians. This project determined the L55M genotypes of paraoxonase 1 (PON1) in 187 people and evaluated associations of PON1 single nucleotide polymorphisms (SNPs), Q192R and L55M, with CAD and T2D in a Mississippian (southern) population. Significant associations were found with PON1 SNPs and race: genotypes LL, LM, QR, and RR showed significant associations with race (p values 0.0000955, 0.0024, 0.00001244, and 0.00001676, respectively), and combined genotypes LMQQ and LMRR were significantly associated with race (p values = 0.0001013 and 0.000473, respectively). While no significant associations were found between PON1 SNPs and CAD (p values > 0.2374), combined genotype LMQQ and genotype LM trended towards the likelihood of having T2D with p values = 0.0723 and 0.0931, respectively, and are suggestive of a potential biomarker for T2D risk.

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DEDICATION

This paper is dedicated firstly to my heavenly Father, God, for allowing me to make it this far. Through all the trials and tribulations, he has been by my side and has carried me out of darkness and into the light of eternal life. All the glory goes to him! Secondly, to my mom, Bobbie J. McDaniel, for her continued, unwavering support and for being a blessing in my life. I LOVE YOU dearly!! And to any person reading this may God continue to grant you grace and mercy.

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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Janice Chambers, Dr. Robert Wills, Dr. Allen Crow, MaryBeth Dail, LaDetra Forrest, and lab members and staff. I also would like to acknowledge the National Institute of Health for partially funding this research.

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CHAPTER I

INTRODUCTION

1.1 Prevalence of type 2 diabetes and cardiovascular diseases

The rates of the chronic diseases, cardiovascular disease (CVD) and diabetes, in connection with obesity, are growing to epidemic proportions in the United States, affecting millions of people. During 1991, a meta-analysis of six studies, reported that over 600,000 deaths in the United States were due to obesity and its complications. Among these complications are said chronic diseases[1]. Obesity leads to a variety of chronic illnesses, including type 2 diabetes (T2D), reproductive, digestive, and cardiovascular diseases and even certain cancers[2]. Mokdad et al. reports, that the prevalence of this condition continues to increase climbing from 7.3% in 2000 to 7.9% in 2001; an 8.2% increase in a year's time[3]. As we experience increases in the rates of obesity there is also an increase in the occurrences of associated diseases, especially CVD and diabetes. Based on information reported by the American Heart Association, more than 2,000 Americans died of CVD each day in 2007. Also in 2007, more than 150,000 Americans who died from CVD were less than 65 years old, with African Americans having the highest rates of hypertension in the world at 44%. In 2008, an estimated 18,300,000 Americans were diagnosed with diabetes mellitus representing 8% of the adult population. In addition to that it was estimated that more than 7,000,000 cases were undiagnosed and almost 37% had prediabetes in U.S. adults [4].

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1.2 Paraoxonase as an at-risk biomarker

In knowing this, policies and programs have been implemented to monitor and to increase awareness of obesity and associated chronic diseases [2], as well as increases in research based on potential molecular level risk factors. Over the past 20 years, a number of studies have emerged implicating paraoxonase 1 (PON1) and its status as a risk indicator for diabetes and cardiovascular diseases, such as atherosclerosis. [5-7] PON1 status refers to PON1's activity levels (i.e., hydrolysis of esters such as paraoxon) and genotype. In some studies functional genotype was determined through activity plots of diazoxon hydrolysis vs. paraoxon hydrolysis when actual genotyping was not possible[8]. This potential biomarker could aid in increasing disease awareness and in instituting preventive measures at earlier points of life. Biomarkers help quantify normal biological processes in an organism and are most commonly introduced into the biological system[9]. What makes this potential biomarker so beneficial is that it is already inside the human body and may be located in DNA.

1.3 Importance of a southern (Mississippi) study population

Despite there being numerous studies conducted on a variety of populations such as Turks[10], Italians and Irish [11], South-western Koreans[12], Mexicans[13] and Egyptians[14], there have been conflicting results in concluding that PON1 polymorphisms have any associations with either chronic disease based on ethnicity. Two studies have been conducted by our lab that have focused on United States Southerners. One of these studies concluded that race (African American and Caucasian) contributed to proportions of PON1 Q192R functional genotypes, and that these differences in proportions might account for the poorer cardiovascular health conditions characteristic of the Southern United States population, especially in the African American population

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which suffers more from CVD than the Caucasian population[15]. In the other, being the first study to report PON1's association with atherosclerosis in a Deep South population, Coombes et al.(2011) concluded that higher levels of PON1 activity, as assessed by the hydrolysis of phenylacetate, decreased the odds of atherosclerosis in a group of African American and Caucasian Southerners; however, the PON1 192 genotype was not found to be significantly associated with atherosclerosis [16].

In 2000, Mississippi was one of the leading states in the U.S. in obesity at an estimated 24.3%, and this rate was highest among African Americans[17]. In the Heart Disease and Stroke Statistics 2011 Update reported by The American Heart Association, Mississippi was among the four highest states in the US in age adjusted death rates with 350 deaths from CVD per 100,000 people[4]. Noting the distinct increase from 2007, cited earlier, to 2011, health statistics would suggest that Mississippians would be an ideal study population to determine what role, if any, PON1 plays in disease risk. Mississippians, as well as other populations with increased rates of diabetes and cardiovascular diseases could benefit greatly from a potential disease risk biomarker because it is an accurate means of earlier detection that is inexpensive and only minimally invasive.

1.4 Diabetes: Type 2 Diabetes Mellitus

Diabetes is characterized by high blood glucose levels caused by deficiencies in insulin production, insulin's mechanisms or a combination of the two. It is classified into 3 classes, Type 1: inability to produce insulin because of destroyed pancreatic β-cells; Type 2: the body does not produce or use what insulin is produced efficiently; and Gestational: diabetes that occurs during pregnancy[18]. T2D accounts for 90-95% of all

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diagnosed diabetes cases[19]. As of 2011, from data collected by the Centers for Disease Control in 2010, over 25 million people in the United States were affected by diabetes. Despite multifactorial epidemiology reports, it is clear that one of the main contributors of diabetes is weight gain [20]. Ford et al. suggested that for every kilogram gained the risk of getting diabetes increases by an estimated 4.5%[21]. According to The National Institutes of Health (NIH)'s clinical guidelines in 1998, having a body mass index (BMI) of more than 30 kg/m^2 classifies a person as being obese and a BMI of 25-29.9 kg/m^2 as being overweight[22]. By these guidelines, there are over 97 million adults in the United States who are considered obese or overweight. Along with weight gain, insulin resistance and insulin sensitivity plus hyperglycemia, all of which have been deemed as results of obesity, also add to the causes of diabetes [23, 24]. In providing a positive correlation between the two, studies have linked the increases in diabetic occurrences with the prevalence of obesity in the United States [3, 17, 25]. Adipose tissue normally functions as a storage house for triglycerides and secretes bioactive molecules[26]. When this storage becomes persistently over nourished and obesity results, adipocytes become dysfunctional and release triglycerides which are then converted to free fatty acids (FFA), which leads to chronic inflammation. This chronic inflammation is due to an infiltration of inflammatory monocyte derived macrophages brought on by enlarged adipocytes. This constant state of inflammation can lead to insulin resistance in adipose tissue[27]. This inflammation increases the levels of circulating FFA which in turn, leads to increased levels in the portal venous system which has been reported to be at the heart of insulin resistance stemming from visceral obesity[20]. Visceral obesity is correlated with a list of factors some of which are independent risk factors for CVD[28].

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1.5 Cardiovascular Diseases: Atherosclerosis

Due to atherosclerosis being one of the two most prevalent forms of CVD and the focus of this thesis, atherosclerosis, coronary heart disease, and coronary artery disease (CAD) will be used interchangeably. The exact pathogenesis of cardiovascular disease, namely atherosclerosis, is not completely clear because CVD is associated with a list of risk factors such as low levels of high-density lipoproteins (HDL)[29], obesity, insulin resistance, hyperglycemia, endothelial dysfunction, and chronic inflammation[20, 28]. The onset of coronary artery disease is usually characterized by lipid peroxidation and the occurrence of atherosclerotic lesions known as fatty streaks which may develop into atherosclerotic plaques. These can prove fatal depending on the stability of the plaque[30]. Fatty streaks develop from increased uptake of oxidized lipids by macrophages and this uncontrolled uptake causes these cells to be packed with lipids in the vessel wall of large arteries. They develop into foam cells and, when they undergo apoptosis, these cells become hard and flatten, thus resulting in fatty streaks [31-33]. These streaks form partly as a result of persistent over-nourished adipose tissue leading to an underlying inflammatory state which increases levels of oxidized low density lipoproteins and release of FFA[31]. As mentioned earlier, increased levels of FFA promote a more inflammatory state and lead to insulin resistance[27]; other recent reports have shown that increased FFA levels can cause vascular endothelial cell dysfunction[34].

In addition, other conditions reported to be observed in insulin resistant states are elevated levels of fibrinogen and thrombin-antithrombin which aid in clot formation of injured endothelium[28], supporting the idea that insulin resistance and CAD are associated. With the prevalence of obesity being at epidemic proportions, the occurrences

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of diabetes are increased and the risk of developing CVD is also heightened. Reported in the 2011 Heart Disease and Stroke Statistics Update, at least 2,200 Americans died every day of CVD with an average of one death every 39 seconds. About 33% of these deaths occurred before the age of 75, which is almost 3 years before the average life expectancy of 77.9 years. In addition, 1 of every 6 deaths was due to CHD. Most of the data reported in the 2011 Update was collected during the years 2007 and 2008[4].

1.6 Paraoxonase 1 (PON1)

1.6.1 Discovery of Paraoxonase

Abraham Mazur was the first to describe an enzyme that could catalyze hydrolysis of organophosphates in animal tissue, during the mid-1940s[35]. Continued research led Aldridge to initially identify this enzyme as paraoxonase in 1953. It was so named because of its ability to hydrolyze paraoxon, the active metabolite of the organophosphate insecticide, parathion[36, 37]. It was also shown to encompass the ability to hydrolyze aromatic esters (A-esterases)[36, 37]. In 1991, paraoxonase was finally reported to be responsible for both activities[38], as well as being linked to antiatherosclerotic properties such as reducing the accumulation of lipid peroxides on low-density lipoproteins (LDL)[39].

Over the past 50 years in human serum paraoxonase research, two other PONs (PON2 & PON3) have been discovered whose genes are located next to PON1 on the long arm of chromosome 7 (7q21.3-q22.1) in humans[40, 41]. Recent studies have shown that the basic function of these paraoxonases (1, 2, & 3) is hydrolyzing lactones [42, 43]. Even though there has been much research conducted on paraoxonases, one member of this multi-gene family, PON1, is the most commonly researched.

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1.6.2 Variations due to PON1 polymorphisms

Studies with humans have shown a wide inter-individual variation in PON1 levels and activities. Variations in activities are due to PON1 polymorphisms or single nucleotide polymorphisms (SNPs)[44, 45], ethnicity[13, 14], health due to diet and lifestyle habits[46-48] or environmental factors, and possibly a combination of them all. These variations cause the efficiency of PON1 protective abilities, such as with organophosphate exposure in a study conducted by Mackness et al. to be reduced[49]. PON1 has 7 important polymorphisms: 2 in the coding region and 5 in the promoter region. The two common SNPs, one a leucine (L)/methionine(M) substitution at position 55 and the other substitution of arginine (R)/glutamine(Q) occurring at position 192, occur in the coding region[44, 45].

1.6.3 Insurgence of research due to PON1's implication in health

Human PON1 is mainly found in the liver where it is synthesized and secreted into the blood where it is associated with high density lipoprotein via the APO-A1 protein[50]. PON1's association with HDL has been demonstrated to have some protective capabilities, health wise, by preventing the oxidation of LDL[39, 49]and reducing oxidative stress in macrophages[51]. Discovering these capabilities caused a surge of research in this area. Though a study conducted by Mackness and Mackness reported that PON1 status is a better predictor than just genotype itself [52], much research has been conducted that sheds light on the participation of SNPs (L55M and Q192R) in a variety of diseases and conditions[12, 17], especially their association with T2D and CVD.

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1.7 Role of PON1 Polymorphisms (L55M and Q192R) in Type II Diabetes & Coronary Artery Disease

1.7.1 Risk factors of CAD and T2D

The etiology of either atherosclerosis or T2D is not very clear; however, some of the factors that contribute to the development of these diseases overlap each other. Factors contributing to atherosclerosis include high blood pressure, low levels of HDL cholesterol, sedentary lifestyle, overweight, family history of heart disease and smoking. Factors contributing to T2D include, obesity, physical inactivity, family history of diabetes, race, fat distribution, pre-diabetic or have had gestational diabetes [22]. CAD is a particular step of atherosclerosis that is characterized by the narrowing of the arteries due to the development of atheromas inside the arterial walls[53]. With this knowledge, the increased need to identify a potential risk marker is very evident. Though there have been numerous studies conducted with PON1, the actual role of its SNPs (L55M & Q192R) in T2D and CAD is not yet clear.

1.7.2 Conflicting Research

For example, a positive correlation was demonstrated in a study conducted on Turkish patients with T2D in comparison with healthy non-diabetic controls. It was determined that PON1 activity was decreased in the presence of T2D. They also suggest that both SNPs influences PON1 activity[10]. Also, in a similar previous study conducted by Mackness et al., a group of 252 Non-Insulin Dependent Diabetes Mellitus (NIDDM) individuals from Manchester Diabetes Centre were subjected to serum analysis, some of whom displayed one or more diabetic complications, low serum PON1 activity was observed; however there were no significant differences in allele frequencies[7].

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Another analysis performed by Oliveria et al. in 2004, tested a population of 352 patients with CAD and 380 high risk controls. Studies showed that only the PON1 L55M polymorphism as being an independent risk factor for CAD [6], however it has been commonly known that the PON1 192R is associated with atherosclerosis [54]. In contrast to the above studies, a meta-analysis of 43 studies, composed of over 11,000 coronary heart disease cases, found no associations between CHD and 2 polymorphisms from the PON1 gene (L55M & T(-107)C) and 1 polymorphism from the PON2 gene (S311C). However, they did find a weak association for the Q192R PON1 polymorphism[55]. Another conflicting analysis during the same year suggested that purified PON1 might lose some of its protective ability when tested using LDL and copper or the free radical generator 2,2′-azobis-2-amidinopropane hydrochloride (AAPH) as the oxidation system [56]. Because PON1 could lose some of its protective abilities in oxidative states, a system in an oxidized state, such as that produced by lipid peroxidation, could limit PON1's protective abilities against atherosclerosis or diabetes. No matter how conflicting these results may be, there are still no studies of which we are aware that analyze Mississippians besides the two studies conducted by our lab described earlier.

1.7.3 Objectives of current research

Mississippi is one of the states with the highest rates of CVD and T2D in association with obesity[3]. Therefore, any potential biomarker to increase at-risk populations' awareness would be beneficial. This study is a continuation of research on samples our laboratory has from a cardiology clinic in Tupelo, MS initially analyzed in Coombes et al [16]. Here, these samples are used to determine if there is a correlation between both of PON1's common SNPs (Q192R, L55M) with T2D and/or CAD which

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could identify a biomarker of risk. This study consists of three objectives. 1) Examine the relationship between both PON1 L55M and PON1 Q192R associations with CAD and T2D. 2) Clarify the association of PON1 polymorphisms with CAD and T2D, also considering race. 3) To develop, if possible, a potential biomarker for preventative measures for at-risk populations.

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CHAPTER II

MATERIALS AND METHODS

2.1 Study Population

Serum samples were previously collected from a cardiology practice, Cardiology Associates of North Mississippi in Tupelo, MS. A total of 200 serum samples from Caucasians (60 male, 60 female) and African Americans (40 male, 40 female) ages 45 and higher were obtained. A 60/40 population ratio was used because it reflects the racial demographic of Mississippi. This population is the same as initially reported upon in a recent study from our laboratory[16]. Protocols were approved by the IRBs of both Mississippi State University and North Mississippi Medical Center. Informed consent was obtained from all participants prior to obtaining demographic and health information or blood samples. All data have been de-identified. Subjects with atherosclerotic vascular disease were counted as positive for disease. Subjects with a clinical diagnosis of T2D were also noted. Race was determined by self-reports.

2.2 DNA Extraction

Genomic DNA was previously isolated from whole blood via the Sigma-Aldrich® GenElute™ Blood Genomic DNA Kit and concentrated via Qiagen's QIAEX®II Gel Extraction Kit following the recommendations of the manufacturers.

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2.3 Chemicals and Reagents

All chemicals and reagents were purchased from New England BioLabs with the exception of AmpliTaq Gold 360 DNA polymerase (Applied BioSystems; Forest City, CA), primers (Eurofins MWG Operon; Huntsville, AL), exACTGene^{TM} PCR dNTP Mix (Promega; Madison, WI), Blue/Orange 6X Loading Dye (Fisher Scientific; Waltham, MA) and SYBR Safe DNA Gel Stain (Invitrogen; Carlsbad, CA).

2.4 PON1 L55M Genotyping

2.4.1 Polymerase Chain Reaction

Following the method received from the Medical Genetics Department of the University of Washington (Jane Ranchalis, personal communication), a region containing the $PON1_{55}$ codon was amplified by a polymerase chain reaction (PCR) to yield a 386 bp product using forward primer: 5' AGAGGATTCAGTCTTTGAGGAAA 3' and reverse primer: 5'CTGCCAGTCCTAGAAAACGTT 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), 1.60 µl of 25 mM $MgCl_2$, 0.160 µl 10 mM dNTPs (Promega), 0.160 µl of each primer at 25 µM, 0.1 µl AmpliTaq Gold polymerase (ABI) at 5U/µl and water sufficient to bring the final volume to 20 µl.

2.4.2 Restriction Digests

Since there is a native NlaIII (NEB) restriction site at codon 55, 10 µl of the PCR product solution underwent overnight digestion. The digestion resulted in a single non-digested 386-bp fragment for individuals having PON1 55LL genotype; 386-, 299-, and 90-bp fragments for individuals with the 55LM genotype and bands of 296- and 90-bp in

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size for individuals who carry the PON1 55MM genotype. These data were combined with the previously determined Q192R genotyping by our lab using restriction fragment length polymorphism assay[16].

2.4.3 Electrophoresis

The digested PCR products were visualized by agarose gel electrophoresis. A 2% agarose gel was prepared by mixing 10g of agarose with 500ml of 0.5X Tris/Borate/EDTA (TBE) running buffer. 0.5X TBE running buffer was prepared by adding 50mls of 10 X TBE stock solution to 950ml of distilled water (dH_2O). 10 µl of each digested PCR product and 10 µl of each undigested PCR product were combined with Blue/Orange 6X loading dye at 1µl of loading dye to every 5µl of sample and loaded next to each other on the gel. Electrophoresis was carried out at 90V using a FisherBiotech Electrophoresis System. Once migration was complete, the gel was stained with either SYBR Safe DNA gel stain following the manufacturer's recommended method or ethidium bromide (EtBr). When EtBr was used at 1µg/µl, the gel was stained for 30 minutes with agitation and de-stained with dH_2O for 20 minutes. If SYBR Safe gel stain was used, the gel was stained for 40 minutes with agitation. After each staining procedure, ultraviolet excitation was used to visualize the bands. Since EtBr is a known carcinogen, the staining solution was prepared by a senior member of the lab. When the senior member was not available, SYBR Safe DNA gel stain was used. Although the SYBR Safe gel stain is less toxic, its expense precluded routine use.

2.5 Statistical Analysis

After the determination of L55M genotypes, these data were combined with PON1 Q192R genotypes determined previously by our lab[16] on the same study

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population. The data underwent statistical analysis. The data were analyzed by Fisher's Exact Test using PROC FREQ in SAS for Windows 9.2 (SAS Institute Inc., Cary, NC) to determine if there were associations between the frequencies of PON1 L55M and PON1 Q192R genotypes. Fisher's Exact Tests were also conducted to determine if there were associations between PON1 genotypes and race. In the analysis of race and PON1 genotypes, the association between race and individual PON1 L55M genotypes, individual PON1 Q192R genotypes, and all possible PON1 55/192 combined genotypes were assessed. Bonferonni's correction was used to adjust for the effect of multiple pairwise comparisons. The strength of association between the occurrence of T2D or CAD and individual PON1 L55M genotypes, individual PON1 Q192R genotypes, and all possible PON1 55/192 combined genotypes was assessed through logistic regression using PROC LOGISTIC in SAS for Windows 9.2 in a series of univariate models.

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CHAPTER III

RESULTS

3.1 Combining PON1 L55M genotyping distributions with PON1 Q192R genotyping distributions

Restriction patterns identifying the PON1 L55M genotypes were visualized on 2% agarose gels (Figure 1). Of the original 200 samples, only 187 yielded results. The data obtained here on the PON1 L55M genotypes were combined with previously determined Q192R genotype data[16]. The distributions of genotypes and combined genotypes within the population are outlined in Table 1 and Table 2, respectively.

3.2 Meeting the objectives

In order to report a clear and concise investigation, four questions were asked: 1) is there an association of PON1 L55M genotypes with PON1 Q192R genotypes; 2) is there an association of PON1 L55M and PON1 Q192R genotypes and PON1 55/192 combined genotypes with race; 3) is there an association of PON1 55/192 combined genotypes and PON1 L55M and PON1 Q192R genotypes with T2D; 4) is there an association of PON1 55/192 combined genotypes and PON1 L55M and PON1 Q192R genotypes with CAD?

3.2.1 Is there an association of PON1 L55M genotypes with PON1 Q192R genotypes?

Overall, an association between the L55M and Q192R genotypes was found with a *p value= 0.000008281* using Fisher's Exact Test. However, a Bonferonni's correction

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was applied to accommodate the effect of multiple pairwise comparisons. Associations were considered significant for *p values<0.0056***.** Tables 3, 4, and 5 outline the associations between each individual Q192R genotype with the presence or absence of each individual L55M genotype to determine which genotypes are more likely to occur together in a subject. In Table 3, only two associations were detected for the absence or presence of LL and it was with the QQ and RR genotypes with significant *p values of 0.000131* and *0.0014*, respectively. In the case of LM genotype (Table 4) there were no significant associations detected. Table 5 displays statistically significant p values for the absence of MM in the presence of QQ *(p value=0.00004285)* or QR *(p value=0.0020).*

3.2.2 Is there an association of PON1 L55M and PON1 Q192R genotypes and PON1 55/192 combined genotype with race?

When all genotypes of each polymorphism are considered, it was determined that both Q192R and L55M, has a significant association with race with *p values of 0.0000005395* and *0.0003286*, respectively. After determining the overall association between Q192R and L55M genotypes and race, analysis of each individual genotype's relationship with race was performed to determine the levels of association within the data set. Significant associations are denoted with different superscripts and have *p values < 0.0167* according to the Bonferonni's correction (Tables 6 & 7). Genotypes LL, LM, QR, and RR all show significant associations with race, *p values 0.0000955, 0.0024, 0.00001244, and 0.00001676*, respectively. An analysis of PON1 55/192 combined genotype was also performed in association with race (Table 8). A Fisher's Exact Test on PON1 55/192 combined genotypes was also performed to determine any association with race. The overall association had a *p value = 0.00000002027*. As previously done, Bonferonni's correction was applied to individually analyze each combined genotype's

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association with race to determine the levels of association and to account for the effect of multiple pairwise comparisons. Significant associations have *p values< 0.0056* (Table 8). Combined genotypes LMQQ and LMRR were the only combined genotypes that showed any statistical significance being less than the calculated Bonferonni's correction with *p values- = 0.0001013* and *0.000473*, respectively. LMQQ are more likely to be Caucasians, whereas LMRR was exclusively linked in this population with African Americans.

3.2.3 Is there an association of PON1 55/192 combined genotypes and PON1 L55M and PON1 Q192R genotypes with T2D?

These associations with T2D were analyzed using Logistic regression modeling. There was no statistically significant association observed *(p values > 0.1530)* on any levels of association, however, combined genotype **LMQQ** and genotype **LM** had p values that were trending towards significance *p values = 0.0723 and 0.0931*, respectively. These associations are outlined in Table 9.

3.2.4 Is there an association of PON1 55/192 combined genotypes and PON1 L55M and PON1 Q192R genotypes with CAD?

Also using Logistic regression modeling, we analyzed these associations and there were no statistically significant associations observed *(p values ≥ 0.2374)*. (Table 10)

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Table 3.1 : Frequencies of Individual Genotypes within a Southern Population

	PON1 Genotypes					
	LL	LM	MM	QQ	QR	RR
Total*	93 (50%)	79 (42%)	15(8%)	68 (36%)	83 (44%)	36 (20%)

*Total number of individual genotypes in population (percentage of population)

Table 3.2 Frequencies of Genotype Combination Distribution within in a Southern Population*

		PON1 192			**Total**
		QQ	QR	RR	
PON1 55	LL	23 (12.3%)	43 (22.99%)	27 (14.44%)	**93**
	LM	32 (17.11%)	39 (20.86%)	8 (4.28%)	**79**
	MM	13 (6.95%)	1 (0.53%)	1 (0.53%)	**15**
Total		**68**	**83**	**36**	**187**

* Data are occurrences of combined genotype in population (% values are of the entire study population)

Table 3.3 Analysis of a Possible Association between PON1 192 Genotypes and PON1 LL Genotype

		PON1 192			
		QQ	QR	RR	**Totals**
PON1 55	LL	23 (34%)	43 (52%)	27 (75%)	**93**
	Not LL	45 (66%)	40 (48%)	9 (25%)	**94**
	p values*	**0.00131****	**0.7691**	**0.0014****	

* P values refer to the comparison of the presence or absence of LL versus the presence or absence of QQ, QR or RR.
** Denotes significance ***(p values< 0.0056).*** Comparisons were made using Fisher's Exact Test with an applied Bonferonni's correction. (% values are of the entire study population)

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Table 3.4 Analysis of a Possible Association between PON1 192 Genotypes and PON1 LM Genotype

		PON1 192			
		QQ	QR	RR	**Totals**
PON1 55	LM	32 (47%)	39 (47%)	8 (22%)	**79**
	Not LM	36 (53%)	44 (53%)	28 (48%)	**108**
	p values*	**0.3564**	**0.2367**	**0.0084**	

* P values refer to the comparison of the presence or absence of LM versus the presence or absence of QQ, QR or RR.
** Denotes significance ***(p values< 0.0056).*** Comparisons were made using Fisher's Exact Test with an applied Bonferonni's correction. (% values are of the entire study population)

Table 3.5 Analysis of a Possible Association between PON1 192 Genotypes and PON1 MM Genotype

		PON1 192			
		QQ	QR	RR	**Totals**
PON1 55	MM	13 (19%)	1 (1%)	1 (3%)	**15**
	Not MM	55 (81%)	82 (99%)	35 (97%)	**172**
	p values*	**0.00004285****	**0.0020****	**0.3101**	

* P values refer to the comparison of the presence or absence of MM versus the presence or absence of QQ, QR or RR.
** Denotes significance ***(p values< 0.0056).*** Comparisons were made using Fisher's Exact Test with an applied Bonferonni's correction. (% values are of the entire study population)

Table 3.6 Frequencies and Comparison of Individual Genotypes with Race

		PON1 L55M Genotypes			
		LL	LM	MM	**Totals**
Race	A	50^{a} (68%)	21^{a} (28%)	3^{a} (4%)	**74**
	C	43^{b} (38%)	58^{b} (51%)	12^{a} (11%)	**113**
Totals		**93**	**79**	**15**	**187**

*Values within columns that have different superscripts are significantly different having p values < 0.0167, obtained using Fisher's Exact Test with an applied Bonferonni's correction. % values are of the occurrence of African Americans or Caucasians within the genotype population

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Table 3.7 Frequencies and Comparison of Individual Genotypes with Race

		PON1 Q192R Genotypes			
		QQ	QR	RR	**Totals**
Race	A	13[a] (18%)	35[a] (47%)	26[a] (35%)	**74**
	C	55[b] (49%)	48[a] (42%)	10[b] (9%)	**113**
Totals		**68**	**83**	**36**	**187**

*Values within columns that have different superscripts are significantly different having p values < 0.0167, obtained using Fisher's Exact Test with an applied Bonferonni's correction. (% values are of the occurrence of African Americans or Caucasians within the genotype population)

Table 3.8 PON1 Combined Genotypes Associations with Race

PON1 55/192	A*	(%)	C	(%)	P values
LLQQ	8	10.8	15	13.3	0.6569
LLQR	25	33.8	18	15.9	0.0072
LLRR	14	23.0	10	8.9	0.0101
LMQQ	3	4.1	29	25.7	***0.0001013****
LMQR	10	13.5	29	25.7	0.0648
LMRR	8	10.8	0	0.0	***0.000473****
MMQQ	2	2.7	11	9.7	0.0805
MMQR	0	0.0	1	1.0	1.000
MMRR	1	1.4	0	0.0	0.3957

*Total number of African Americans was 74 and Caucasians was 113. The ***p values* < 0.0056*** are significant, obtained using Fisher's Exact Test with an applied Bonferonni's correction. The % values are percentages of combined genotypes that were either African Americans or Caucasians in this population.

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Table 3.9 Analysis of Combined and Individual Genotypes with Type 2 Diabetes

PON1 55/192 Combined Genotypes	**P Values***	Totals (% values)**
LLQQ	**0.8955**	8 (35%)
LMQQ	**0.0723***	7(22%)
MMQQ	**0.1739**	7(54%)
LLQR	**0.4984**	17(40%)
LMQR	**0.4431**	12(31%)
MMQR	**0.9910**	1(100%)
LLRR	**0.5812**	11(41%)
LMRR	**0.4060**	4(50%)
MMRR	**0.9915**	0(0%)
PON1 Genotypes		
LL	**0.3827**	36(39%)
LM	**0.0931***	23(29%)
MM	**0.1530**	8(53%)
QQ	**0.4293**	22(32%)
QR	**0.8868**	30(37%)
RR	**0.4330**	15(42%)

*These trended towards significance. No statistically significant p values were observed $p \geq 0.1530$, which were obtained using Logistic Regression. Significance was based on p values ≤ 0.05.

**The total number that was positive for Type 2 Diabetes. % values are of the percentages within a genotype population that had T2D. There were 67 diabetics and 119 non diabetics. Due to missing demographic data, 2 observations were not included in the analysis.

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Table 3.10 Analysis of Combined and Individual Genotypes with Coronary Artery Disease

PON1 55/192 Combined Genotypes	P Values*	Totals (% values)**
LLQQ	**0.4357**	10(43%)
LMQQ	**0.6477**	17(55%)
MMQQ	**0.8375**	7(54%)
LLQR	**0.2963**	18(44%)
LMQR	**0.2687**	23(59%)
MMQR	**0.9907**	1(100%)
LLRR	**0.7627**	14(54%)
LMRR	**0.2415**	2(29%)
MMRR	**0.9907**	1(100%)
PON1 Genotypes		
LL	**0.2374**	42(47%)
LM	**0.4260**	42(55%)
MM	**0.4738**	9(60%)
QQ	**0.9421**	34(51%)
QR	**0.8556**	42(52%)
RR	**0.8869**	17(50%)

*No statistically significant p values were observed $p \geq 0.2374$, which were obtained using Logistic Regression. Significance was based on p values ≤ 0.05.
**The total number in population that was positive for CAD. % values are of the percentages within a genotype population that had CAD. There were 93 subjects with CAD and 89 subjects without. Due to missing demographic data, 6 observations were not included in the analysis.

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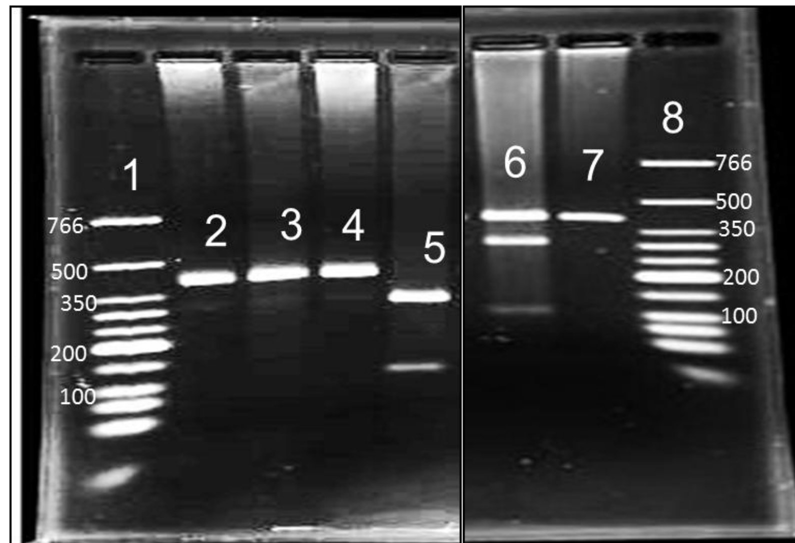

Figure 3.1 Determination of PON1 L55M Genotyping through Agarose Gel

2% agarose gel visualized by ethidium bromide staining. Lanes 1 & 8: Low Molecular Weight Ladder for size confirmation. Lanes 2, 4 & 7: Undigested PCR products serving as controls. Lane 3: 55LL- no digestion with band at 386-bp Lane 5: 55MM- incomplete digestion with bands at 296- and 90-bp fragments and Lane 8: 55LM-complete digestion with bands at 386-, 299-, and 90-bp fragments.

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CHAPTER IV

DISCUSSION & CONCLUSION

4.1 Importance of a Mississippi (southern) population

Coronary artery disease and T2D are the leading causes of mortality and morbidity in the U.S. especially Mississippi. In 2011, 350 deaths per 100,000 people were attributed to heart disease and in 2010 there were 254 deaths per 100,000 people[57]. T2D death rates in Mississippi jumped from 24.7 deaths per 100,000 people in 2008 to 31.2 deaths per 100,000 people in 2010[58]. These rates are reported to be higher in African Americans than Caucasians. African Americans had a rate of 45 deaths per 100,000 to Caucasians' 17 deaths per 100,000 due to diabetes. In regards to heart diseases, African Americans death rate was 295 deaths per 100,000 to Caucasians 246 deaths per 100,000. Noting this increase in death rates shows that there is a great need for a potential biomarker for at-risk individuals. These statistics and Mississippi's current health conditions, along with Mississippi having a racial demographic of 60/40 Caucasians to African Americans, make Mississippi an ideal study population for developing a possible risk biomarker. A biomarker such as this is accurate, inexpensive, minimally invasive, and can be detected way before disease onset.

4.2 PON1's role in CAD and T2D

PON1 has been analyzed in current literature as having associations with CAD and T2D, however it has been reported that variations in PON1 polymorphisms affects PON1 activity levels. This means that having a certain polymorphism or a combination of

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polymorphisms would either increase or decrease PON1's protective capabilities. There have been many genotyping studies but this investigation is among one of the first studies investigating a Mississippi or a Southern population. The others were also generated from this laboratory [15, 16]. This study investigates PON1 and its common polymorphisms (L55M and Q192R) with the occurrence of T2D and CAD within a population that is mainly African American and Caucasian.

4.3 Addressing the objectives

In this present study we address the objectives outlined in the text earlier: 1) examine the relationship between both PON1 L55M and PON1 Q192R associations with CAD and T2D; 2) clarify the association of PON1 polymorphisms with CAD and T2D, also considering race; and 3) identify, if possible, a potential biomarker for preventative measures for at-risk populations.

4.3.1 Relationship between PON1 SNPs

We examined the relationship between PON1 L55M and PON1 Q192R using Fisher's Exact Test. As in some other studies [59], all possible combinations of the Q192R and L55M were observed. In agreement with other studies[13], we found that LL (50%) genotype was the most frequently occurring genotype and MM (8%) was the least frequently occurring genotype. The most frequent combined genotype was LLQR (23%), LMQR (20.86%) and the least were MMRR and MMQR both occurring at < 1% of the population. This confirms previous studies that found MM to be a rare genotype[11, 59].

In regards to the occurrence of Q192R genotypes versus the likelihood of the occurrence of L55M genotypes, our data show that QQ is most likely to be paired with LM genotype even though LM did not yield any significant associations (Table 4). Tables

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1 and 2 show that the QQ genotype is not significantly associated with either of the homozygous forms of L55M. The significant association that was observed is between the RR and LL genotypes. This significant association suggests that if the RR genotype occurs at position 192 there is a more likely chance of LL occurring at the 55 position. Although the combined genotype LLQR is the most frequently occurring combined genotype in this population, a majority of all of the other subjects being positive for the LL genotype are also positive for the RR genotype in this population, which is in agreement with the above finding.

4.3.2 Relationship between PON1 SNPs and Race

This study reports significant associations with PON1 SNPs and race. Only four individual genotypes (Table 6) proved to be statistically significant with race with the LL and RR genotypes being associated with African Americans and the QR and LM genotypes being significantly associated with Caucasians. Our study agrees with the results of Davis et al., who reported that African Americans have a greater chance of being RR than did Caucasians [15]. Carriers of the R allele have been known to be at increased risk of vascular complications[60], however this study did not report allele frequencies.

Even though there was no significant association found within this population with the MM genotype, said genotype has been found in many other studies that have subject populations of color, Brazilian[6], Mexicans[13] and even North-West Indians[61]. This study found that the majority of the MM genotypes were found in Caucasians, though this could be due to the genetic complexity of a southern population.

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4.3.3 Relationship between PON1 SNPs and CAD and T2D

In contrast with some studies [61, 62]and in accord with others[6, 10, 63], some of which were based on PON1 activity levels, this study found no significant associations between PON1 L55M and PON1 Q192R with CAD or T2D. There were however one combined genotype (LMQQ) and one individual genotype (LM) that showed a trending towards a significant association with T2D, meaning subjects with these genotypes may be at greater risk for T2D; more subjects are needed to verify this. In contrast with Altuner et al. [64], Q192R was not a strong predictor for either disease though L55M may have been a predictor for T2D with an increased sample size.

4.4 Conclusion

In conclusion, several factors, including sample size, interracial population, or there not being a biomarker to be developed could account for inconsistencies within this study as well as other studies reported in PON1 research, whether it be genetics based, activity based, or a combination of both. Therefore, to elucidate PON1s relationship with these diseases, as well as other diseases, future studies have to provide a clear connection. Increased sample size and statistical models which incorporated demographic and clinical variables might more effectively detect associations between disease occurrence and PON1 genetics.

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