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## The Impact of Organochlorine Pesticides and Lipid Biomarkers on Type 2 Diabetes Mellitus

Paul Robert Eden

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THE IMPACT OF ORGANOCHLORINE PESTICIDES AND LIPID BIOMARKERS  
ON TYPE 2 DIABETES MELLITUS

By

Paul Robert Eden, Jr.

A Dissertation  
Submitted to the Faculty of  
Mississippi State University  
in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy  
in Environmental Toxicology  
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2012

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THE IMPACT OF ORGANOCHLORINE PESTICIDES AND LIPID BIOMARKERS  
ON TYPE 2 DIABETES MELLITUS

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Type 2 diabetes mellitus (T2DM) is classified as a metabolic disorder characterized by hyperglycemia that results from defects in insulin action and/or secretion, and currently affects 8.3% of the US population according to the CDC's 2011 National Diabetes Fact Sheet. Several contributing factors have been identified to development of this disease. Published evidence indicates type 2 diabetes mellitus (T2DM) patients display lower overall paraoxonase activity and that this may be partially due to genetic variations in the paraoxonase-1 (PON-1) gene. Some bioaccumulative organochlorine (OC) pesticides have been shown to contribute to increased T2DM prevalence. In addition, these OC compound levels have been associated with alterations in adipocyte cytokine levels as well as increased inflammatory markers.

Three hundred blood samples with clinical and demographic information were obtained from two US Air Force hospitals. A total of 151 non-diabetics and 149 T2DM subjects were evaluated for PON-1 activity, PON-1 Q192R and L55M genetic polymorphisms, OC compound concentrations, inflammatory marker levels and adipokine concentrations. PON-1 activity, using diazoxon as the substrate, was decreased

in the T2DM subjects. Some of the PON-1 genetic polymorphisms tested were also associated with decreased PON-1 activity. OC compound levels were increased in the T2DM subjects. The non-diabetic subjects possessing elevated DDE and trans-nonachlor were associated with increased inflammation, a common hallmark of early T2DM development. Additionally, elevated OC levels were seen in association with altered adipokine concentrations.

Overall, a decrease in the antioxidant properties of PON-1 as well as factors contributing to chronic low level inflammation such as elevated OC plasma concentration appear to be significant contributors to T2DM prevalence in the population studied.

## DEDICATION

I wish to dedicate this research to my wife Tori, for her patience and support in my pursuit of my graduate education.

## ACKNOWLEDGEMENTS

I want to first thank Dr. Janice Chambers. Throughout this entire process you have been supportive of my achieving this goal as well as helping guide me when my investigation hit some speed bumps. Your guidance was truly an inspiration and without your leadership I would never have achieved this lifelong goal. I would also like to thank my other committee members; Dr. Allan Crow, Dr. Matthew Ross, Dr. Howard Chambers, and Lt Col (Dr.) Eric Olsen. Each of you helped me at different stages of my project to keep me on track and to look outside the box when necessary. Your insights were critical to the success of my project.

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## CHAPTER I

### INTRODUCTION

As of 2011 there are 25.8 million people in the United States affected with type 2 diabetes mellitus (T2DM) and an additional estimated 79 million people with prediabetes [1]. This accounts for 8.3% of the country's total population and 26.9% of people over the age of 65. It is estimated that 346 million people worldwide currently have either type 1 or type 2 diabetes and the World Health Organization estimates that deaths from diabetes will double between 2005 and 2030 [2]. This dramatic increase has been the focus of intense scrutiny by the medical and research communities. The Centers for Disease Control estimate that the total cost for diabetes in the United States for 2007 was \$174 billion and the Department of Defense spent over \$124 million on 160,386 cases of diabetes in that same year [3].

Several possible associated contributors to T2DM development have been identified with exposure to persistent organic pollutants being one of these. Organochlorine (OC) pesticides such as *p,p'*-DDE and transnonachlor have been linked to increased prevalence of Type 2 Diabetes in epidemiological studies [4-5] and the fact that detectable amounts of OCs are found in the majority of healthy populations [6] lend relevance to this possibility.

Paraoxonase-1 (PON-1), an HDL-associated lactonase, has been shown to afford protective effects against atherosclerosis [7] and demonstrates lower activity in type 2 diabetics compared to non-diabetics [8]. Polymorphisms in both coding and promoter

regions of PON-1 have produced variations of enzyme activity in regards to oxidation of low density lipoproteins (LDL) [9].

Several adipokines have been identified in adipose tissue recently and some have shown to have significant contributions to insulin sensitivity and glucose metabolic regulation. Leptin, a product of the *ob* gene, has been shown to have a blunted effect in type 2 diabetics as well as a role in insulin regulation. Adiponectin is a potent insulin sensitizer as well as having a role in decreasing inflammatory markers. Amylin is generated parallel to insulin in the pancreas and has been demonstrated to have an increased concentration in type 2 diabetics. Overall these adipokines are some of the key adipocyte hormones currently associated with type 2 diabetes.

### **Diabetes Basics**

Diabetes, from the Greek word “siphon”, has been a known disease for hundreds of years. Physicians were unable to do anything to combat this epidemic and therapies varied widely, such as prescribing a diet of jelly of viper’s flesh, broken red coral, and fresh flowers of blind nettles [10]. Up to the early 20<sup>th</sup> century physicians’ only way to identify the disease was to literally taste the urine to see if it was “sweet”. Fortunately, the discovery of insulin as well as the development of more sophisticated glucose testing has enabled providers to bring this disease under some control. The most frequently mentioned diabetic diseases are prediabetes, Type 1 diabetes mellitus, gestational diabetes, diabetes insipidus and type 2 diabetes mellitus.

Prediabetes, also called impaired glucose tolerance, is a condition that is classified by the American Diabetes Association by a fasting blood glucose of >100 mg/dL but less than 125 mg/dL. Approximately 11% of people with prediabetes develop frank type 2

diabetes mellitus within 3 years of identification with prediabetes [11]. A person with prediabetes carries a 1.5x risk of cardiovascular risk compared to a person with normal glucose. In addition, some research has indicated that higher hemoglobin A1c and obesity can increase the likelihood that prediabetics will become frank diabetics and decreased physical activity and increased BMI are associated with conversion from normal fasting glucose to a prediabetic state [12].

Type 1 diabetes mellitus is an autoimmune disorder frequently called insulin-dependent diabetes (IDDM) and reflects a lack of endogenous insulin production. This is a result of “organ-specific immune destruction of the insulin-producing  $\beta$  cells in the islets of Langerhans within the pancreas” [13]. The reasons for the immune destruction are still largely unknown. Some models indicate that environmental factors, high sun exposure as a small child, and genetic predisposition are at work [13]. In regard to the genetic disposition of IDDM development in mouse models it has been shown that the destruction of the  $\beta$  cells can be due to immune regulation dysfunction resulting in increased autoreactive  $CD4^+$  and  $CD8^+$  T cells as well as autoantibody producing B cells [13]. This disease often afflicts those under the age of 18 and requires injections of exogenous insulin throughout the person’s lifetime to maintain glycemic control. Loss of glycemic control can result in acute metabolic conditions such as ketoacidosis as well as chronic complications including cardiovascular events and kidney failure.

Gestational diabetes is a special class of diabetes that is seen in approximately 18% of pregnant women, according to the American Diabetes Association [14]. This equates to 135,000 pregnancies a year in the United States alone [15]. Women who have not previously suffered from T2DM sometimes develop this disease, although it is not fully known why. Some speculate that this may be due to the hormonal changes in a



woman caused by placental growth that interferes with insulin regulation of blood glucose. Because this disease does not have a direct causative factor most women are asked to perform an oral glucose tolerance test, typically around the 24<sup>th</sup> week of pregnancy, in order to identify the disease and treat prior to significant impact on the mother and infant. According to the CDC women who have had gestational diabetes have a roughly 35-60% higher chance of developing type 2 diabetes within the following 10-20 years [1]. A meta-review of 20 studies indicated that women who had gestational diabetes have a risk ratio of 7.43 in developing type 2 diabetes later in life [15].

Diabetes insipidus is a disease classified as an extreme electrolyte imbalance that presents with an overabundance of dilute urine production. The kidneys are unable to retain water, either from a decrease in the release of antidiuretic hormone (ADH) or an inability in the kidneys to respond to released ADH [16]. The symptoms associated with this disease are uncontrollable thirst, dry skin, dehydration and constipation. There are no relationships to other types of diabetes aside from early references to extreme urine output between diabetes mellitus and diabetes insipidus.

Type 2 diabetes mellitus, also called adult-onset diabetes and non-insulin-dependent diabetes mellitus, is classified as a metabolic disorder demonstrating an impairment in carbohydrate metabolism due to defects in insulin action or production [17]. Classification of an individual with this disease depends largely on the circumstances at the time of diagnosis, including current medications and other possible confounding medical conditions such as hypertension, alcohol consumption, or obesity. This classification reflects a “combination of resistance to insulin action and an inadequate compensatory insulin secretory response” [18]. Hepatic glucose production is generally increased while insulin’s ability to slow this process is retarded. In addition,

glycogen synthesis in muscle is significantly inhibited, thus reducing the ability of the body to effectively remove the excess glucose from the blood stream [19]. Free fatty acid release from adipose tissue additionally reduces insulin's ability to shepherd glucose into cells [20].

Pathogenically, as an individual gains adipose tissue through advancing age, sedentary lifestyle, diet or secondary chronic inflammation the body's insulin sensitivity decreases preventing efficient transport of glucose from the blood and liver. This is not typically discovered early by medical professionals at this stage since the plasma glucose levels are maintained in the normal range due to a compensatory insulin increase [21]. This continues on for months to as long as decades before eventually insulin resistance increases to a point that a steady-state of glucose in the blood stream cannot be maintained and the individual transitions to impaired glucose tolerance status. This moderate hyperglycemia may be observed during typical physical examination laboratory analysis and might catch the notice of the individual's medical provider. Eventually the ability of the pancreatic  $\beta$ -cells to compensate for insulin resistance fails and deterioration and impaired function of the cells' ability to generate insulin occurs. The  $\beta$ -cells ultimately begin to fail and/or insulin cannot be produced by the body in quantities sufficient to metabolize normal glucose intake. This would be considered a vastly simplified case; however the majority of typical T2DM subjects would fall under this scenario.

Approximately 97% of diabetes mellitus patients have T2DM [22]. Most literature indicates a higher prevalence of T2DM in males than in females [1, 22]. In addition, African-Americans are 77% more likely to develop T2DM than non-Hispanic whites, while Hispanics are 66% more likely and Asian Americans are 18% more likely

than non-Hispanic whites to develop T2DM [1]. Residence in an urban location lends itself to development of the disease due to the “western life-style characterized by an unhealthy diet, obesity, and lack of physical activity” [22-24]. While the number of new cases of T2DM in the United States rose from 1.6 million in 2007 to 1.9 million in 2010 it is worth noting significant differences between the ages of the new cases. In 2007 approximately 17.2% of new cases were between the ages of 20-39 while 50% of new cases were between the ages of 40-59 and 32.8% of new cases were ages 60 or higher. In 2010 the percentage of new T2DM cases between the ages of 20-39 has increased to 24.4%, 55.2% of new cases were for ages 40-59 and only 20.4% of new cases were for people 60 or older. This reflects a trend seen in recent articles indicating a younger age of diagnosis of T2DM [25].

Clinical diagnosis of T2DM is typically based on one of three methods. A fasting blood glucose level of  $>125$  mg/dL on two occasions, random blood glucose level of  $>200$  mg/dL with accompanying symptoms of type 2 diabetes mellitus (polyuria, blurred vision, polydipsia, fatigue, weight loss) or a positive oral glucose tolerance test [26]. An oral glucose tolerance test (GTT) consists of a patient fasting for at least 8 hours prior to ingestion of a standard glucose load of 75g, typically by drinking a beverage with the specific glucose content. Blood draws are collected just prior to glucose ingestion and at one and sometimes two hours post loading. A glucose level of  $>140$  mg/dL indicates impaired glucose tolerance while the criteria for T2DM is  $>199$  mg/dL [26] as seen in the figure 1.1 [27].

Thus far there has not been a single causative factor that is conclusively linked to all T2DM development; several contributing factors have been identified as playing a role in the disease process. These contributing factors include but are not restricted to

older age, race, genetics, obesity, pregnancy, autoimmune destruction of pancreatic beta cells, toxic environmental agents, and even a lack of vitamin D [18, 22-23, 28-30].

Passing on of genetic predisposition for T2DM has been seen in 25-50% of cases down familial lines [22]. One study discovered that within close family members such as parents or siblings of diabetics that 10-30% of those family members also suffered from diabetes. This is in stark contrast to the non-diabetic members of this study who had 1-6% of their family members were impacted by diabetes.

Obesity is frequently seen not only as a result of but as a contributing factor of type 2 diabetes mellitus. Insulin hypersecretion has been shown to be a result of obesity and insulin regulates lipogenesis as well as glucose uptake [31]. In addition, higher lipid infiltration in the skeletal muscle, liver, and pancreatic  $\beta$ -cells has been correlated with higher insulin resistance in several studies [32]. Obesity is also frequently associated with metabolic syndrome, a syndrome characterized by obesity, dyslipidemia, hyperglycemia, and hypertension [4]. There are currently no universally accepted guidelines for metabolic syndrome. However, the National Cholesterol Education Program has presented recommended diagnosis guidelines of waist circumference greater than 40 inches for men and 35 for women, triglycerides above 150 mg/dL, HDL less than 40 mg/dL for men and less than 50 mg/dL for women, blood pressure above 130/85 mm Hg and fasting glucose equal to or greater than 100 mg/dL [33]. A patient presenting with at least 3 of the above characteristics classifies that person as having metabolic syndrome. In at least one study it was found that those meeting the guidelines for metabolic syndrome were almost nine times as likely to develop diabetes than those that did not meet the guidelines [34].

A well-established theory relating obesity to T2DM comes from Randle et al [20]. This group proposed that non-esterified fatty acid (NEFA) concentrations affect the glucose/fatty acid cycle [20, 35]. This mechanism, also called Randle's Cycle, stated that NEFA levels "negatively impacted insulin-mediated glucose uptake" [35] as a result becoming a risk factor for T2DM. Essentially as the person's level of adipose tissue rises, the amount of free fatty acids interferes with glucose metabolism and the resultant hyperglycemia contributes to adult-onset diabetes development. Free fatty acid increases in conjunction with increased triglycerides in  $\beta$ -cells have also been shown in animal studies to precede  $\beta$ -cell failure [21].

Exposure to environmental toxicants such as persistent organic pollutants (POPs) has been associated with diabetes development. Organochlorine compounds such as chlordane derivatives as well as DDE, in addition to pesticide contaminants such as dioxin, have been found to be associated to T2DM prevalence [23, 36]. These compounds are found to increase low level chronic inflammation which has been found to interfere with insulin signaling and result in decreased insulin sensitivity to hyperglycemic states [37]. The compounds themselves do not directly affect insulin action; however, their presence increases the inflammation which drives many of the signaling pathways insulin uses to direct glucose into tissues.

A confounding factor for type 2 diabetes mellitus is that this disease can affect so many organs that identifying a single cause can be difficult. Symptoms can include joint pain, kidney dysfunction, vision problems such as glaucoma and cataracts [38], peripheral neuropathy that can result in foot ulcerations and amputations [18], and cardiovascular symptoms including higher incidence of atherosclerosis [39]. This list is by no means comprehensive.

## **Adipocyte involvement in T2DM**

Adipocytes, the fat cells that make up adipose tissue, excrete small amounts of adipocytokines and adipokines; however due to their abundant connections to the systemic circulation and mass in reference to the body mass they can exhibit a significant overall effect on body functions. There are over 25 different adipokines affecting physiological functions such as immune response, homeostasis, and lipid and insulin metabolism [32]. With regard to obesity's impact on T2DM development, the interaction of several adipokines secreted from and interacting with adipose tissue have been found to show significance. These peptides act throughout the body exerting paracrine and endocrine effects. The primary adipokines focused on for this project were adiponectin, leptin, and amylin and these compounds impact, among other things, energy balance, lipid metabolism, and insulin sensitivity [32]. The most abundant adipokine in human adipose tissue is adiponectin, an endogenous insulin sensitizer.

Adiponectin, also called Acrp30, apM1, AdipoQ and GBP28 [40] is a 244-amino acid protein whose gene is located on chromosome 3q26 [41] and shares some structural similarity to tumor necrosis factor alpha. The protein typically circulates either in a high molecular weight or low molecular weight form and it is believed by some groups that the high molecular weight form has a higher association to insulin resistance. This adipokine, the most abundant protein generated by adipocytes, is primarily secreted from white adipose tissue even though it is found at lower levels in most tissues of the body. Normal levels of this compound in the blood are 5-30  $\mu\text{g/mL}$ , or approximately 1000 times as high as the subject's leptin concentrations. A meta-analysis revealed that higher concentrations of adiponectin are associated with lower risk of T2DM [41] and conversely that a low concentration of adiponectin was associated with increased insulin

resistance. In addition, levels of adiponectin have been found to be inversely proportional to adipose in the abdomen.

There are several mechanisms that have been proposed to explain adiponectin's action in regards to T2DM including "suppression of hepatic gluconeogenesis, stimulation of fatty acid oxidation in the liver, stimulation of fatty acid oxidation and glucose uptake in the skeletal muscle, and stimulation of insulin secretion" [42-43]. In addition, adiponectin has also been proposed to activate adenosine monophosphate protein kinase (AMPK) [40]. This mechanism primarily stimulates glucose uptake but has also been seen to decrease gluconeogenesis in the liver, agreeing with previous studies cited. These effects are found to occur via the receptors AdipoR1 and AdipoR2 [44]. AdipoR1 is found throughout the body while AdipoR2 is focused primarily in the liver. Adiponectin's anti-inflammatory impact has been primarily associated with increased nitric oxide production and activation of nitric oxide synthase. A significant consideration regarding adiponectin and inflammation is its relationship to TNF- $\alpha$ . It has been shown that adiponectin and TNF- $\alpha$  regulate each other's synthesis [45], that is, as TNF- $\alpha$  goes up as a result of inflammation, adiponectin production decreases and vice versa. Therefore if chronic low level inflammation is present then adiponectin production, and the anti-inflammatory contributions it makes, will respectively decrease. Adiponectin has also been associated to higher HDL and lower serum triglyceride levels [41], which could be significant in relation to antioxidant HDL proteins such as paraoxonase-1.

Leptin, another key adipokine, regulates food intake and energy expenditure and under normal conditions reduces appetite by activating receptors in the satiety center of the hypothalamus [46]. Leptin is from the Greek word *leptos* and is a 167 amino acid,

16kD cytokine released from adipocytes which signals the body that sufficient energy stores have been released, ceasing the need for additional intake. It is encoded from the *ob* gene and has been called the “starvation signal” [32], lowering sex and growth hormones in response to fasting. Leptin is excreted in quantities directly related to the amount of body fat, an important consideration when discussing obesity’s relationship to T2DM [47]. In studies of mice overexpressing leptin it was found that white and brown adipose tissue completely disappeared [48]. Human hepatocyte studies have shown that leptin impacts several insulin signaling pathways, including “modulation of tyrosine phosphorylation of the insulin receptor substrate-1 and enhancement of insulin’s ability to suppress phosphoenolpyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis” [49]. Liu et al. reported that leptin inhibited glycogen synthesis by up to 45% in muscles of a mouse model [50], which would seem to support this concept. In addition, leptin activates several signal transduction pathways such as Janus kinase signal transducer and activator of transcription 3 (JAK-STAT3) as well as phosphatidylinositol 3-kinase (PI3K), both linked to caloric intake and homeostasis of glucose [47]. Leptin has demonstrated a blunted response in obese people, possibly due to a resistance in this segment of the population. Some studies have attempted to administer leptin as a therapeutic agent, however due to the high levels most obese people maintain the effect is minimal. The mechanism of leptin resistance has not yet been elucidated. There is some conflict between research groups whether there is a direct effect of leptin on insulin secretion. One study indicated that based on an examination between insulin sensitivity and leptin levels that approximately 40% of the variability in leptin concentrations among those with similar BMI was due to insulin sensitivity [51]. In support of this study was a recent examination of 1994 NHANES data by Bandaru and Shanker in which an



association between leptin and increased T2DM prevalence was observed [52]. However, when body mass index was added to the multi-variable model leptin's statistical significance disappeared. Other studies have supported the position that elevated leptin levels have an inhibitory action on insulin sensitivity [50]. Additionally, some research has shown that insulin acts directly at the adipocyte in promoting leptin secretion as well as gene expression [51]. In contrast, there are studies that have asserted that leptin secretion inhibits insulin, mostly at the site of insulin production in the pancreas [53-54]. This information does not necessarily reflect a contradiction and could in fact demonstrate a second way in which hyperleptinemia increases T2DM development.

Leptin has also been examined in recent studies in association to paraoxonase-1 (PON-1) levels as well as other physiological factors that could contribute to metabolic disorders. In vitro and in vivo studies have shown that leptin has a significant inverse correlation with PON-1 activity and that as leptin levels have increased, a decrease of PON-1 activity, in some cases as high as 30%, is noted [42, 55-56].

Amylin, also called islet amyloid polypeptide, is produced in the  $\beta$ -cells of the pancreas simultaneously with insulin. This 37-amino acid molecule is generated as the 89-amino acid proamylin and then post-processing occurs in the pancreas which results in formation of the amylin molecule [57]. The function of amylin primarily is to inhibit gastric release of glucose, slowing the transfer and generation of glucose from the hepatic region. Presumably this is so after a meal there is not too high a spike in glucose for insulin to be able to maintain homeostasis. In cases of increased amylin concentrations cell apoptosis in the pancreatic  $\beta$ -cells increases by causing "toxic amyloid particles" to aggregate in the pancreas, resulting in beta cell defect and resultant insulin secretion degradation [57]. These deposits have been found in up to 90% of type 2 diabetes

mellitus patients. However, there has been a great deal of debate on this issue as some researchers have made the case that instead of these amyloid deposits causing the death of the pancreatic cells that the death of the cells themselves cause the deposits to form.

One amylin study that is frequently cited showed that these amylin particles cause membrane leakage through interaction with ion channels, supporting the argument that amylin can contribute to increased pancreatic  $\beta$ -cell apoptosis [58]. In addition, when applied extracellularly to the pancreatic cells amylin has been demonstrated to initiate several apoptotic pathways such as JNK and p38 kinase [59]. A differing opinion occurred as a result of a mouse study that showed that it was the increase in amyloid area, and not the deposits themselves, that was cytotoxic. [59]. A more recent study in which the researchers administered pharmacological doses of amylin to healthy and T2DM Wistar rats showed that amylin normalized glycogen content in the T2DM cohort. In addition, response to insulin was more pronounced in both the normal and T2DM groups, indicating that amylin may have involvement in glucose transport [60]. There are still a number of issues to fully elucidate in regards to this cytokine.

Additionally, there is evolving work being performed on some minor adipokines such as ghrelin. Ghrelin is a 28 amino acid peptide secreted from the stomach, with peak levels prior to meals, suggesting that it is involved in the stimulation for food intake. According to recent literature ghrelin drives “appetite and food intake, increases body weight, [and] inhibits insulin action and secretion” [40]. This is significant because it promotes weight gain by reducing the use of fat as a metabolic fuel, a key consideration when trying to lose weight or reduce excess free fatty acids in the adipose tissue. Ghrelin is reduced by consumption of glucose or lipids, but not water and is thought to be

reduced by rising leptin levels [61]. This peptide has been linked as an independent factor in type 2 diabetes mellitus, although the mechanism has not yet been fully identified [62].

### **Inflammation and T2DM**

Inflammation has as much to do with type 2 diabetes as it does with hyperglycemia. Some of the contributing factors to development of T2DM such as dyslipidemia and hyperglycemia can cause several inflammatory factors and pathways to either activate or increase in expression. This in turn causes disruptions in insulin signaling pathways as well as increases in adiposity that then contribute to more inflammatory and proinflammatory responses in a vicious cycle. This stress typically results in, among other things, activation of the JNK pathway which in turn activates NF- $\kappa$ B. Research has indicated that monocytes from T2DM patients secrete elevated levels of these pro-inflammatory cytokines and that monocytes from the peripheral blood are already activated [63]. This reinforces theories of inflammation and insulin resistance as a cycle in which higher insulin resistance produces circumstances that lend themselves to increased low-level inflammation and the inflammation then blocks insulin signaling causing insulin resistance and so on.

Some of the most common inflammatory cytokines discussed in regards to T2DM development are monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6) as seen in figure 1.2 [64].

The adipocyte itself is capable of secreting MCP-1 and IL-6 while recruited macrophages secrete TNF- $\alpha$  [65-66]. There are several other inflammatory markers, including several in the interleukin series, which play a role in inflammation, however the three previously mentioned are, in my opinion, the most significant. Most research has

focused on the inflammatory response that is generated as the adipocytes become overfilled with lipid and low-level chronic inflammation results [67] while others have refuted this theory [68], instead implying that increases in inflammatory markers are associated with the increase in fat mass seen in obesity and not insulin sensitivity.

MCP-1, also called MCP-1/CCL2, is a cytokine that is responsible for recruitment and regulation of macrophages in many parts of the body. It is a 13kDa molecule, 76 amino acid cytokine that is found both in adipocytes and endothelial cells [69]. In the case of T2DM this molecule is secreted from adipocytes partially as a result of hypertrophy in the adipocyte. In addition, lipid peroxidation end products such as 4-hydroxy-2-nonenal and other lipid peroxidation products have been shown to directly stimulate MCP-1 production [70]. Interleukin 6 is a 26kDa protein that has broad specificity and inflammatory characteristics that relate to cell differentiation to active forms. This cytokine is generated by several immune response cells including macrophages, B and T cell lymphocytes, and endothelial cells [71]. IL-6 induces cytotoxic T-cell growth and most importantly stimulates many types of cell differentiation, including the differentiation of monocytes to macrophages. IL-6 levels have been shown to spike during hyperglycemic events, common for T2DM patients [72]. In addition, in vivo studies of IL-6 have demonstrated that infusion of this cytokine induces gluconeogenesis and resulted in hyperglycemia [67]. TNF- $\alpha$  is a proinflammatory cytokine that has been identified as having involvement in not only affecting insulin signaling pathways [73] but also helping to upregulate the NF- $\kappa$ B pathway which results in higher production of additional inflammatory mediators such as IL-6, IL-8, IL-1 $\beta$ , and MCP-1 [74].

The increase in TNF- $\alpha$  and IL-6 found in T2DM subjects specifically interferes with insulin signaling pathways through JNK-1-mediated serine phosphorylation, preventing GLUT-4 expression and subsequent glucose uptake by adipocytes [68]. In addition, these two cytokines promote lipolysis and increase FFA in circulation, further increasing glucose production in the liver and the resultant insulin resistance [75]. Conversely, in a mouse study that removed TNF- $\alpha$  there was an improvement in insulin sensitivity [73].

TNF- $\alpha$  has also demonstrated an ability to directly upregulate NF- $\kappa$ B via the IKK- $\beta$  pathway. Excess caloric intake can also cause stress to the endoplasmic reticulum, resulting in additional activation of NF- $\kappa$ B via the IKK- $\beta$  pathway. ER stress as a result of exposure to environmental toxicants, reactive oxygen species, and prostaglandins can also cause activation of NF- $\kappa$ B pathways [73] resulting in increased inflammatory marker expression. An example of some of the molecular pathways seen in this inflammation is seen in figure 1.3 [73].

One question that has been raised regarding T2DM and inflammation and has not been fully answered is whether the inflammation causes the insulin resistance and resultant T2DM or if the hyperglycemic state causes inflammation. Lipotoxicity also presents its own set of questions in regard to increase in cellular inflammation and its contribution to insulin resistance. Research seems to indicate that it is the excess of caloric intake that causes fundamental changes in monocyte/macrophage functions, bringing about the elevation of some of the previously mentioned cytokines [63].

## **Organochlorine Pesticides**

As obesity has been related to T2DM in several studies, the presence of lipophilic environmental toxicants in the body has frequently been considered a possible source for the increasing development of systemic diseases, including T2DM. In this respect organochlorine (OC) pesticides are ideal candidates. These compounds have a high resistance to degradation and high lipophilicity [76] which make them ideal for bioaccumulation in a normal person's adipose tissue. These chemicals were employed in agriculture and insect control from the 1940s to the 1970s. Due to ecological considerations and later chronic human toxicity concerns these chemicals have been banned in many countries for the past 30 years [77]. However, use of these pesticides outside of the US is still not tightly controlled [37] and some are still employed in vector control. A fairly recent study of soil concentrations in some agriculture-heavy US states demonstrated significant measurable level of multiple OC compounds, demonstrating that their presence remains even decades after the end of use of these compounds [78].

Organochlorine pesticides include chlorinated diphenylethane derivatives such as DDT and its analogues; the cyclodienes chlordane, aldrin, dieldrin, heptachlor, endrin; hexachlorocyclohexanes such as lindane; and others such as mirex, toxaphene and chlordecone [77]. These compounds were mostly introduced in the 1940's and maintain an environmental presence either through runoff after administration, wastes from factories manufacturing these compounds, or from landfills where contaminated wastes were deposited [79]. Based on a review of the scientific literature three of the most common compounds that have been associated with increased prevalence of T2DM are the DDT derivative DDE, transnonachlor and oxychlordane.

The compound *p,p'*-DDE is a breakdown product of 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl) ethane (DDT) [80]. DDT was originally created sometime in the 19<sup>th</sup> century, however it was pioneered primarily by Paul Muller, a researcher at J R Geigy pharmaceuticals [81]. He and his team discovered the extremely potent insecticidal ability of DDT, earning him the Nobel Prize. The compound was quickly employed to remove malarial parasites in many parts of the world as well as a broad insecticidal compound in most parts of the world. It was determined, however, over time, that this compound did not break down to inert byproducts but instead remained almost intact in the soil for as long as decades. This resulted in the eventual banning of DDT products in the US in 1972. DDT, DDE and other breakdown products of DDT have been found in all parts of the world, including the Arctic, Antarctic, and all parts in between [80]. The chemical structure of DDE is found in figure 1.4 [82].

Chlordane was an organochlorine pesticide used in the US until the 1980's as an insecticide that contained over 120 separate compounds [83]. Among the most common components of chlordane is *trans*-nonachlor, a constituent that has been shown to maintain a half-life of approximately 10-20 years. Oxychlordane is a minor component of chlordane, also possessing the longer half-life. Both of these products have been identified in multiple studies as having an association to increased T2DM incidence [36, 84]. The chemical structure of each is found in figures 1.5 and 1.6 [82].

An event that has merited particular scrutiny in regard to OC involvement with metabolism is the Vietnam War program Operation Ranch Hand. This program involved US Air Force personnel spraying the jungles of Vietnam and Laos with over 11 million gallons of defoliant and herbicides, primarily Agent Orange, between 1962 and 1971 in order to defoliate parts of the jungle terrain. Agent Orange consists of a 1:1 mixture of

2,4 dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) [85]. A key component discovered in analysis of Agent Orange is dioxin. Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD) is not an herbicide itself, but rather a contaminant of 2,4,5-T that occurs when the herbicide is heated above 160°C during the manufacturing process. At the end of the war the US government commissioned the Air Force Health Study to examine the soldiers exposed to these defoliants and determine if there were any significant health effects [86]. One of the interim conclusions of this study has been an association between serum dioxin levels and increased T2DM prevalence. Several researchers have examined the relationship between T2DM and dioxin among military veterans [23, 85-88]. In 2006 a research team examined the molecular basis for increased T2DM in relation to dioxin exposure and discovered that an increase in NF-κB expression was seen in relation to increased dioxin body burden [89]. This increased expression of a key proinflammatory regulator demonstrated that as inflammatory markers increase in concentration there is a resultant reduction in insulin sensitivity as previously described. A second study of interest involves an explosion at a chemical plant in Seveso, Italy, in 1976. This explosion resulted in contamination of TCDD in a large section of northern Italy with the resultant observational studies indicating an increase in T2DM within the population in relation to serum dioxin levels [90-91].

The ubiquitous presence of OC compounds in soil and water around the world has resulted in measurable levels of some of these compounds in a significant majority of people, both those with T2DM and healthy alike. Park et al. found trans-nonachlor and *p,p'*-DDE in 100% of a 100 person study of patients with metabolic syndrome in South Korea [92]. Monitoring studies of healthy individuals by Charlier and Plomteux noted over 82% of a 251-person study contained measurable levels of DDT or HCB [93] while



Jakszyn et al. noted detectable *p,p'*-DDE in 98% of a 953 healthy subject study of 5 regions of Spain [6]. These observations are significant as the trend of increasing T2DM seen over the past several decades coincides with the introduction of these compounds to the environment. It is overzealous to lay the progression of this disease solely at the doorstep of these compounds; however it is at least suggestive that their presence in human blood could contribute to development of this systemic disease.

The mechanism for organochlorine pesticide damage to insulin regulation and stimulation has not been identified explicitly as there are several organ and molecular systems involved and thus many possible causes of the damage to the endocrine system. In one study Sprague-Dawley rats were exposed to lipophilic persistent organic pollutants (POPs) for 28 days via various diets including high fat and high POP levels. In the high fat diet it was found that the POPs “exacerbated the deleterious metabolic effects...indicating that the presence of environmental organic contaminants may influence the outcomes of food and dietary products” [37]. The POPs used were mixtures that the researcher identified as being equivalent to those found in crude salmon oil. The POP mixtures for organochlorines utilized a mixture of hexachlorobenzene, cis-chlordane, beta-endosulfan, trans-nonachlor, and toxaphene [37]. This study theorized that POPs may activate pregnane X receptors or other nuclear receptors which in turn “induce the regulation of genes involved in the inflammatory pathway, mitochondrial function, lipid oxidation, and lipogenesis” [37].

Research into the association between a person’s body burden of OC and T2DM has become a source of interest for several labs in the past several years [5, 36, 94-96]. One of the earliest studies that indicated a possible association between T2DM and OC exposure was by Morgan et al. who looked at morbidity and mortality in occupational

exposure between 1971-1977 [94]. This study could not make a definitive association but pointed towards cardiovascular disease and diabetes as possibly having an association to occupational OC exposure. Some studies have associated increased prevalence of prediabetes in relation to T2DM exposure [97].

Increases in adiposity are frequently linked to increased T2DM prevalence. It is commonly seen in obese T2DM subjects that their disease decreases in severity or even goes away in the case of significant weight loss. Some labs have shown that increased OC concentrations in the blood are associated to increased adiposity [98-99], a key component in both T2DM and metabolic syndrome development. A study by one of the leading groups in this field showed that while elevated OC compounds are associated with increased incidence of T2DM that obesity was only weakly associated with diabetes [36]. One novel study examined blood levels of OC compounds before and after drastic adipose reduction seen in bariatric surgery. This study found that at one year post surgery for morbidly obese individuals that the blood levels of OC compounds went up 388%, [100] a significant indication of the lipophilic nature of these chemicals. While there have been findings that associate some OC compounds with T2DM development other studies have shown that while polychlorinated biphenyl (PCB) compounds are associated with increased adiposity that *p,p'*-DDE was not [101]. This may show that the relationship between OC levels and T2DM is more complicated than simple obesogenic properties.

Increases in adiposity also raise the question as to how these lipophilic compounds impact the adipose once they are sequestered from the blood. This concept is fairly new and has generated a great deal of excitement in evolving theories of T2DM development. A recent article demonstrated that exposure of adipocytes to DDE as well as other OC compounds produced increases in leptin and adiponectin while also

increasing fatty acid uptake when exposed over time [102]. These actions can contribute to hypertrophy of the adipocytes and the previously discussed inflammatory response as a result.

Upregulation of inflammation pathways has been considered to be one of the primary ways that OC compounds contribute to T2DM development. In vitro studies of human serum and DDT demonstrated that this OC compound activated the complement system as well as inducing TNF- $\alpha$  and nitric oxide in macrophages [103-104]. These actions add to oxidative stress in the endoplasmic reticulum as well as activation of the NF- $\kappa$ B pathway, both of which contribute significantly to low-level chronic inflammation seen in T2DM development. In addition to this contribution to oxidative stress some OC compounds lower defenses against selected pathogens at low levels [105].

A study that has been used extensively in examining the association between OC compound levels and T2DM is the National Health and Nutrition Examination Survey (NHANES). This is a US Government survey designed to look at the nutritional status of children and adults as well as examining overall health in the population in order to identify broad epidemiological issues. This program began in the 1960's and has led to several reviews of the data regarding OC compounds and T2DM association. Lee et al. have reported from the 2002 NHANES data that trans-nonachlor and oxychlorane were positively associated with an increased homeostasis model assessment of insulin resistance (HOMA-IR) and risk of T2DM [4, 106]. This study also stated that those subjects with low persistent organic pollutants had the lowest prevalence of T2DM, even with a body mass index (BMI) above 30 kg/m<sup>2</sup>. There are conflicting reports whether polychlorinated biphenyls (PCBs) are linked to type 2 diabetes mellitus with some

advocating an impact on T2DM development [95] and others indicating that there is no association between T2DM and these compounds [96].

### **Paraoxonase-1 and Diabetes**

Obesity's association with incidence of type 2 diabetes mellitus has frequently been linked through and to hyperlipidemia [107]. PON-1, an-HDL-associated lactonase, has been shown in several studies to provide antioxidant properties that inhibit LDL lipid peroxidation frequently found in atherosclerosis [108-112]. In addition, it is this lipid peroxidation that contributes to low-level chronic inflammation seen in development of T2DM.

The paraoxonase family of enzymes was initially identified by Dr. Abraham Mazur, who while studying hydrolysis of organophosphorous compounds, found that various human and rabbit tissues hydrolyzed diisopropyl fluorophosphate [113]. Aldridge further investigated this phenomenon, noting that a division of the esterases existed. One category, the B-esterases, were inhibited by interaction with substrates while the other, A-esterases, were capable of hydrolyzing the substrates [114]. Paraoxonase was further classified into PON-1 and two related enzymes PON-2 and PON-3 [115]. The three paraoxonase genes are located adjacent to each other on the human chromosome 7 [116] and share approximately 70% similarity at the nucleotide level. However, only PON-1 has been demonstrated to have the ability to hydrolyze organophosphates such as paraoxon [117].

The PON-1 gene has been mapped to human chromosome 7q21-22, proximal to the gene for cystic fibrosis and encoding for a 43-45kDa protein [118-120]. Harel et al. were the first to determine the structure of PON-1 through x-ray crystallography [121].

This determination revealed a “six-bladed  $\beta$ -propeller” with each blade containing 4 strands. This examination also revealed that PON-1 is anchored to HDL particles via an “aromatic belt” consisting in tryptophan and tyrosine side chains [121] and, more importantly, that PON-1 is anchored on HDL via ApoA-I [122]. This attachment to HDL is very tight ( $K_D < 1\text{nmol/l}$ ), which is important as in vitro studies indicate that the Apo-A-I-bound PON-1 has a much higher lactonase activity than the free molecules [122]. In addition, some single nucleotide variations in PON-1 have a higher affinity for HDL than others, leading to increased lactonase ability and stability [123]. PON-1 is generated primarily in the liver, the tissue containing the highest PON-1 expression, although reverse transcriptase PCR of other tissues (muscle, brain, adipose, hyaline cartilage) in mice reflect PON-1 mRNA [124]. As most HDL is synthesized in the liver as well this would lend credence to this assumption.

Paraoxonase-2 (PON-2) is a protein in the same family as PON-1; however it has subtly different properties. The protein itself possesses a mass of approximately 44kDa and is expressed in most tissues, including macrophages [116]. PON-2 has been shown in research to possess some antioxidant capabilities and even a low level of prevention of LDL oxidation such as seen in PON-1, although on a significantly smaller scale. A 2010 study that indicated PON-2 plays a role in preventing macrophage assimilation of triglycerides as well as retarding macrophage oxidative stress in hyperglycemic conditions [125]. PON-2 protein is “undetectable in HDL, LDL or cell supernatants by Western blot but appears to remain intracellular, associated with plasma membrane” [116].

Paraoxonase 3 (PON-3) is, like PON-1, associated with HDL. This 40kDa protein is expressed primarily from the liver and has been associated to LDL oxidation [116].

Research has indicated a much higher activity of PON-3 than PON-1, in some cases as high as 100 times higher, in preventing LDL oxidation [110]. In addition, PON-3 expression has been shown to reduce adiposity and some of the effects of atherosclerosis [126]. However, it is found in much lower circulating levels than PON-1 and has been the least characterized due to its inability to hydrolyze paraoxon and phenyl acetate, a standard test of activity of the PON-1 molecule [127].

Several studies performed throughout the 1960's and 1970's showed bi- and in some cases tri-modal activity levels of PON-1 [128]. These activity variations were studied by many labs in order to correctly identify PON polymorphisms [129]. The use of a two substrate analysis was pioneered by La Du et al. [130]. A comparison of paraoxon hydrolysis versus that of phenyl acetate separated low from high PON-1 rates [131]. However, there was a middle ground not fully explained by the separation of homozygotes for this polymorphism. Use of a diazoxon/ paraoxon ratio was better at clearing up the three phenotypical variations of the PON-1<sub>192</sub> polymorphism [131] (homozygous high, heterozygous, homozygous low). It is important to note that PON activity as opposed to concentration was examined mainly because initial studies using PON concentration indicated that it was activity, not concentration that was associated with disease states [8]. The assays used substrates that PON-1 hydrolyzed and measured concentrations of end products in order to determine activity. The reaction for the three previously mentioned paraoxonase activity assays are seen in figure 1.7 [132]:

Both the parathion and diazinon assays produce measurable concentrations of p-nitrophenol and 2-isopropyl-6-methyl-4-hydroxypyrimidine (IMHP), respectively, while the phenyl acetate assay produces phenol as its end product.

Over 160 genetic single nucleotide polymorphisms (SNP) for PON-1 have been identified thus far, some in the coding region and some in the promoter region [133]. These polymorphisms have been shown to be responsible for varying levels of activity in hydrolyzing certain pesticides or LDL oxidation. Adkins et al. and Humbert et al. identified one of the most prominent, a single base pair polymorphism at the “amino acid residue 192 of the 355 amino acid protein” [119, 134-135]. This represents a single amino acid difference between glutamine (Q) to arginine (R) [136]. The (R) phenotype hydrolyzed paraoxon more rapidly while the (Q) phenotype hydrolyzed the substrate at a slower rate. In contrast the (Q) phenotype hydrolyzes sarin and soman more rapidly than the (R) phenotype [133]. Diazoxon, another organophosphate, demonstrated slower hydrolysis for the (R) phenotype than the (Q) phenotype and so a comparison of the two substrates was able to demonstrate the separation of the three phenotypes (QQ, RR, and QR) [134].

Another common coding polymorphism seen with paraoxonase-1 is the Met-Leu<sub>55</sub> polymorphism. The PON-1<sub>55</sub> SNP demonstrates a variation between a methionine (M) and a leucine (L). This additional coding SNP has been linked to LDL-associated oxidation [137] and has been shown to predict cardiovascular disease in “multiple studies including one where the PON-1<sub>192</sub> polymorphism did not predict disease” occurrence [138]. However, there have been other studies that have disputed the theory of the PON-1<sub>L55</sub> genotype being associated with cardiovascular disease [139]. Both PON-1 coding polymorphisms have been shown to have “strong linkage disequilibrium” in several populations [139-140] and so have value in co-analysis scenarios. Other polymorphisms, particularly in the promoter regions of the PON-1 gene have also been identified by previous studies. The most commonly mentioned of these, PON-1<sub>-108</sub>, PON-1<sub>-909</sub> and

PON-1<sub>1741</sub> have been suggested to be associated with changes in PON-1 serum concentration or activity [124, 141].

The measured PON-1 activity can help determine some of the gene polymorphisms as well as determine if the disease state is affecting the protein activity. There are several modulating factors for PON-1 activity that could have influence on PON-1's role in T2DM. Several antibiotics cause a dose-dependent decrease in PON-1 activity including Rifamycin, ampicillin, ciprofloxacin, and clindamycin phosphate [142]. Subjects taking aspirin demonstrated an increase in PON-1 activity, although this could be due to the anti-inflammatory properties of aspirin preventing retardation of PON-1 activity [133]. Lifestyle factors such as smoking reduce PON-1 activity, while alcohol demonstrates an increase with moderate consumption and a reduction in PON-1 activity with heavy drinking. Antioxidants have been shown to increase PON-1 activity [133], however this may be due to the antioxidants protecting PON-1 from inactivation caused by oxidative stress. Additionally, older age and male gender have both been identified as factors that typically show lower enzyme activity levels. PON-1 is not susceptible to classical induction as seen with phenobarbital or other Cytochrome P450 (CYP) enzyme inducers.

One additional factor that may contribute to low PON-1 activity in association with T2DM is glucose inhibition of PON-1 itself. Recent research has revealed that incubation of serum with high glucose concentrations resulted in a reduction in PON-1 activity [143-144]. This is particularly relevant when discussing hyperglycemic disorders such as T2DM. Some research has indicated that PON-1 expression is upregulated as a result of this glycation, possibly as a compensation to decreased PON-1 activity [143].



Decreased PON-1 expression has been associated with type 2 diabetes mellitus patients in multiple studies [109, 145-147]. However, as T2DM impacts multiple organ systems identifying a single role of PON-1 is elusive. Hofer et al. found that while overall there was an association between the PON-1 192 genotype and diabetes that the PON-1 55 genotype influenced urinary albumin loss [145]. Abbott et al. has stated that peripheral neuropathy associated with T2DM has a specific role in suppressing PON-1 activity and further that the structure of HDL in diabetic patients is “compositionally abnormal” and may affect paraoxonase binding to HDL [8]. This research implies that the neuropathy does not directly impact suppression of PON-1 activity but possibly reduces the body’s capacity to detoxify lipid peroxides, thus leading to interference with PON-1 activity.

Oxidative stress is another contributing factor for T2DM development that has been previously discussed. However, PON-1 displays a significant contribution in reducing oxidative stress. PON-1’s primary role in relation to antioxidation is to prevent LDL oxidation which can lead to oxidative stress increases. Oxidation of LDL particles is a primary contributor to their being “scavenged” by monocytes, resulting in foam cells that accumulate below the epithelial lining of blood vessels. This contributes to oxidative stress through reactive oxygen species formation and lipotoxicity. These oxidative stress increases contribute to low-level inflammation frequently seen in T2DM. Additionally, hyperglycemia directly induces oxidative stress [148], so under low PON-1 activity circumstances there is additional oxidative stress from the decreased protection. Also, PON-1 has been associated with increased lipid hydroperoxide levels, an oxidative stress measure [149]. Interestingly, some research has shown that oxidative stress actually inactivates PON-1’s antioxidant effects [150], but that PON-2 increases in antioxidant capabilities.

Inflammation is intimately linked to oxidative stress, and these pathways are also closely linked to T2DM development as previously discussed. PON-1, in addition to preventing LDL oxidation commonly seen in the formation of foam cells and resultant increases in oxidative stress, inhibits monocyte to macrophage differentiation via a peroxidase-like function of the molecule. [151]. Additionally, Mackness et al. found that hepatic PON-1 inhibits endothelial cell MCP-1 production, MCP-1 being a key pro-inflammatory cytokine [152]. An in vitro study looking at  $\beta$ -cell involvement with PON-1 observed an increase in  $\beta$ -cell production of insulin when increased concentrations of PON-1 were added [148]. This study also identified that the surface sulfhydryl group on PON-1 was necessary for this additive effect and that association to HDL and PON-1's catalytic activity were not significant factors to  $\beta$ -cell stimulation. The authors speculate that there may be some involvement with PON-1 and insulin biosynthesis, which if true would certainly be an additional link between T2DM and PON-1.

The questions raised by T2DM become more numerous every day. Each published experiment brings the diabetes community closer to fully elucidating the nature of this multi-systemic disorder. My pursuits in this regard have focused on 4 aspects of the disease progression. The first is an examination of organochlorine compounds found in a military population of non-diabetic and T2DM subjects. This was followed by testing of common inflammatory markers in the non-diabetic subjects. In addition, I performed enzyme measurements of three of the most commonly discussed adipokines in order to examine their relevance to T2DM development as well as possibly identify an association between concentrations and that of plasma OC compounds. Finally, I examined one of the anti-inflammatory HDL markers primarily associated with T2DM development,

paraoxonase-1. I analyzed enzymatic activity using a variety of substrates as well as an examination of genetic polymorphisms believed to have influence on PON-1 activity.

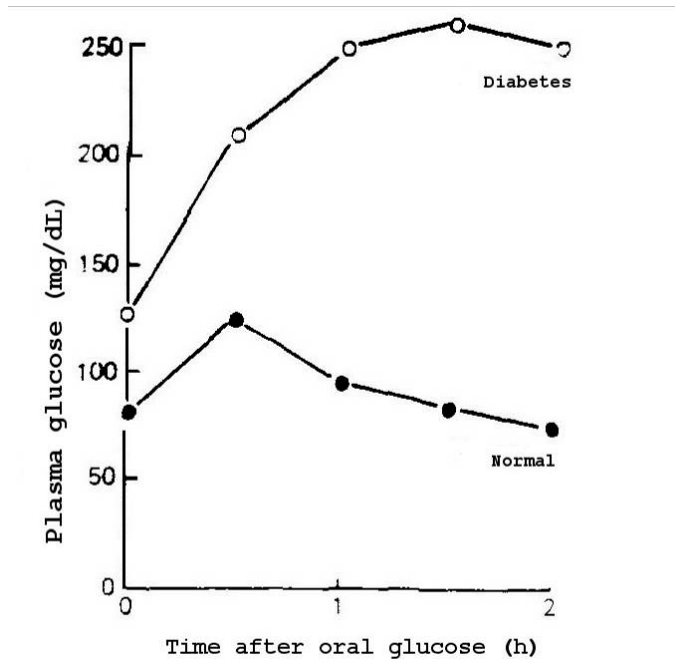


Figure 1.1 Glucose tolerance test for type 2 diabetes and non-diabetic subjects. [27]

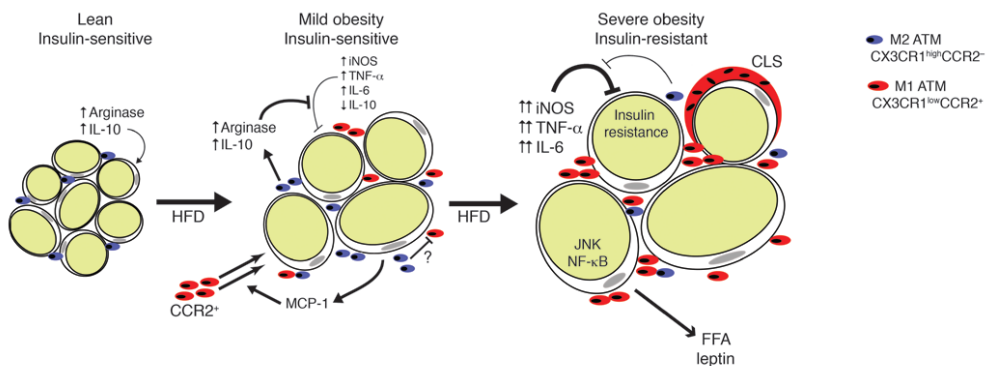


Figure 1.2 Adipocyte-derived inflammatory markers. [64]

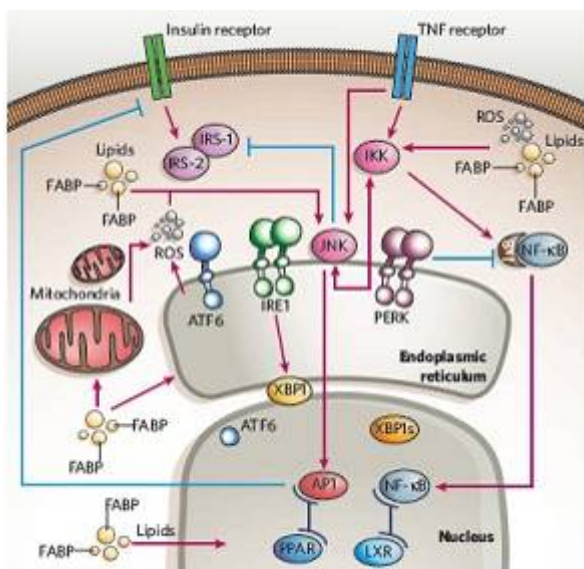


Figure 1.3 Molecular pathway of inflammation [73]

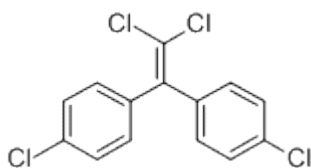


Figure 1.4 Organochlorine pesticides: structure of DDE. [82]

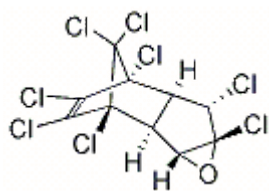


Figure 1.5 Organochlorine pesticides: structure of Oxychlorthane [82]

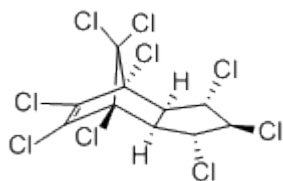


Figure 1.6 Organochlorine pesticides: structure of Transnonochlor [82]

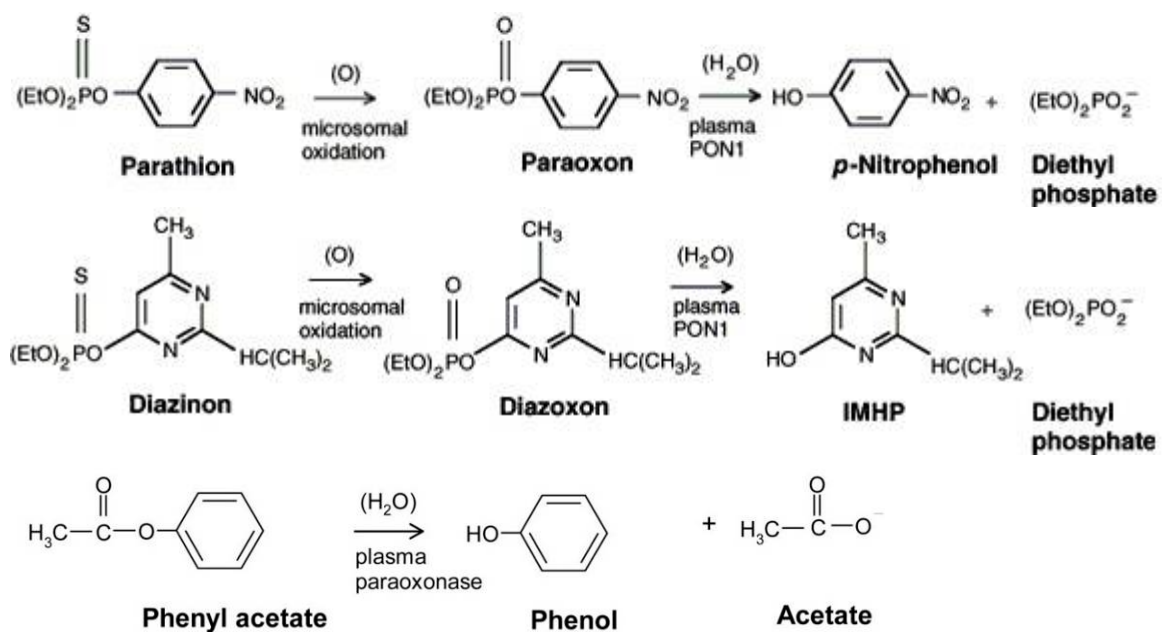


Figure 1.7 Structures of paraoxonase-1 substrates [132]

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CHAPTER II  
ASSOCIATION OF ORGANOCHLORINE PESTICIDE PLASMA  
CONCENTRATION AND ADIPOKINES WITH PREVALENCE  
OF TYPE 2 DIABETES MELLITUS

**Introduction**

Incidence of type 2 diabetes mellitus (T2DM) in the United States has been significantly increasing over the past several decades (Cowie et al., 2009). The Centers for Disease Control and Prevention (CDC) estimated in 2010 that 8.3% of the US population, or 25.8 million people, are currently diagnosed with T2DM with an additional 79 million people estimated to have prediabetes (CDC, 2011). The contributing factors to T2DM development include obesity, age, dietary glycemic intake, and sedentary lifestyle. According to the CDC as of 2009 about 26.7% of the US adult population were reported as being obese and researchers have stated that 60-90% of T2DM cases are related to obesity or weight gain (Anderson et al., 2003). The specific cause of the disease has been elusive due to the multi-factorial etiology as well as contributing factors to disease development ranging from environmental toxicants to genetic susceptibility to geographical residence (Adeghate et al., 2006).

Initial linkages between OC compounds and metabolic process abnormalities were provided by the Air Force Health Study. This study focused on 2,3,7,8-tetrachlorodibenzodioxin (TCDD) that occurred in the herbicide Agent Orange used

extensively during the Vietnam War (Longnecker and Michalek, 2000) and was subsequently linked to an association with T2DM development.

Organochlorine pesticides (OC) include DDT and its metabolites, cyclodienes including chlordane and its derivatives, and hexachlorocyclohexanes such as lindane. Organochlorine pesticides, found to be highly bioaccumulative and lipophilic (Ennaceur et al., 2008), were predominately used up until the 1970's in the US and still maintain a background presence in the soil of many agricultural areas both in and out of the United States (Bidleman and Leone, 2003; Jiang et al., 2009). This previous heavy usage and persistence contributes to the possibility of bioaccumulation either by workers or on the food crops that are grown there. While most OC pesticides have been banned in the United States for over 30 years, use of these pesticides outside of the US is still not tightly controlled (Ruzzin et al., 2010) and some are still employed in vector control. These compounds have been linked to an increase of lipid dysfunction and resultant development of metabolic disorders such as T2DM (Rignell-Hydbom et al., 2007; Ukropec et al., 2010). Turyk et al. (2009) performed a long-term incidence study of OC body burden in comparison to diabetes development and over the course of 20 years those with a higher body burden of 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE) had a significantly higher rate of T2DM. Some studies have demonstrated that these compounds are present in a very high percentage of the non-diabetic population as well, with this segment of the population displaying a higher level of prediabetes in those with higher body burdens of OC compounds (Ukropec et al., 2010). Additionally, Lee et al. (2006, 2007) have done extensive analysis on the National Health and Nutrition Examination Survey results on plasma levels of OC compounds and have identified increased levels of OC pesticides with metabolic regulation dysfunction.

Several adipokines, particularly adiponectin and leptin, play important roles in metabolic regulation (Arora and Arora, 2008; Gnacinska et al., 2010). These adipokines have been shown to alter insulin sensitivity, energy metabolism, and lipid regulation. In addition, adipocyte dysfunction causes an increase in inflammatory processes and a resultant increase in several inflammatory cytokines (Shoelson et al., 2006). Leptin is a 16kD adipokine encoded from the *ob* gene that regulates food intake and energy expenditure and under normal conditions reduces appetite by activating receptors in the satiety center of the hypothalamus (Arora and Arora, 2008). It has also been called the “starvation signal” (Ronti et al., 2006), lowering sex and growth hormones in response to fasting. Human hepatocyte studies have demonstrated that increased leptin concentrations impact several insulin signaling pathways, including tyrosine modulation of the insulin receptor substrate-1 (IRS-1) pathway and enhancement of insulin suppression of phosphoenolpyruvate carboxykinase (Askari et al., 2010). Adiponectin, also called Acrp30, apM1, AdipoQ and GBP28 (Sonnnett et al., 2010) is a 244-amino acid protein whose gene is located on chromosome 3q26 (Ziemke and Mantzoros, 2010) and shares some structural similarity to tumor necrosis factor alpha. This adipokine, the most abundant protein generated by adipocytes, is primarily secreted from white adipose tissue and low levels have been associated with T2DM through several possible mechanisms including stimulation of hepatic fatty acid oxidation, activation of adenosine monophosphate protein kinase and stimulation of insulin secretion (Konscos et al., 2010; Li et al., 2009; Sonnnett et al., 2010).

With the increasing percentage of the US population suffering from T2DM and the potential link between body burden of OC compounds and development of diabetes,

the aim of this study was to study possible associations between T2DM and body burden of OC pesticides within diabetic and non-diabetic populations.

### **Methods and Materials**

Study subjects consisted of 149 T2DM subjects and 151 non-diabetic subjects. All samples were collected at either Keesler AFB Medical Center (Biloxi, MS) or Wright-Patterson AFB Medical Center (Dayton, OH). Pre-existing samples were used for 251 subjects while the additional 49 subjects were enrolled (following their written informed consent). All diabetic subjects were diagnosed either through self-identification at the time of study enrollment or confirmation through electronic medical record ICD-9 codes for T2DM. One hundred eighteen out of the 149 T2DM subjects were on glycemic control medications. Statin and/or lipid-controlling medications were not captured during the data collection process. All study subjects were either Caucasian or African-American and between the ages of 18-65. Subjects from other racial groups or that could not have race identified were excluded from this study. Subjects outside of the age range were excluded in order to prevent enrollment of a very young non-diabetic population and a very old T2DM population. Institutional Review Boards at Keesler AFB, Wright-Patterson AFB, and Mississippi State University approved the study protocol. All information and samples supplied to Mississippi State University for analysis were de-identified. Demographic information is displayed in Table 2.1.

Fasting heparinized blood samples were collected and tested on the day of collection for glucose and total lipids (HDL, LDL, triglycerides, and total cholesterol). The respective hospital laboratories performed the clinical chemistry analysis using Roche Hitachi Cobas 6000 and the Siemens Dimension RxL Max chemistry analyzers.

The testing results from the medical charts were then provided to the study staff after removing all patient information other than the study number. The plasma was separated from the formed elements and all blood samples were frozen at  $<-20^{\circ}\text{C}$ .

Analysis of OC compounds was performed on a gas chromatography/mass spectrometer following organic solvent extraction. The methodology was developed initially by DPX labs (Columbia, SC) for analysis of pesticides in fruit and vegetables and was adapted for our analytical purposes. An internal standard of  $\text{C}^{13}$  *p,p'*-DDT at  $0.01\ \mu\text{g}/\text{mL}$  and  $\text{C}^{13}$  *trans*-nonachlor at  $0.01\ \mu\text{g}/\text{mL}$  in hexane was added to 1mL of subject plasma. Some samples had less than 1mL available; those were processed in the same manner but the volume of plasma was noted and the final concentration for analysis was adjusted to reflect the proper concentration. The sample was then vortexed for 30 seconds followed by 5 minutes of settlement time. Two mL of acetonitrile was added to the specimen to precipitate proteins and other potentially interfering substances. The mixture was vortexed for 1 minute and then centrifuged at 4000 rpm for 11 minutes with the resultant supernatant pipetted into a clean test tube. Two mL deionized water were added to the supernatant and the specimen was vortexed for 30 seconds. The supernatant/water was then aspirated into a disposable pipette extraction (DPX) column for 30 seconds. The DPX column is a solid-phase extraction device that utilizes reverse phase mechanisms to extract and isolate the OC compounds onto sorbent stored in the tip of the column. Using a 10mL syringe the liquid was then forced out of the column and discarded. Wash solution (0.5 mL, 33% acetonitrile/ $\text{H}_2\text{O}$ ) was aspirated into the DPX column followed by a discard of the wash; this step removed additional protein and artifacts from the column. Elution solvent (1mL) consisting of 50/50 ethyl acetate/hexane (EtAc/hex) was then aspirated into the column. After waiting for 10-15 seconds the



eluent was ejected into a clean glass conical tube. This step was performed twice and the eluents combined. The eluent was then blown down under a N<sub>2</sub> stream to achieve dryness. Once dryness was achieved the specimen was resuspended with 100 µL of 50/50 EtAc/hexane and again blown down to dryness under a N<sub>2</sub> stream. The specimen was then resuspended with 100 µL of 50/50 EtAc/hex. If the specimen was less than 1000 µL at the beginning of the procedure, a proportional amount of 50/50 EtAc/hexane was added in the last step. This final amount of solution (100µl) was then placed into a gas chromatography (GC) vial with insert and analyzed using a 2 µL injection volume.

Concentrations of the target compounds were determined by examining the peaks of interest on an Agilent Technologies 6890N gas chromatograph connected to a 5975C triple-axis mass spectrometer. The gas chromatograph separated the compounds and the concentrations of each were measured by the mass spectrometer. Internal standard extraction efficiency was based on percentage of known compound measured in each sample run. This was used to take into account variations in sample size and analyzer/extraction efficiency for each study sample. Areas under the curve were converted to ng/mL utilizing a standard curve of each of the studied OC compounds composed of data points between 1 ng/mL and 500ng/mL. The standard curve was generated based on known quantities of the standards being added to pooled plasma samples that had already been determined to initially contain none of the measured compounds. Total lipids from subject clinical testing were converted to g/L of plasma based on calculations provided by Bergonzi et al. (Bergonzi et al., 2009). The concentrations of the three OC compounds under study were expressed as ng of DDE, *trans*-nonachlor, or oxychlordan per gram of fat. Non-detectable samples were assigned one half the level of quantitation as seen in previous research (Jakszyn et al., 2009)

Inflammatory marker testing was performed on all available non-diabetic subjects; sufficient plasma was available for 129 of the 151 non-diabetic samples. In addition, 61 non-diabetic subjects also had their study plasma tested for insulin using enzyme-linked immunosorbent assay (ELISA). These test panels were performed using the Millipore Milliplex map kit for human metabolic hormone (Cat. No. HMH-34K) on a Luminex 200 microtiter plate reader. Twenty-five microliters of heparinized study plasma or standard was added to the appropriate wells and processed as specified in the assay procedure (pub date 04/14/09). Analysis was performed for a panel including TNF- $\alpha$ , IL-6, glucagon, MCP-1, insulin, and leptin. All results were reported in pg/mL. Results below the limit of detection based on the standard curve were reported as one half of the level of detection.

Adipokine ELISA testing was performed using the Millipore Human Amylin 96-well plate assay (Cat. No. EZHA-52K) using 50 $\mu$ L of subject plasma per well run in duplicate. Leptin was measured using the Millipore Human Leptin 96-well plate assay (Cat. No. EZHL-80SK), using 25 $\mu$ L of subject plasma per well run in duplicate. Adiponectin was measured via the BioVendor Human Adiponectin ELISA, High Sensitivity 96-well kit (Cat. No. RD191023100). The samples were diluted 300x per the assay instructions (version 98 140311 15). All samples were tested as specified in the respective assay procedures and included both controls and standard curves for each plate run.

All data analysis was performed using SAS version 9.2 (SAS Institute, Cary, NC). Demographic analysis was performed using logistic regression for statistical significance with significance identified as  $p \leq 0.05$ . Logistic regression with odds ratio (OR) was used for measuring significance based on T2DM status for all OC compounds measured.

Comparisons between T2DM and non-diabetic subjects within categorical data sets utilized a Student's t-test to detect significant differences between the cohorts. In addition a multivariable model containing age, gender, race, adiponectin, leptin, amylin, BMI, lipid results, and the measured OC compounds was constructed. Comparisons based on race and gender also utilized logistic regression with appropriate odds ratios. Increasing OC plasma concentration calculations were based on quartiles and performed using analysis of variance. In addition a dummy variable for each quartile was generated and logistic regression was used comparing each quartile of OC compounds to a referent, in this case the quartile with the lowest concentration. All adipokine ELISA testing was analyzed using logistic regression to analyze the association these hormones had with T2DM prevalence. Data are displayed with standard error of the mean unless otherwise stated.

## **Results**

The T2DM group was significantly older and had a higher BMI than the control group (Table 2.1). Fasting glucose was significantly higher in the T2DM population even though the majority (118/149) were taking medication for their diabetes. The mean glucose for T2DM subjects not taking T2DM-related medications was 109.7 mg/dL while T2DM subjects taking medication for their diabetes was 140.4 mg/dL ( $p=0.002$ ). Elevated plasma insulin was also significantly higher in T2DM subjects. With regard to differences based on gender there was no significant difference in BMI, glucose, insulin, and triglycerides (all  $p \geq 0.20$ ) between males and females. Overall females had a significantly higher total cholesterol than males ( $181.2 \pm 3.2$  mg/dL vs  $165.5 \pm 3.1$  mg/dL, respectively) as well as HDL ( $52.8 \pm 1.1$  vs  $43.3 \pm 0.9$  mg/dL, respectively),

producing a  $p \leq 0.001$  in a Student's t-test. There was a statistical difference between higher age and higher BMI in the non-diabetic group ( $p=0.001$ ) but not in the T2DM group ( $p=0.46$ ). African-American race was found to be statistically associated with increased T2DM prevalence ( $p=0.002$ , odds ratio=2.41), and to higher BMI with African-Americans measuring  $33.2 \pm 0.7$  and Caucasians measuring  $31.2 \pm 0.4$  ( $p=0.013$ ). Triglycerides were significantly lower in African-Americans ( $102.6 \pm 7.2$  mg/dL) than Caucasians ( $150.7$  mg/dL  $\pm 6.0$ ) ( $p \leq 0.001$ ) and total cholesterol was lower in African-Americans ( $163.3$  mg/dL  $\pm 4.6$ ) than Caucasians ( $175.7$  mg/dL  $\pm 2.6$ ) ( $p=0.02$ ).

A total of 81.3% (244/300) of the subjects possessed a measurable level of at least one of the target pesticides based on our testing protocols. The majority of those subjects with measurable OC compounds were for *p,p'*-DDE alone. A total of 46.6% (69/148) of subjects possessing one of the three measured OC compounds were type 2 diabetic. In addition, 58.7% (44/75) of subjects with two of the target compounds were type 2 diabetic and 61.9% (13/21) of subjects with measurable concentrations of all three target compounds were diabetic. Out of those subjects negative for all three tested compounds 41.1% (23/56) had T2DM.

Overall *p,p'*-DDE was significantly associated with the T2DM population ( $319.2 \pm 40.6$  ng/g lipid) versus the non-diabetic population ( $139.2 \pm 19.2$  ng/g lipid) ( $p \leq 0.001$ , OR= 1.021/ 10 ng/g lipid increase). *Trans*-nonachlor was also statistically associated with T2DM, with a mean of  $15.5 \pm 1.2$  ng/g lipid for T2DM subjects versus  $10.8 \pm 0.6$  ng/g lipid in the non-diabetic subjects ( $p=0.001$ , OR= 1.046/ 1 ng/g lipid). Oxychlordan was not significantly associated with T2DM ( $22.9 \pm 2.1$  ng/g) compared to the non-diabetic subjects ( $21.3 \pm 2.2$  ng/g) ( $p=0.61$ ). Figure 2.1 graphically demonstrates the difference between the T2DM and non-diabetic populations for each of the 3 tested OC compounds.

The T2DM odds ratio in relation to DDE plasma concentration increased with higher plasma concentrations to 1.76 (95% CI 0.92-3.34) and 4.42 (95% CI 2.21-8.87) for the 50-75<sup>th</sup> percentile (Q3) and the 75-100<sup>th</sup> percentile (Q4), respectively. It should be noted that the mean plasma levels of *p,p'*-DDE are an order of magnitude higher in the third and fourth quartiles than the plasma levels of *trans*-nonachlor and oxychlordanes in the third and fourth quartiles. *Trans*-nonachlor demonstrated a similar rise in T2DM likelihood based on the plasma concentration with an OR of 2.20 (95% CI 1.14-4.24) and 4.35 (95% CI 2.20-8.60) for Q3 and Q4, respectively. Oxychlordanes plasma concentration produced an OR of 2.67 (95% CI 1.34-5.15) and 1.72 (95% CI 0.90-3.29) for Q3 and Q4, respectively (Table 2.3).

African-American subjects demonstrated 2.4-fold higher levels of DDE ( $419.9 \pm 80.2$  ng/g) compared to the Caucasian subjects in this study ( $173.9 \pm 17.0$  ng/g) ( $p=0.004$ ). *Trans*-nonachlor was higher, although not significantly, in African-Americans ( $14.0 \pm 1.54$  ng/g) than in Caucasians ( $12.8 \pm 0.72$  ng/g) ( $p=0.48$ ). Oxychlordanes was also higher in African-Americans ( $24.8 \pm 3.6$  ng/g) than in Caucasians ( $21.3 \pm 1.7$  ng/g) ( $p=0.39$ ). There was not a statistically significant interaction between race and any of the measured compounds to increased T2DM prevalence ( $p \geq 0.10$  for all 3 OC pesticides). Within the racial demographic, African-American T2DM subjects had approximately 2.3 times as much plasma DDE on average than their non-diabetic counterparts ( $p=0.03$ ). African-American T2DM subjects also had 49% higher *trans*-nonachlor and 2% higher oxychlordanes levels than their non-diabetic counterparts ( $p=0.05$  and  $0.95$ , respectively) (Figure 2.2). Caucasian T2DM subjects contained approximately 87% more DDE than their non-diabetic counterparts ( $p=0.002$ ). In addition, Caucasian T2DM subjects had 42% higher concentrations of *trans*-nonachlor ( $p=0.003$ ) and 6% higher oxychlordanes

levels ( $p=0.72$ ) than non-diabetic Caucasians (Figure 2.2). All three OC compounds were significantly associated with lower cholesterol and LDL ( $p\leq 0.05$  in all cases), however we were not able to capture information regarding statin medications taken. There was not a significant difference between males and females for any of the 3 OC compounds tested using a standard t-test ( $p\geq 0.40$  in all 3 cases) and gender was not statistically associated with T2DM in this population ( $p=0.35$ ).

There was a trend towards significance ( $p=0.06$ ) between *trans*-nonachlor and male gender to increased T2DM but not with DDE ( $p=0.15$ ) or oxychlordane ( $p=0.17$ ). BMI showed a statistical trend for increased DDE ( $p=0.07$ ) but not for *trans*-nonachlor ( $p=0.42$ ) or oxychlordane ( $p=0.99$ ). Increased age was statistically associated with increased DDE and *trans*-nonachlor ( $p\leq 0.001$ ) but not oxychlordane ( $p=0.20$ ). After developing a multivariable model containing age, race and BMI *trans*-nonachlor was associated with increased T2DM ( $p=0.05$ , OR=1.03/ ng/g increase) while DDE ( $p=0.19$ ) and oxychlordane ( $p=0.80$ ) were not associated.

When comparing the first quartile to the fourth quartile increases in leptin, TNF- $\alpha$ , and MCP-1 were all significantly associated with increased plasma concentration of DDE ( $p<0.001$ , 0.03, and 0.01, respectively) (Table 2.4) while glucagon and IL-6 were not found to be associated with increased DDE comparing Q1 to Q4 ( $p=0.50$  and 0.72, respectively). When incorporated into a model containing all 5 metabolic markers, leptin and MCP-1 were still associated with increased DDE concentrations ( $p=0.0002$  and 0.01, respectively). Separating out the subjects into quartiles based on DDE levels resulted in an increase in leptin concentration of approximately 172% between the 0-25<sup>th</sup> percentile (Q1) and the 75-100<sup>th</sup> percentile (Q4). Additionally, TNF- $\alpha$  increased approximately 41% and MCP-1 increased 66% between Q1 and Q4 of DDE plasma concentration. There

was no statistical association between insulin and DDE in the 61 non-diabetic subjects ( $p=0.53$ ); since there was an incomplete data set for this analyte, it was not included in additional models. Blood leptin concentrations were positively associated with increased levels of *trans*-nonachlor ( $p\leq 0.001$ ) while IL-6 was negatively associated with increasing *trans*-nonachlor concentration ( $p=0.04$ ). MCP-1, glucagon and TNF- $\alpha$  were not associated with increased *trans*-nonachlor levels ( $p\geq 0.40$  in all cases). A multivariable *trans*-nonachlor model containing the previously mentioned 5 metabolic markers identified only leptin as still maintaining statistical significance ( $p=0.01$ ) while the other 4 analytes were not statistically significant ( $p$  values  $\geq 0.19$ ). Oxychlordane did not demonstrate a significant association either individually ( $p\geq 0.16$  in all cases) or in a multivariable model ( $p\geq 0.09$ ).

Increased leptin demonstrated a statistical association with T2DM prevalence ( $p=0.02$ ), while decreased adiponectin was associated with T2DM subjects ( $p=0.03$ ) (Figure 2.3). Amylin was not significantly associated with T2DM ( $p=0.40$ ); however in this study amylin was lower in the T2DM population. A core model containing age, adiponectin, leptin, and BMI, both DDE and *trans*-nonachlor were found to be significantly associated with prevalence of T2DM ( $p=0.05$  and  $0.04$ , respectively). However, when race was added to the multivariable model the significance for DDE decreased ( $p=0.18$ ) while *trans*-nonachlor retained a trend towards significance ( $p=0.06$ ). Oxychlordane was not significant in either of these two scenarios ( $p=0.93$  without race added and  $p=0.77$  with race included).

## Discussion

While all of these compounds have been banned in the United States and most of the world for over 30 years they are still prevalent in the soil and due to their lipophilic nature bioaccumulate in the adipose tissue. Many areas of the United States still maintain a baseline of these compounds as evidenced by measurements of common foods purchased from grocery stores in Texas; this study measured concentrations of *p,p'*-DDE as high as 2.3 ng/g (Schechter et al., 2010). In comparison to the 2003-2004 NHANES study the mean DDE concentration for the T2DM subjects in our study was slightly higher than the NHANES population's geometric mean (319 ng/g vs 268 ng/g, respectively) (CDC, 2009) while the non-diabetic study subject mean was significantly lower at 139 ng/g. T2DM subjects in this study possessed on average less *trans*-nonachlor than the NHANES mean (15.5 ng/g vs 16.9 ng/g, respectively) while the non-diabetic subjects contained even less than their T2DM counterparts (10.8 ng/g). The NHANES mean was not specific for T2DM subjects but included both T2DM and non-diabetic subjects. In addition, only 54 total subjects had detectable levels of *trans*-nonachlor. Oxychlorane in the T2DM subjects and non-diabetics were found to be approximately equivalent to the 75<sup>th</sup> percentile of the NHANES data (19.9 ng/g); however only 85 total subjects had measurable levels so it is possible that our analysis was not as sensitive as the method employed by the CDC. It is not stated what percentage of the overall NHANES population were non-detectable for this compound, however many of the sub-populations indicate lower than level of detection as high as 50% percentile so it is assumed that the percentage nationwide of people carrying this compound is relatively low. For mean calculation purposes non-detects were measured as one-half the level of quantitation and lipid adjusted. The high percentage of the



population that had quantifiable levels of at least one of the 3 OC compounds under study agrees with previous studies including those that examined healthy populations (Jakszyn et al., 2009). In addition, Figure 2.1 demonstrates that the separation between T2DM and non-diabetic subject OC compound levels becomes more pronounced with increased concentrations of each compound; this observation was reinforced by the increasing odds ratios seen in Table 2.3.

The study population was obtained from two United States Air Force (USAF) medical centers, one in the southern United States and the other in the midwestern United States. The clients seen at these facilities consisted of active duty military members, retired military members, or their families. However, due to the fact that all samples were collected from USAF military bases, these results do not necessarily reflect the local populations. Military members and their families change station every 3-4 years on average and it is likely that the majority of the subjects had not been living in these locations for their entire lives. We were unable to obtain home of record information from the subjects. Occupation was also not captured during the course of this study so occupational exposure to these compounds could not be ascertained.

Clinical measurements of HDL, LDL, total cholesterol and triglycerides were not statistically associated with T2DM status in this case; however there were no available data regarding the use of statins, a common class of cholesterol-lowering medications. Statistically it even appeared that elevated total cholesterol level was actually protective of T2DM development. However, a common first step in treatment of T2DM is to alter the subject's diet and exercise regimen. It is this presumed change in diet, along with possible prescriptions of cholesterol-lowering statins, that are more likely the cause of the lower cholesterol by T2DM subjects. Overall, 61.8% of the male subjects and 63.7% of

the female subjects were identified as clinically obese (BMI  $\geq$  30), although when examined based on T2DM status only 48.3% of the non-diabetic subjects were obese while 77.2% of the T2DM subjects were, so the assumption that a majority of the T2DM subjects are prescribed statins seems reasonable. Glucose was statistically higher in the T2DM population even though the majority of the subjects in this cohort were taking medication to control their hyperglycemia. These high glucose levels could reflect an increased rate of uncontrolled diabetes in the subject population or a failure to follow their physician's orders regarding control of their hyperglycemia when providing a fasting blood sample. Approximately 10% of all T2DM subjects had fasting glucose results of  $>200$ mg/dL. Hemoglobin<sub>A1C</sub> results were not obtained so a determination of long-term hyperglycemia versus failure to properly fast for this blood draw could not be evaluated.

Although the DDE concentrations were much higher than the other tested OC compounds, the association with T2DM was more significant with *trans*-nonachlor when each OC compound was added to a multivariable model containing age, gender, BMI, and race (Table 2.5). This result may be due to the higher overall mean of DDE found in the African-American population and its biasing effect on statistical analysis. An association of oxychlordane with T2DM was not present; however all 3 tested OC compounds were higher in African-Americans than in Caucasians for this study population. The statistical significance of the association between BMI and two of the measured OC compounds agrees with literature indicating larger adipose stores can sequester higher amounts of the pesticides in question (Hue et al., 2006). Lower levels of cholesterol and LDL were associated with increased concentrations of all 3 OC compounds; however these data were not significant in analysis due to the lack of

information regarding statin medications used by the subject population. It is noteworthy that the T2DM subject population had lower levels of most lipids than the non-diabetics, likely due to statin medications or other lifestyle adjustments prescribed by their primary physicians upon T2DM diagnosis. Because the OC compound concentrations were adjusted for plasma total lipid levels the absolute levels of the OC compounds in plasma were even higher in the T2DM group than in non-diabetic subjects.

Univariate logistic regression indicated a statistical association between older age and either DDE or *trans*-nonachlor concentration, though it is of interest that subjects as young as 20 years old tested positive for both oxychlordan and DDE. The average BMI for subjects under 30 was in the overweight range ( $26.6 \pm 1.2$ ) so it is possible that the increased adiposity of the subjects increased the chance of accumulating sufficient quantities of the measured compounds to be detected. The age of the study subjects was restricted to no more than 65 years old in order to prevent analysis to be biased by a significantly older T2DM cohort than the non-diabetic cohort. The CDC states that while overall 8.3% of the US population is affected by T2DM that among US residents above the age of 65 the rate goes up to 26.9% of the population (CDC, 2011).

Previous research has established that many inflammatory markers are increased in T2DM (Yang et al., 2009), which makes sense as T2DM is as much a disease of inflammation as it is metabolism. It was hypothesized that more of a difference in inflammatory marker concentrations would be seen in non-diabetics between low and high OC compound body burdens, and that testing might identify pre-diabetic states of inflammation and their possible association to the OC compounds under study. The most significant findings were the increases in MCP-1, TNF- $\alpha$ , and leptin between the lowest and highest quartiles of DDE plasma concentrations in non-diabetic subjects, agreeing

with previously published in vitro studies (Alegría-Torres et al., 2009). The increase in MCP-1 concentrations seen here may reflect a possible progression towards higher insulin resistance seen in T2DM development as well as the resultant recruitment of increased numbers of monocytes that in turn express higher levels of TNF- $\alpha$ . Additionally, the significant increase in TNF- $\alpha$  demonstrates inflammatory cytokine signaling that has both been associated with T2DM development (Plomgaard et al., 2007) and shown to assist in amplifying a proinflammatory state. It is unknown why an increase in IL-6 levels with increased DDE concentrations was not seen in this study. Leptin was increased almost three-fold between Q1 and Q4 of DDE concentrations in the non-diabetic samples tested, strongly correlating to the blunted response this hormone typically displays in T2DM (Askari et al., 2010).

Some laboratory studies have identified specific increases in some adipokines such as leptin when the adipocytes are exposed to OC compounds (Howell and Mangum, 2010) and it is possible that the plasma concentrations of these chemicals have a more specific role in T2DM than simply assisting development of a pro-inflammatory state. Leptin has been indicated as a limiter of triglyceride accumulation in the liver and skeletal muscle as well as protecting the pancreatic  $\beta$ -cells from lipid accumulation (Rabe et al., 2008). These results lend credence to the theory that increased body burdens of some OC compounds not only increase incidence of T2DM via increase in general low-level inflammation but can also have direct impact on adipokine function. Additional study on impact of OC compounds on adipocyte function is needed in order to better determine if plasma concentrations of these chemicals can be used to identify higher risk of T2DM in non-diabetic subjects.

The adipocyte itself is capable of secreting monocyte chemoattractant protein-1 (MCP-1) and interleukin 6 (IL-6) while recruited macrophages secrete tumor necrosis factor alpha (TNF- $\alpha$ ). Some research has indicated that these cytokines influence insulin sensitivity (Kouyama et al., 2007; Plomgaard et al., 2007) while others have refuted this theory (Carey et al., 2004), instead implying that increases in inflammatory markers are associated with the increase in fat mass seen in obesity and not insulin sensitivity. The increased levels of TNF- $\alpha$  and IL-6 found in T2DM subjects could specifically interfere with insulin signaling pathways through JNK-1-mediated serine phosphorylation, preventing GLUT-4 expression and subsequent glucose uptake by adipocytes (Carey et al., 2004). In addition, these two cytokines promote lipolysis and increase FFA in circulation, further increasing glucose production in the liver and the resultant insulin resistance (Gustafson, 2010). MCP-1, a cytokine that is secreted from adipocytes partially as a result of enlarged adipocyte size, further perpetuates the cycle of inflammation by recruiting activated macrophages that then secrete TNF- $\alpha$ . The adipokine results seen in Figure 2.3 generally agree with previous research between non-diabetic and T2DM populations (Bremer et al., 2011; Tanizawa et al., 1997). Adiponectin was decreased overall in the T2DM subjects and leptin was increased. Amylin was actually lower in the T2DM subjects, although not significantly and in conflict with other studies (Cai et al., 2011) indicating a higher amylin concentration in T2DM subjects. It is unclear as to the cause of this in our study subjects; however not all of the samples were tested (77/300 total) due to limited amount of plasma available and the limited results could account for some of the variation in the results of this test.

## Conclusions

The results of this study indicate that increased plasma concentrations of some organochlorine compounds are associated with increased prevalence of T2DM. DDE and *trans*-nonachlor both demonstrated significant associations with increased prevalence of T2DM in univariate and some multivariable analysis models. When analyzing inflammatory marker and other biochemical factors in non-diabetics, it appears that increased OC compound plasma concentration may drive some analytes towards a state of metabolic disorder similar to that seen in type 2 diabetics. This increased inflammatory state coupled with abnormal adipokine concentrations can possibly indicate an earlier stage of T2DM development. Overall, OC compound plasma concentration can be a significant contributing factor to increased levels of insulin resistance seen in T2DM and provide clues to possible higher risk indicators and earlier detection of T2DM development.

Table 2.1 Demographic and clinical laboratory value information for study population via univariate logistic regression (n=300) with standard error of mean

	<b>Non-Diabetic</b>	<b>Type 2 Diabetic</b>	<b>Odds Ratio per unit for T2DM</b>	<b>Confidence interval</b>	<b>p-value</b>
Number of subjects (male/female)	151 (79/72)	149 (86/63)	1.244 (male)	0.79-1.96	0.35
Age (years)	48.7±0.9	56.3±0.6	1.094/yr	1.06-1.13	<0.001
Race (African American/Caucasian)	23/128	45/104	2.4 (AA)	1.37-4.24	0.002
Body Mass Index (BMI)	30.0±0.5	33.3±0.4	1.1/unit BMI	1.07-1.18	<0.001
Cholesterol (mg/dL)	188.7±3.0	156.9±2.9	0.98/ 1 mg	1.02-1.03	<0.001
Triglycerides (mg/dL)	133.1±7.1	146.6±7.2	1.002/mg	1.00-1.04	0.19
HDL (mg/dL)	49.7±1.0	45.4±1.2	0.97/ mg	0.96-0.99	0.006
LDL (mg/dL)	117.2±2.5	90.1±2.3	0.97/mg	0.96-0.98	<0.001
Glucose (mg/dL)	94.6±1.1	133.6±4.1	1.06/mg	1.04-1.08	<0.001
Insulin (µU/mL)*	11.6±1.2	20.9±3.1	1.05/1 µU	1.01-1.09	0.02

HDL=High Density Lipoprotein, LDL=Low Density Lipoprotein \*Insulin n=134

Table 2.2 Ion extraction mass and gas chromatograph retention time (minutes)

	<b>Ion Extraction Mass</b>	<b>Retention Time (minutes)</b>
<i>p,p'</i> -DDE	246.0003	9.87
<i>trans</i> -nonachlor	406.787	9.585
oxychlorane	386.8052	8.89
IS- <i>trans</i> -nonachlor	416.82	9.579
IS-DDT	247.04	11.842

IS=Internal Standard

Table 2.3 Odds Ratios for Type 2 diabetes mellitus based on quartile of OC compound plasma concentration

		Q1	Q2	Q3	Q4	p-value for trend
<i>p,p'</i> -DDE	Mean (ng/g)	8.66	49.25	180.37	675.41	
	T2DM/non-diabetics	30/45	23/51	40/34	56/19	
	Odds Ratio	Referent	0.676	1.765	4.421	<0.001
	Confidence Interval		0.344-1.329	0.921-3.380	2.205-8.866	
		Q1	Q2	Q3	Q4	
<i>Trans-nonachlor</i>	Mean (ng/g)	6.88	8.67	10.20	26.65	
	T2DM/non-diabetics	26/50	30/43	40/35	53/23	
	Odds Ratio	Referent	1.342	2.198	4.346	<0.001
	Confidence Interval		0.690-2.608	1.140-4.235	2.197-8.598	
		Q1	Q2	Q3	Q4	
Oxychlordanes	Mean (ng/g)	7.03	8.91	11.99	60.35	
	T2DM/non-diabetics	29/46	34/41	47/28	39/36	
	Odds Ratio	Referent	1.315	2.663	1.718	0.026
	Confidence Interval		0.687-2.520	1.337-5.149	0.898-3.288	

Q1=<25<sup>th</sup> percentile, Q2=26-50<sup>th</sup> percentile, Q3=51-75<sup>th</sup> percentile, Q4=>75<sup>th</sup> percentile  
 \*trends analyzed using ANOVA analysis between quartiles of the target compound



Table 2.4 Metabolic hormone and inflammatory marker ELISA testing based on *p,p'*-DDE plasma concentration (pg/mL)

		IL-6	Leptin	Glucagon	TNF- $\alpha$	MCP-1
Q1	Mean	11.0	11602	48.5	5.6	179.1
	SE	2.7	1823	10.8	0.6	14.3
Q4	Mean	8.8	31601	51.3	7.9	300.0
	SE	2.6	5795	10.6	0.9	15.9
p-value		0.72	<0.0001	0.51	0.03	0.01

Q1=<25<sup>th</sup> percentile *p,p'*-DDE, Q4=>75<sup>th</sup> percentile *p,p'*-DDE; p-value represents logistic regression over all tested samples.

Table 2.5 Comparison of DDE and *trans*-nonachlor multivariable models using logistic regression including odds ratio (OR) and confidence intervals

	<i>trans</i> -nonachlor			DDE		
	p value	OR	Conf Int	p value	OR	Conf Int
DDE	-----	-----	-----	0.16	1.001	1.00-1.002
<i>trans</i> -nonachlor	0.05	1.028/ng	1.00-1.06	-----	-----	-----
Age	<0.0001	1.088	1.05-1.13	<0.0001	1.086	1.05-1.12
Gender (F)	0.25	0.734	0.43-1.24	0.20	0.708	0.42-1.20
BMI	0.0003	1.099	1.04-1.16	0.0005	1.095	1.04-1.15
Race (B)	0.004	2.587	1.35-4.96	0.01	2.369	1.21-4.64

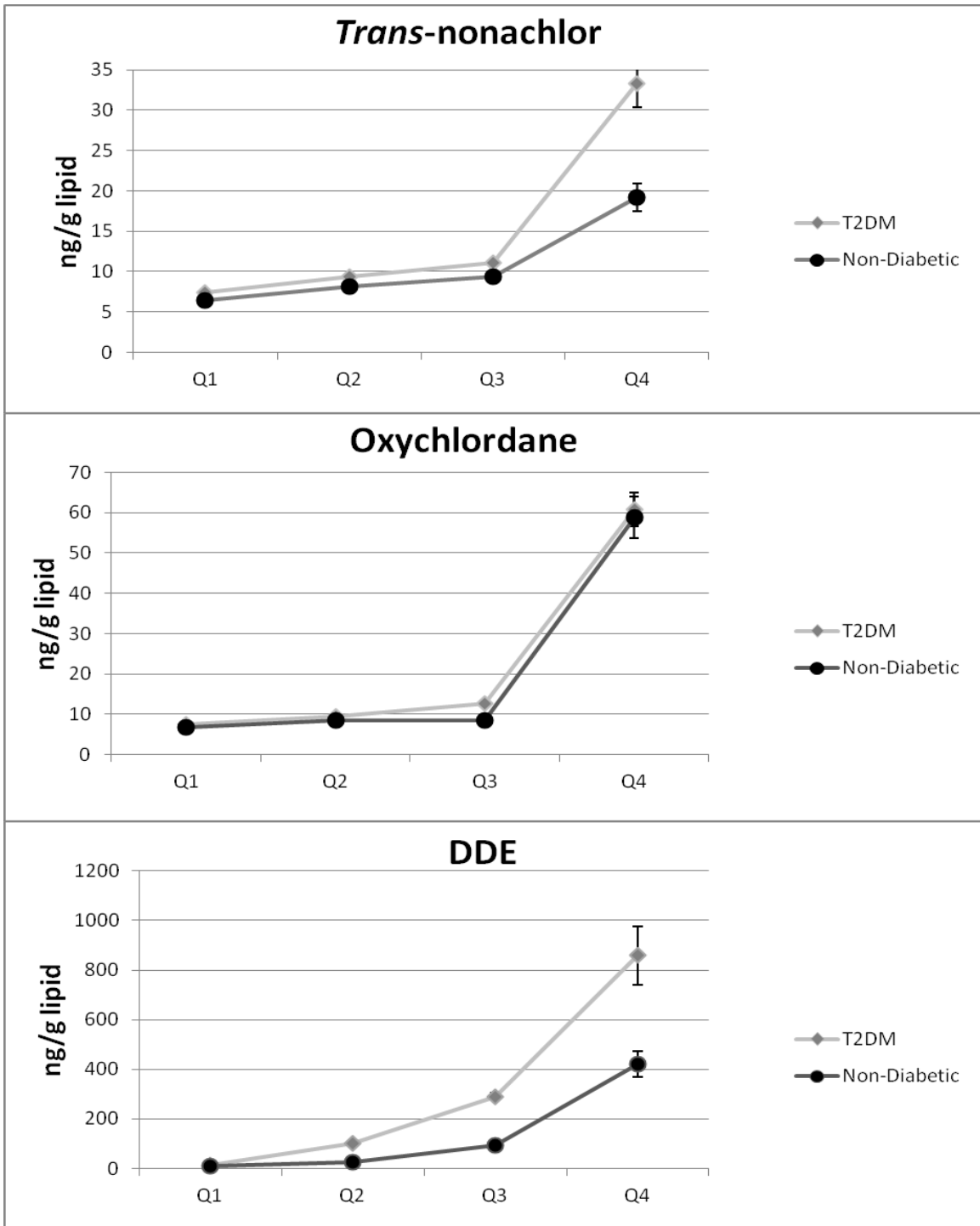


Figure 2.1 Measurement of mean OC compound concentrations in subject plasma by quartile. All results are expressed as ng/g lipid and include standard error of the means, some of which are not visible beyond the diameter of the symbol. (n=36-38 for each quartile)

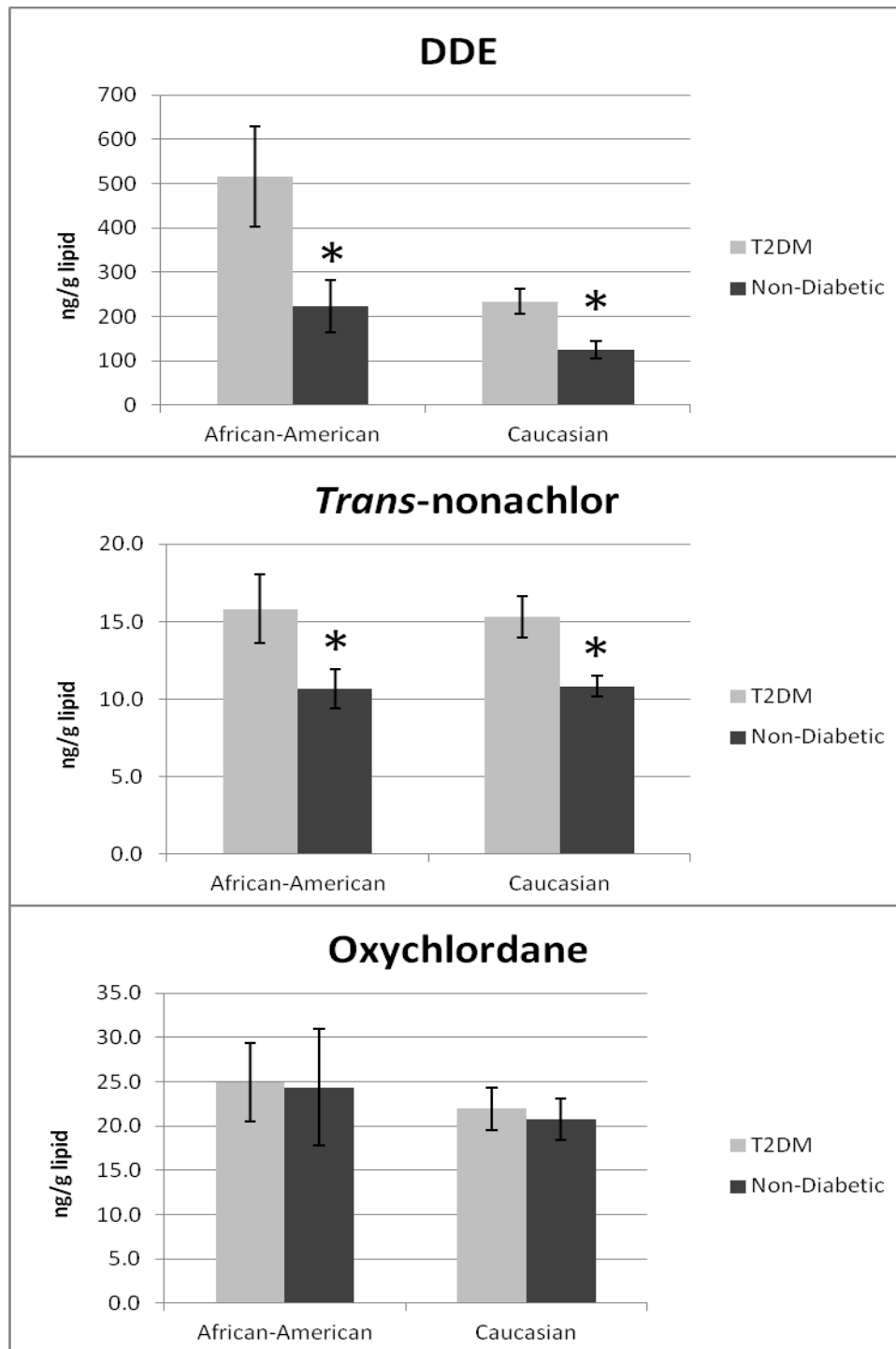


Figure 2.2 Measurement of OC compound concentrations by race including T2DM and non-diabetic study subjects. Asterisk indicates statistical significance ( $p \leq 0.05$ ). All results are expressed as ng/g of lipid. (n=45 African-American, T2DM; n=23 African-American, non-diabetic; n=104 Caucasian, T2DM; n=128 Caucasian, non-diabetic)

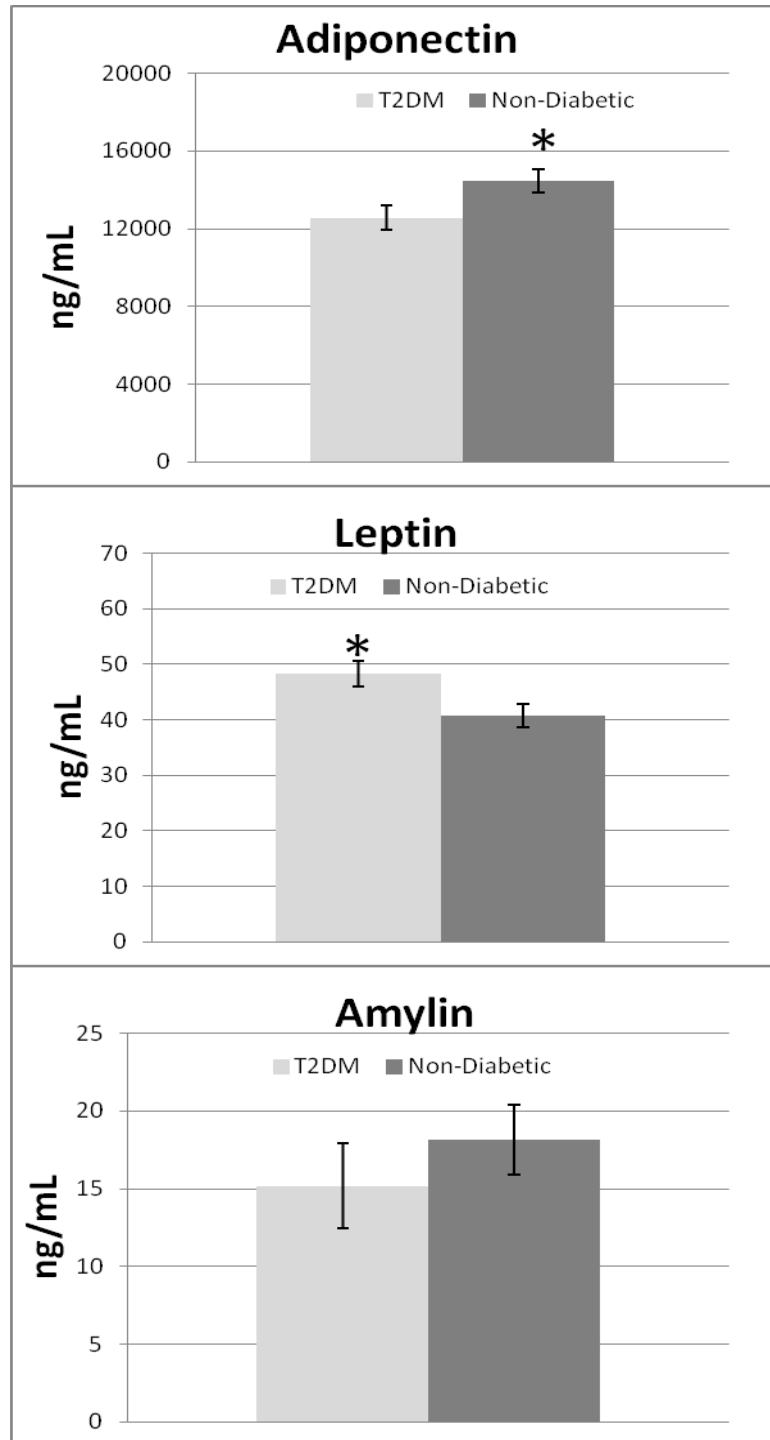


Figure 2.3 Plasma adiponectin, leptin and amylin levels in T2DM and non-diabetic subjects. Asterisk indicates statistical significance ( $p \leq 0.05$ ). All results are expressed as ng/mL

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CHAPTER III  
ASSOCIATION OF PARAOXONASE-1 ACTIVITY AND GENE POLYMORPHISMS  
WITH TYPE 2 DIABETES MELLITUS

**Introduction**

In 2010 over 1 million people in the United States between the ages of 45-64 were diagnosed with type 2 diabetes mellitus with an additional 465,000 people between the ages of 20-44 diagnosed (CDC, 2011). In 2005-2008 an additional 35% of US adults above the age of 20 were considered pre-diabetic based on their hemoglobin<sub>A1C</sub> levels (CDC, 2011). Paraoxonase-1 (PON-1) is an enzyme associated with high density lipoprotein (HDL) that hydrolyzes some organophosphate compounds as well as affords protection against low-density lipoprotein (LDL) oxidation (Furlong et al., 2010). This capacity to protect against lipid oxidation has been extensively studied and higher PON-1 activity has been linked to protective effects against several systemic diseases including atherosclerosis, cardiovascular disease and type 2 diabetes mellitus (Camps et al., 2009). The main cause of mortality in T2DM is cardiovascular disease, an important consideration since lower PON-1 activity is also seen in atherosclerosis (Durrington et al., 2001). PON-1 activity has been shown to be lower in T2DM patients (Abbott et al., 1995; Altuner et al., 2011; Ergun et al., 2011). In addition, recent research has shown that incubation of serum with high glucose concentrations over a period as short as 7 days resulted in a reduction of PON-1 activity (Ikeda et al., 2008; Mastorikou et al., 2008)

over time. Lower PON-1 activity when in the presence of high glucose concentrations may at least partially explain why PON-1 activity is lower in T2DM subjects.

The PON-1 gene has been mapped to the long arm of chromosome 7q21-22 and contains over 160 known genetic single base pair polymorphisms, some in the coding region and some in the promoter region (Costa et al., 2005). These polymorphisms have been shown to be responsible for producing variations in PON-1 activity. Two of the most prominent polymorphisms are at amino acid positions 192 and 55. The Q192R polymorphism represents a single base pair variation between glutamine (Q) and arginine (R) while the L55M polymorphism contains either leucine (L) or methionine (M). Both genetic polymorphisms have been implicated as having impact on development of T2DM (Mackness et al., 1998). Earlier studies have indicated that the PON-1<sub>55</sub> polymorphism has an impact on PON-1 activity level in T2DM patients (Mackness et al., 2002; Mackness et al., 1998); however more recent findings dispute this (Altuner et al., 2011).

Population studies have reflected differences in allele frequencies for PON-1 genotypes among various racial and ethnic groups. Our laboratories have shown that African-Americans display a higher proportion of the PON-1<sub>192RR</sub> genotype than Caucasians (Coombes et al., 2011; Davis et al., 2009) while a recent study consisting of Korean subjects displayed no presence of the PON-1<sub>192RR</sub> genotype at all (Shin, 2009). In addition, this Korean study reflected a less than 1% presence of the PON-1<sub>55MM</sub> genotype. As previously stated some of these genetic polymorphisms are suggested to contribute to increased susceptibility to development of T2DM as well as other systemic diseases characterized by increased LDL oxidation such as atherosclerosis.

Typical measurement of PON-1 activity and determination of functional genotype for the Q192R polymorphism is performed utilizing enzymatic measurement of PON-1's

ability to hydrolyze two organophosphorous compounds in a high salt solution, the high salt being used to increase PON-1 activities and separate the Q192R genotypes on an activity plot (Furlong et al., 2010). The most common substrates used for these studies are the highly toxic organophosphates paraoxon and diazoxon, the active metabolites of the insecticides parathion and diazinon, respectively. The PON-1<sub>192R</sub> genotype hydrolyzes paraoxon more rapidly and hydrolyzes diazoxon less efficiently than the PON-1<sub>192Q</sub> genotype. These differences allow separation of the three PON1<sub>192</sub> functional genotypes when plotted, and can be used when actual genotyping is not possible. Additionally, arylesterase activity is frequently measured using phenyl acetate as a substrate. This measurement, whose activity levels are not dependent upon PON-1<sub>192</sub> genotype (Richter et al., 2008), has been utilized as a measurement of overall PON-1 activity.

PON-1 polymorphism genotyping is most commonly performed using PCR restriction fragment length polymorphisms (PCR-RFLP) (Altuner et al., 2011) followed by acrylamide gel electrophoresis and visualization using ethidium bromide (Flekac et al., 2008). This process is preceded by digestion using specific restriction enzymes and amplification using PCR to generate sufficient DNA product. This process can be very time consuming, particularly when faced with a large number of study samples. An alternative to this process is the use of real-time PCR (RT-PCR) melting curve analysis. This process can generate accurate genotypes for PON-1 polymorphisms and reduce contamination concerns due to its single-step process and the fact that the test is prepared and performed in the same container. After obtaining genomic DNA from routine blood samples a thermal cycler can be used to distinguish single base pair differences for up to 32 samples within a 75 minute time frame (Pocsai et al., 2003).

This experiment was conducted to investigate the possible association of T2DM with PON-1 activity and variation in the Q192R and L55M single base pair polymorphisms. Fasting glucose as well as insulin, BMI, lipid profiles, age, sex, and race, which were obtained from the patient charts, were also evaluated as possible contributing factors.

### **Materials and Methods**

The study population consisted of 300 subjects (149 T2DM/151 non-diabetic controls) who were patients at either Keesler Air Force Base (AFB) Medical Center (Biloxi, MS) or Wright-Patterson Air Force Base Medical Center (Dayton, OH). Institutional Review Boards at Keesler AFB or Wright-Patterson AFB, and Mississippi State University approved the study protocol. All information and samples supplied to Mississippi State University for analysis were deidentified. The voluntary, fully informed consent of the 49 recruited subjects at Keesler AFB used in this research was obtained as required by 32 CFR 219 and AFI 40 402, Protection of Human Subjects in Biomedical and Biochemical Research. See the previous chapter for a description of the demographic breakdown as well as sample collection methods.

Genomic DNA isolation was performed using the QiagenDNeasy Blood & Tissue Kit® and quantified using a NanoDrop ND-1000. Samples were normalized with molecular-grade water to 25µg of DNA. The primers employed for PON-1<sub>55</sub> were PON-1<sub>55</sub>F and PON-1<sub>55</sub>R and generated a 138bp PCR product. Primers employed for PON-1<sub>192</sub> were PON-1<sub>192</sub>F and PON-1<sub>192</sub>R and generated a 151 bp PCR product. The detection set used for PON-1<sub>55</sub> consisted of a 3'-fluorescein-labelled anchor and a 5'-LC Red 640-labelled sensor probe. The detection oligonucleotide sets used for PON-1<sub>192</sub> consisted of

a 3'-fluorescein-labelled anchor and a 5'-LC Red 705-labelled sensor probe. The sequences of primers and probes as well as applied fluorochromes used on the Roche LightCycler 2.0 are in Table 3.1. Both the custom primers and fluorescently labeled probes were synthesized by Roche Applied Science (Indianapolis, IN). PCR and melting curve analysis were performed in 19 $\mu$ L volumes in glass capillaries on a Roche LightCycler 2.0. After an initial denaturation of 95°C for 10 minutes, 50 cycles of PCR were performed using a temperature profile of 94°C for 1 second, 57°C for 10 seconds and 72°C for 10 seconds with a temperature transition rate of 20°C/second. Fluorescence was measured during each annealing step to provide real-time amplification results. At the end of the amplification a melting curve was generated by ramping temperature from 42°C to 80°C with a temperature transition rate of 0.1°C/second after establishing the 42°C baseline for 2 minutes. Fluorescence intensity was monitored continuously during this slow heating process. Melting curves were converted to melting peaks by plotting the negative fluorescence derivative against temperature similar to previous research (Pocsai et al., 2003). Control material provided by Roche Biosciences was used to verify the PON-1<sub>55</sub> procedure while previously sequenced samples from an earlier study (Coombes et al., 2011) were tested for PON-1<sub>192</sub> to verify the procedure.

Paraoxon was a gift of Howard Chambers (Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University) and was synthesized as previously described (Chambers et al., 1990). Paraoxon hydrolysis was measured spectrophotometrically in microtiter plates as described by Richter and Furlong (Richter and Furlong, 1999) and modified in our laboratories (Davis et al., 2009). Heparinized plasma samples were diluted using Tris-HCl (0.1M, pH 8.0) with 2mM CaCl<sub>2</sub> and run in paired triplicates (a total of 6 wells) and a second set of triplicates using

1mM EDTA instead of CaCl<sub>2</sub> to eliminate PON-1 activity. The samples were incubated for 5 minutes at 37°C following which paraoxon in ethyl alcohol (1.2mM final concentration) was added. After 20 minutes of incubation the reaction was terminated with the addition of 50µL of 20mM EDTA plus 2% Tris base solution with SDS and the 4-nitrophenol was quantified at 405nm. Data were expressed as micromoles paraoxon hydrolyzed per minute per liter of plasma.

Diazoxon was purchased from ChemService (West Chester, PA). Ten microliters of heparinized plasma were added to a test tube containing 975 µL of a calcium buffer solution of Tris-HCl (0.1M, pH 8.0) with 2mM CaCl<sub>2</sub> and incubated for 5 min at 37°C. After incubation 10µL of 0.2M diazoxon in ethyl alcohol was added and the contents of the test tube were immediately vortexed and transferred to a UV cuvette for measurement. Release of 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP) was continuously monitored at 270nm for 2 minutes. Samples were run in triplicate and the results were expressed as micromoles diazoxon hydrolyzed per minute per liter of plasma. Paraoxon hydrolysis activity was plotted against diazoxonase activity in Figure 3.2.

Arylesterase activity was measured using phenyl acetate as the substrate. Ten microliters of heparinized plasma was added to 990µL of a calcium buffer containing Tris-HCl (0.1M, pH 8.0) with 2mM CaCl<sub>2</sub>. Test tubes containing this reaction volume were prepared in duplicate with a third test tube containing 10µL of heparinized plasma and 990µL of 1mM EDTA buffer described above to correct for non-PON-1 mediated hydrolysis. All test tubes were incubated at 37°C for 10 minutes prior to addition of the substrate. After incubation 10µL of 50mM phenyl acetate in ethanol were added. Immediately after vortexing for 5 seconds the sample was transferred to a UV cuvette and

the released phenol was measured spectrophotometrically at 270nm for 2 minutes to measure the release of phenol. The results were expressed as micromoles of phenyl acetate hydrolyzed per minute per liter of plasma.

SAS version 9.2 (SAS Institute, Cary, NC) was used for data analysis.

Demographics were analyzed utilizing logistic regression to determine statistical association with T2DM, with significance measured at  $p < 0.05$ . Comparisons within demographic measurements were made in some cases, in which a t-test was utilized to determine if the differences were statistically significant. In addition, multivariable logistic analysis was performed to incorporate demographic traits in association with T2DM to establish a core statistical model. The model included age, gender, BMI, cholesterol, triglycerides, HDL, LDL, race, PON-1 genotype and PON-1 activity testing (paraoxon hydrolysis, diazoxonase and arylesterase). PON-1 genotypes and activity were also examined using logistic regression and analysis of variance in order to determine significant association with T2DM. Analysis of variance was performed on each PON-1 allele distribution to determine if statistical association was found between the different PON-1 genotypes examined in this study. The combined PON-1 genotypes were separated out in the dataset using a dummy variable and then analyzed using logistic regression to determine significant association with T2DM. All results include standard error of the mean unless otherwise indicated.

## **Results**

As previously reported in Chapter 2, higher age, African-American race, BMI, glucose, and insulin were statistically associated with the T2DM population ( $p < 0.02$  for all previously stated categories). The non-diabetic subjects had higher cholesterol and

HDL than their diabetic counterparts ( $p=0.01$  and  $<0.001$ , respectively). See previous chapter for complete table of demographic information.

Paraoxon hydrolysis activity was not statistically associated with increased T2DM prevalence in this study population; however using diazoxon as the substrate produced a significant association with T2DM in univariate analysis ( $p=0.02$ ) (Table 3.2). The PON-1<sub>192RR</sub> genotype and the PON-1<sub>55LL</sub> genotype displayed the highest enzyme activity while the PON-1<sub>192QQ</sub> genotype and the PON-1<sub>55MM</sub> displayed the lowest PON-1 activity when paraoxon and phenyl acetate assays were employed (Table 3.3). When diazoxon was utilized as the substrate, a similar trend was seen with regard to PON-1<sub>55</sub> genotype; however within the PON-1<sub>192</sub> genotype the QQ genotype was highest and the RR was the lowest, opposite of that seen in the other PON-1 activity assays (Figure 3.3). This order of highest to lowest activity was identical for both the non-diabetic and T2DM populations within all three substrates tested. There was a statistically significant association between higher age and lower PON-1 activity with the substrates paraoxon ( $p=0.03$ ), diazoxon ( $p=0.04$ ) and phenyl acetate ( $p=0.04$ ). African-Americans displayed higher arylesterase and paraoxon hydrolysis activity than Caucasians ( $p=0.05$  and  $<0.001$ , respectively) (Table 3.2). In addition, females displayed higher PON-1 activity via diazoxonase and arylesterase ( $p\leq 0.001$  and  $0.01$ , respectively) while not with paraoxon hydrolysis ( $p=0.37$ ). When modeled based on an outcome of T2DM the PON-1<sub>192RR</sub> genotype demonstrated a statistical association with T2DM subjects ( $n=37$ ) compared with non-diabetics ( $n=19$ ) ( $p=0.01$ , odds ratio=2.3) utilizing logistic regression (Table 3.4). African-Americans demonstrated a higher association with the PON-1<sub>192RR</sub> genotype (51.5%) than did Caucasians (9.1%) ( $p<0.001$ , odds ratio=10.66). Each combined PON-1 genotype was made into a dummy variable and analyzed for association with T2DM



prevalence (Table 3.5). Within combined PON-1 genotypes the RRLM genotype was most significantly associated with type 2 diabetes ( $p=0.03$ , odds ratio=2.08) while the QQMM genotype was the most associated with protective effect against T2DM ( $p=0.02$ , odds ratio=0.66).

The mean melting point temperatures for both the PON-1<sub>55</sub> and PON-1<sub>192</sub> genotypes are shown in Figure 3.1. The PON-1<sub>192</sub> genotype displayed an average melting curve peak at  $61.1^{\circ}\text{C} \pm 1.1$  for the (R) homozygous allele while the (Q) homozygous allele showed a peak of  $66.3^{\circ}\text{C} \pm 0.6$ . The heterozygous allele showed reduced intensity on both melting peak temperatures as seen in the figure. The PON-1<sub>55</sub> genotype had an average melting curve peak at  $61.9^{\circ}\text{C} \pm 0.9$  for the (L) homozygous allele and  $66.3^{\circ}\text{C} \pm 0.9$  for the (M) homozygous allele. Again the heterozygous allele had reduced fluorescence on both melting peak temperatures.

Gene frequencies were consistent with the Hardy-Weinberg equilibrium for the PON-1<sub>55</sub> allele; however, the PON-1<sub>192</sub> allele departed from equilibrium based on T2DM status. PON-1<sub>192</sub> allele frequency was within equilibrium for non-diabetic subjects. The frequency distribution overall for the PON-1<sub>192</sub> polymorphism was QQ (0.48), QR (0.33), and RR (0.19). The frequency distribution overall for the PON-1<sub>55</sub> polymorphism was LL (0.38), LM (0.48) and MM (0.14). The RR/LL genotype displayed the highest overall paraoxon hydrolysis activity among the combined PON-1<sub>192</sub> and PON-1<sub>55</sub> genotypes ( $179.9 \text{ U/L} \pm 70.9$ ) while the QQ/MM genotype produced the lowest activity ( $27.5 \text{ U/L} \pm 14.9$ ).

When age, race, gender, BMI, HDL, LDL, total cholesterol, and triglycerides were analyzed in a multivariable stepwise model with significance at  $p=0.05$ , BMI ( $p=0.01$ ), age ( $p \leq 0.001$ ), African-American race ( $p=0.01$ ), and LDL ( $p \leq 0.001$ ) were all

identified as significantly associated with T2DM prevalence. When diazoxon was incorporated into a multivariable model containing race, gender, and triglycerides a trend towards association with T2DM was noted ( $p=0.10$ ); however, when age or other clinical lipid measurements were added, PON-1 activity with this substrate fell out of the model. When accounting for race, age, and BMI the PON-1<sub>192RR</sub> and PON-1<sub>192QR</sub> genotypes displayed a trend toward significant association with T2DM ( $p=0.09$  and  $0.10$ , respectively) while other PON-1 genotypes measured including combinations of the PON-1<sub>55</sub> and PON-1<sub>192</sub> genotypes were not associated with increased T2DM prevalence ( $p\geq 0.10$  in all cases). PON-1 activity was not significant for any of the three substrates tested when the core model was applied ( $p\geq 0.30$  in all cases); however, when a model of age, BMI and gender was applied to paraoxon hydrolysis activity a trend towards association was seen ( $p=0.09$ , OR=1.041/10 U/L) while this trend was not observed for diazoxonase ( $p=0.36$ ) or for arylesterase ( $p=0.26$ ).

## Discussion

The decrease in PON-1 activity with increasing age agrees with previous research (Costa et al., 2005) as does significantly higher PON-1 activity in females compared to males (Costa et al., 2011). Many PON-1 studies utilize salt-fortified buffers in order to enhance PON-1 activity. This increased activity can then be plotted using a dual substrate plot (e.g., diazoxonase and paraoxon hydrolysis) in order to separate out the three variations of the PON-1<sub>192</sub> functional genotype (Furlong et al., 2010). Since determination of a functional genotype was not necessary for this study, PON-1 activity was measured using non-salt-stimulated buffers to produce activity closer to *in vivo* levels. This may explain some of the less distinct separation of individuals on the

paraoxon hydrolysis activity vs. diazoxonase plots and is similar to research of others using non-salt buffers (Yilmaz et al., 2010). However, when paraoxon hydrolysis activity was plotted against diazoxonase activity reasonably good separation between the PON-1<sub>192</sub> genotypes was observed (Figure 3.2).

In agreement with previous research (Harangi et al., 2002; Pocsai et al., 2003) the use of RT-PCR melting curve analysis enabled a rapid identification of single nucleotide polymorphisms for PON-1 and required minimal preparation after genomic DNA extraction from the white blood cells. On average the PCR cycles and melting curve analysis were completed in less than 80 minutes in contrast to performing the PCR reaction, enzymatic digestion and analysis using an agarose gel. Both melting curve analyses produced a temperature difference of approximately 5°C between the two homozygous base pair sets, demonstrating the high specificity of the detection probes to the target DNA sequences.

The difference in PON-1 genotypes among various ethnic groups is noteworthy when associating the genotype to a disease state. Some studies have indicated that the PON-1 genotype distribution among different ethnic populations can shift the emphasis involving disease prevalence by artificially exaggerating genetic subsets common or rare to a particular ethnic group. This emphasis on particular genetic PON-1 variations can have an impact when comparing genotypes to risk markers for disease. A recent study involving 988 individuals in Korea found zero PON-1<sub>192RR</sub> individuals (Shin, 2009) and a study in the Netherlands of 201 Caucasians produced zero RR/LM and RR/MM genotypes and only 9% RR/LL genotypes (Leus et al., 2000). These differences in ethnic populations can make development of a model for disease risk more challenging due to selection bias. Due to the method of collecting samples that were de-identified we were

not able to identify the race of the subject prior to sample collection so an even distribution of Caucasian and African-American subjects was not possible. However, even taking into account gender, age, PON-1<sub>192</sub> genotype, and lipid measurements, race was still considered a significant factor in T2DM prevalence ( $p=0.02$ , odds ratio=2.7). This agrees with previous research indicating African-Americans have a higher prevalence of T2DM than Caucasians (CDC, 2011). A total of 18.7% of subjects in the present study possessed the PON-1<sub>192R</sub> alloform. While only 5% of the study population was found to have the RR/LM genotype it was still statistically associated with T2DM.

The fact that in both the diabetic and non-diabetic populations paraoxon hydrolysis activity as well as arylesterase activity was highest in the PON-1<sub>192RR</sub> and PON-1<sub>55LL</sub> genotypes and lowest in the corresponding opposite homozygous genotype implies that the effect of the two polymorphisms may be linked. Out of the 18 possible combinations of PON-1 genotypes and T2DM status examined, the five highest paraoxonase activity levels were all PON-1<sub>192RR</sub> genotypes. The high proportion of the same PON-1<sub>192</sub> genotype displaying higher paraoxon hydrolysis activity implies that the PON-1<sub>192</sub> genotype may have a significantly higher impact on overall PON-1 activity than the PON-1<sub>55</sub> genotype. While some literature indicates a significant association by the PON-1<sub>55</sub> genotype with T2DM (Ergun et al., 2011) the impact of this genotype in this study appears to be more minimal.

While PON-1 genotypes have been shown to significantly impact PON-1 activity, there are several other mechanisms that have been found to alter PON-1's ability to hydrolyze oxidized LDL particles and protect against some diseases. Previous studies have indicated that HDL function is decreased as a result of glycation frequently seen in hyperglycemic events of T2DM (Hedrick et al., 2000). In addition, studies have shown

that glycation of PON-1 in T2DM subjects results in decreases in PON-1 activity which could lead to increased susceptibility to cardiovascular disease (CVD) (Mastorikou et al., 2008). Abnormalities in lipids as a result of liver disease, and diseases such as HIV (Daminelli et al., 2008) have also been implicated in possible changes to PON-1 activity.

### **Conclusions**

The results of this study indicate that lower PON-1 activity may have an association with T2DM development and some genetic variations of PON-1 can influence overall PON-1 activity. Some studies have indicated an inconsistency with using only genotype to determine disease risk. Use of both genetic analyses of PON-1 SNPs as well as measurement of PON-1 activity supports previous studies showing an increased association between lower PON-1 activity and T2DM development. The present data suggest that the PON-1<sub>192RR</sub> polymorphism is less protective against development of T2DM and also that this genetic marker is more prevalent in African-Americans. These data suggest that while PON-1 genotyping alone is certainly helpful in identifying possible risks for T2DM development, that PON-1 activity measurement by diazoxonase hydrolysis is also highly recommended. Measurement of PON-1 protein concentrations might enable additional evaluation of the effects of genotype plus environment on PON-1 enzyme activity and its impact on disease.

Table 3.1 Sequence of PCR primers and hybridization probes

<b>Primers/Probes</b>	<b>Sequences</b>
PCR Primers	
PON1-192F	5'-TATTGTTGCTGTGGGACCTGAG-3'
PON1-192R	5'-CCTTCTGCCACCACTCGAAC-3'
PON1-55F	5'-CCTGCAATAATATGAAACAACCTG-3'
PON1-55R	5'-CTAGAACACAGAAAAGTGAAAGAAAAC-3'
Probes	
PON1-192 Sensor	5'-CCCCTACTTACAATCCTGGGAGAT-FL-3'
PON1-192 Anchor	5'-LC705-ATTTGGGTTTAGCGTGGTCGTATGTTG-3'
PON1-55 Sensor	5'-CTCTGAAGACATGGAGATACTGCC-FL-3'
PON1-55 Anchor	5'-LCRed640-ATGGACTGGCTTTCATTAGCTCTGTGAGGT-3'

Table 3.2 Paraoxonase-1 (PON-1) activity levels by Type 2 Diabetes Mellitus (T2DM) status, race and gender.

<b>PON-1 activity</b>	<b>T2DM</b>	<b>Non-Diabetic</b>	<b>p-value</b>
Paraoxonase	83.0 ± 11.1	77.0 ± 10.0	0.36
Diazoxonase	7469 ± 140	8287 ± 151	0.02
Arylesterase	22710 ± 352	22623 ± 308	0.88
N=	151	149	
	<b>African-American</b>	<b>Caucasian</b>	<b>p-value</b>
Paraoxonase	123.4 ± 15.7	67.3 ± 9.5	<0.001
Diazoxonase	6969 ± 136	8147.8 ± 148	0.01
Arylesterase	23678 ± 339	22369 ± 324	0.05
N=	68	232	
	<b>Male</b>	<b>Female</b>	<b>p-value</b>
Paraoxonase	75.2 ± 4.2	85.8 ± 5.3	0.37
Diazoxonase	7514 ± 191	8330 ± 297	<0.001
Arylesterase	22195 ± 382	23243 ± 401	0.01
N=	165	135	

Activities are expressed as micromoles substrate hydrolyzed per L plasma, means ± SE. p-values are calculated by univariate logistic regression.

Table 3.3 Paraoxonase-1 (PON-1) activity levels in all subjects by PON-1<sub>192</sub> and PON-1<sub>55</sub> genotype

		N=	Paraoxonase Activity (U/L)	Diazoxonase Activity (U/L)	Arylesterase Activity (U/L)
PON-1 <sub>192</sub>	QQ	145	37.9 ± 1.7	8758 ± 266.0	21532 ± 400.0
	QR	99	94.7 ± 3.8	7446 ± 278.6	22850 ± 494.5
	RR	56	162.9 ± 6.4	6377 ± 217.5	25278 ± 460.6
r <sup>2</sup>			0.83	0.19	0.12
PON-1 <sub>55</sub>	LL	113	113.8 ± 5.9	8158 ± 297.0	24134 ± 406.9
	LM	143	67.6 ± 3.8	7809 ± 236.7	22459 ± 384.8
	MM	44	33.5 ± 3.7	7401 ± 435.5	19569 ± 784.6
r <sup>2</sup>			0.42	0.10	0.13

p<0.001 in ANOVA among the three genotypes after statistically adjusting for race and gender with the exception of diazoxonase activity in the PON-1<sub>55</sub> genotype (p=0.33).

Table 3.4 PON-1 genotype distribution for T2DM and non-diabetic populations

	T2DM		Non-Diabetic		p-value	OR	Conf Interval
	N=	%	Number	%			
PON-1 <sub>192</sub>							
QQ	68	45.6	77	51	0.35	0.81	0.51-1.27
QR	44	29.5	55	36.4	0.2	0.73	0.45-1.19
RR	37	24.8	19	12.6	0.01	2.3	1.25-4.21
PON-1 <sub>55</sub>							
LL	61	40.9	52	34.4	0.25	1.32	0.83-2.11
LM	72	48.3	71	47	0.82	1.05	0.67-1.66
MM	16	10.7	28	18.5	0.06	0.53	0.27-1.02

p-value represents logistic regression between T2DM and non-diabetic study groups.

Table 3.5 Combined PON-1<sub>192</sub> and PON-1<sub>55</sub> genotype and association to T2DM status via univariate logistic regression

PON-1 Genotype	T2DM subjects (%)	Control subjects (%)	p value	OR	Confidence Interval
QQLL	19 (6.33)	11 (3.67)	0.12	1.37	0.93-2.00
QQLM	36 (12.00)	39 (13.00)	0.74	0.95	0.74-1.23
QQMM	13 (4.33)	27 (9.00)	0.02	0.66	0.47-0.94
QRLl	18 (6.00)	25 (8.33)	0.27	0.83	0.60-1.15
QRlM	25 (8.33)	28 (9.33)	0.48	0.90	0.67-1.21
QRMM	2 (0.67)	1 (0.33)	0.56	1.43	0.43-4.76
RRLl	24 (8.00)	16 (5.33)	0.16	1.27	0.91-1.79
RRLM	12 (4.00)	3 (1.00)	0.03	2.08	1.09-3.95
RRMM	1 (0.33)	0 (0.00)	0.99	743.61	<0.001->999.99



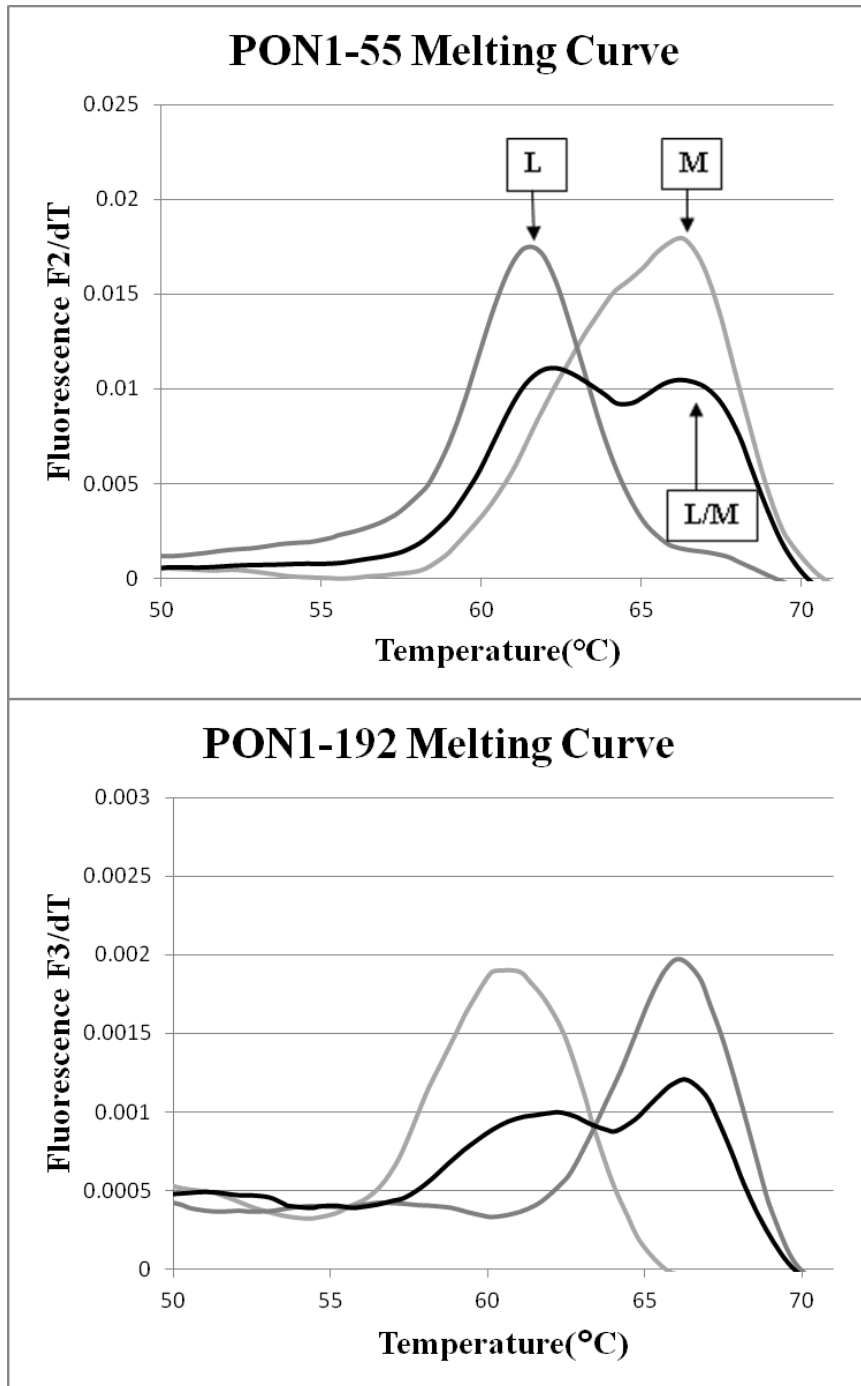


Figure 3.1 Average melting peaks of PON-1<sub>55</sub>(a) and PON-1<sub>192</sub>(b) polymorphisms obtained by real-time PCR. The melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence against temperature. The darkest line indicates the heterozygote. Each melting curve represents one individual with a representative melting curve for the respective allele.

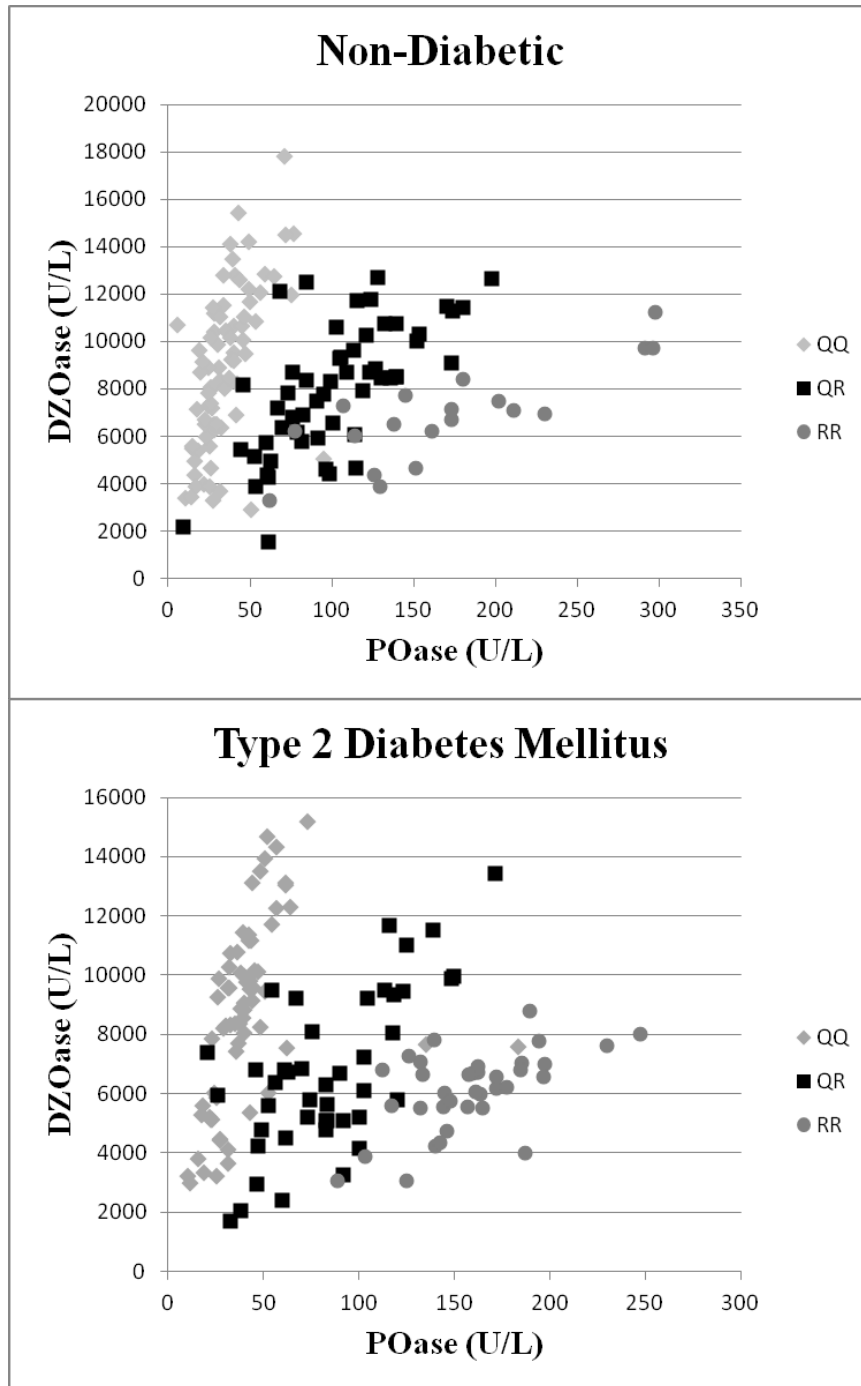


Figure 3.2 Paraoxon hydrolysis activity (POase) is plotted against diazoxonase activity (DZOase). All results are expressed as (U/L). PON-1 genotypes were obtained from real time PCR melting curve analysis.

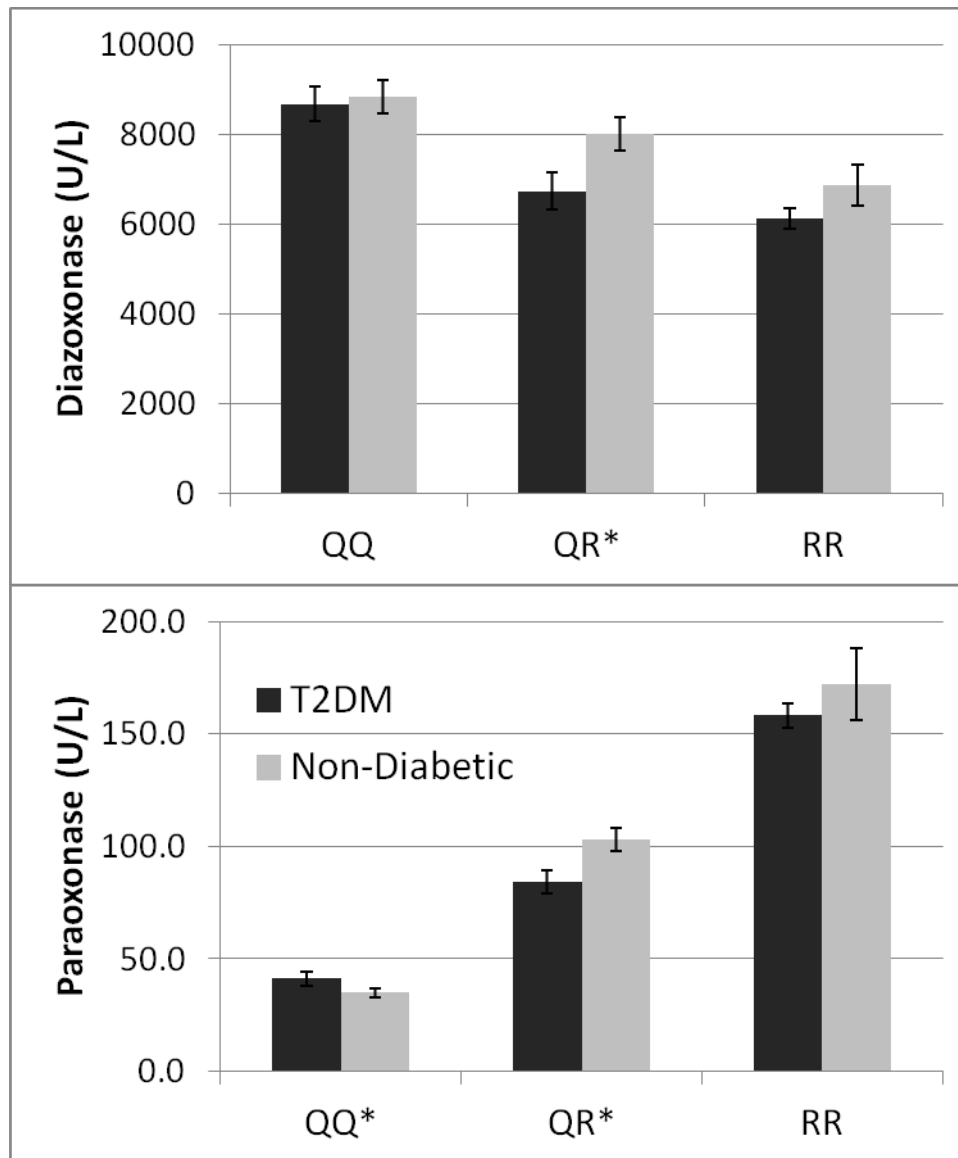


Figure 3.3 Graphical representation of paraoxon hydrolysis activity and diazoxonase activity sorted by PON-1<sub>192</sub> genotype. All results are expressed in (U/L). p<0.05 indicated with an asterisk.

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## CHAPTER IV

### CONCLUSIONS

Type 2 diabetes mellitus is an epidemic that affects millions of people around the world and is expected to continue to rise over the next decade (WHO 2011). There have been literally dozens of research and clinical medical laboratories studying this epidemic and so far no solitary causative agent has been identified. The disease appears to be caused primarily by a combination of hyperglycemia and chronic inflammation. Both of these factors are influenced by a whole host of causes, including environmental factors, age, genetic variations, lifestyle, and toxicant exposure. Excess inflammation, as a result of the body trying to maintain homeostasis and correct metabolic irregularities, is the most important causative factor in my opinion. Hyperglycemia frequently results in excess lipid being deposited in the adipose and this hypertrophy of the adipocytes results in increased inflammation. This inflammation then prevents the body from being able to properly remove excess glucose, thus resulting in the excess being deposited in the adipose, and so forth. Additionally, the introduction and consequent widespread use of high-fructose corn syrup has increased the weight gain seen with excess caloric intake and directed that excess weight to the abdominal region where the white adipose tissue resides (Bocarsly et al. 2010).

This project began with an examination of the ability of paraoxonase-1 (PON-1), a potent antioxidant, to retard that inflammation and as a result reduce Type 2 diabetes mellitus (T2DM) prevalence. In addition, organochlorine compounds (OC) such as p,p'-

DDE, trans-nonachlor and oxychlorodane were assessed for their possible role in promotion of T2DM prevalence as well as their being possible promoters of chronic inflammation seen to be a contributing factor to T2DM development (Plomgaard et al. 2007; Yang et al. 2009). This chronic inflammation has been viewed as a frequent cause of disruption of insulin receptor substrate-1 (IRS-1) pathways and results in the inability of insulin to properly shepherd glucose into the tissues. This metabolic disruption subsequently results in a shift of that glucose into the adipose tissue, disrupting several adipokines whose roles include maintaining insulin sensitivity and preserving metabolic homeostasis. Some literature disputes this association between insulin sensitivity and inflammation (Carey et al. 2004), instead arguing that they contribute to adiposity, itself a contributing factor to T2DM.

The analysis of paraoxonase-1 (PON-1) activity was an important first step in this project as its anti-inflammatory role is considered a key contributing factor to T2DM development. This study demonstrated that the most commonly used substrate, paraoxon, produced results that indicated a higher level of PON-1 activity in T2DM subjects. While this average was not significantly higher than that of the non-diabetics it does go against what the majority of scientific literature indicates. This may have been due to a number of possible of reasons.

The subject population was overwhelmingly Caucasian and as such had a high percentage of the PON-1<sub>192QQ</sub> genotype. This genotype demonstrated a higher PON-1 activity with this substrate in the T2DM subjects. In addition, the population was collected from military base medical centers. While there is no scientific evidence of this it is possible that the subjects in the study enjoyed a higher level of fitness due to their occupations and as a result a slower progression of T2DM. Also the high limit for age in



this study was set at 65 and could also reflect earlier progression in the T2DM subjects. Rosenblat et al., among others, has pointed out that when PON-1 is incubated in serum with elevated glucose concentrations that the PON-1 activity is reduced over time (Mackness et al. 2008). This reduction is as high as 15-20% more than control serum incubated over the same time period. This reduction is significant in the case of T2DM since it is more likely that T2DM subjects will have longer episodes of hyperglycemia compared to their non-diabetic cohorts and that this elevation in blood glucose could result in the decrease in PON-1 activity seen in T2DM subjects. However, when using the substrate diazoxon the typical depression in PON-1 activity seen in T2DM subjects was observed. Phenyl acetate hydrolysis showed no statistical difference between the T2DM and non-diabetic populations. I believe this could be caused by a couple of contributing factors. Aspirin and statin usage both upregulate PON-1 mRNA and while neither set of medications were tracked in the study patients it seems possible, based on the cholesterol levels in the T2DM vs the non-diabetic subjects, that increased statin usage in the T2DM subjects contributed to higher PON-1 activity levels. In addition, elevated TNF- $\alpha$  downregulates PON-1 activity and I would speculate that possibly increased OC compound blood concentrations could contribute to elevated TNF- $\alpha$  and thus skew PON-1 activity.

PON-1 genotype is considered by some labs to be more of an indicator of contribution to T2DM than the overall activity itself. In our study we concluded that the PON-1<sub>192RR</sub> genotype was the most highly associated with T2DM development. This genotype was found to be significantly higher in African-Americans, an ethnic group that has been associated with higher T2DM incidence. The PON-1<sub>55</sub> genotype was not found to be individually associated with T2DM prevalence, however when a combination of the

PON-1<sub>192</sub> and PON-1<sub>55</sub> genotypes was examined the RRLM combined genotype was statistically indicated as having the highest association with T2DM. This may be confounded by the significance that the PON-1<sub>192RR</sub> genotype already has, however. In line with that consideration, the QQMM genotype was statistically associated with non-diabetic subjects.

Detection of organochlorine compounds revealed that the majority of the subjects, including both T2DM and non-diabetic subjects, had measurable levels of at least one of the three target compounds in our study. This agrees with multiple studies that show that even decades after the United States stopped using these compounds as pesticides that they maintain a baseline concentration in our soil and water tables (Bidleman et al. 2003; Jiang et al. 2009). The lipophilic nature of these compounds has resulted in most, if not all, people maintaining a level of these in our adipose tissue and, while there has been no direct mechanism identified, it is believed that these compounds contribute to the low level of chronic inflammation found in many of the study subjects. DDE presented with the highest concentrations, which makes sense when you examine the amounts of each of these pesticides that were employed in the US agricultural regions. However, trans-nonachlor was identified as having a more significant association with T2DM development and increased inflammation.

Examination of inflammatory marker levels is important in early identification of a chronic inflammatory state that could contribute to T2DM as well as other inflammatory diseases such as rheumatoid arthritis. TNF- $\alpha$ , MCP-1 and leptin were all significantly higher in non-diabetics with elevated levels of some of the studied organochlorine compounds. There is no mechanism specifically linking OC plasma concentration with T2DM; however, I would speculate that the bioaccumulation of these

OC compounds promote reactive oxygen species formation which would in turn promote TNF- $\alpha$  and MCP-1. Both of these markers have been linked to insulin resistance through serine phosphorylation of steps of IRS-1, contributing to T2DM development. In addition to elevated inflammatory marker levels, increased OC plasma concentration was associated with abnormal adipocyte results.

The lipophilic nature of the OC compounds leads me to presume that higher levels of these compounds were in the adipose tissue than that found in the circulating blood. This is supported by a recent study that examined gastric bypass surgery that resulted in a decrease in adipocyte mass and a correlating increase in blood OC levels (Hue et al. 2006). Increased levels of leptin and decreased levels of adiponectin were seen in the T2DM population studied here. Leptin, a hormone that tells the body when it is full, is blunted in T2DM subjects based on other research and the same was found in our study. Adiponectin, a potent insulin sensitizer, was decreased, also agreeing with previous research. Amylin was not found to be significantly increased in the T2DM subjects; however, this may be due to the age of the population. Some research indicates that amylin formation happens as the pancreatic  $\beta$  cells undergo apoptosis and amyloid deposits are produced. The relatively young age of the population could indicate that the T2DM disease progression had not gone that far for most of the subjects and so insufficient amylin would be generated. These adipokines, as well as other results of the experiments in this project, merit further study.

Early identification of T2DM is critical to staving off the disease from progressing from a simple inflammatory state to prediabetes to frank T2DM. Additional experiments that investigate inflammation in healthy as well as diseased populations at younger ages would enable identification of contributing risk factors. In addition, long

term study of inflammatory marker build-up would give insight as to timelines for people to develop disorders based on lifestyle and other demographic factors. Given that the study subject population was a military population a comparison of equivalent subjects from a civilian hospital would help identify if lifestyle was a more or less important factor in T2DM. It would be assumed that the lifestyle has a role to play given the geographic concentration of T2DM in the southern portion of the US. Animal models could be very helpful in some of these examinations, particularly in determining if the OC compounds versus overall obesity, hyperglycemia, or genetic factors present as substantial risk factors for T2DM. Further study of adipokine involvement in T2DM would also be of great benefit. Most of the adipokines have been studied only recently and many of their mechanisms of action have not yet been fully elucidated. Animal and cell models for leptin as well as some of the lesser known cytokines like ghrelin might tease out some of the details as to the involvement these compounds have in insulin sensitivity.

In conclusion, T2DM is a multi-systemic disorder that develops as a result of not one particular factor but a host of contributing causes. Some of these causes, such as sedentary lifestyle and excess caloric intake, can be managed by the patient. There are others, though, that many people do not have control over such as the amount of pesticide residue in their food and genetic variations that make one more susceptible to the biochemical mechanisms of T2DM. By identifying these risk factors and enabling the clinical practitioners to spot them at an earlier stage it gives the patient a better chance at preventing development of the disease. The World Health Organization predicts that deaths from diabetes will double by 2030 (WHO 2011) and T2DM is already the 7<sup>th</sup> most common cause of death in the United States (CDC 2011) . Continued research as to

causative factors in T2DM not only helps the physicians treating but the scientists looking for solutions, both pharmacologic and lifestyle.

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