ABSTRACT

ALLEN, HENGAMEH G. Role Of Membrane Lipids in Developing Insulin Resistance Diabetes Mellitus Type II in Caucasians and African Americans. (Under the direction of Dr. Jonathan C. Allen.)

Insulin resistance in diabetes (DMII) can result from membrane lipid (PM) changes. Membrane lipids play a major role in hormonal signal transduction and in appropriate amounts of such molecules may lead to either decreased, or increased membrane fluidity. Therefore, in this study we determined whether PM differences exist between African Americans (BL) and Caucasians (W) and if differences contribute to impaired insulin binding (IB) in DMII. **Methodology:** Subjects were recruited from Caucasian Control (CC) (n=10), African American Control (AC) (n=10), Caucasian Diabetics (CD) (n=5), African American Diabetics (AD) (n=10) groups. The diabetics were type II diabetics on daily insulin injections (age and sex-matched in both racial groups). The evaluations consisted of: three day dietary record, serum total cholesterol (TC), triglyceride (TRG), very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL), glucose (Glu), hemoglobin A_{1C} (A1C), insulin (Ins) levels and RBC membrane lipid composition including, fatty acids, phospholipids (PL), cholesterol (CH) and RBC insulin binding (IB), **RESULTS:** Cholesterol intake did not show any correlations with

any blood constituents, membrane lipids or membrane properties. The macronutrient intake did not show a significant correlation with blood lipid levels, membrane properties and the anthropometric measurements as expected. The triglyceride levels were higher in diabetics (P <0.01). The lipoprotein evaluation indicated significant differences in VLDL (P <0.01), LDL (P <0.05) and HDL (P <0.0001) levels between control and diabetic subjects. There were no racial differences seen among the four groups. Diabetics had higher CH and it correlated with Glu (r= 0.65, P<0.05) and IB (r= -0.61, P <0.05). The CH / PL revealed strong correlation between LDL (r=0.42, P <0.01) and HDL (r= -0.56, P <0.05). The PM trans fatty acid levels (TFA) were highest in AD (P <0.01), but no correlations with IB & Glu. The PM saturated / polyunsaturated ratio (S / P) was higher in diabetics (P <0.05) correlating with insulin level (r= 0.42, P <0.01) & IB (r= -0.45, P <0.05), but no correlations with serum lipids occurred. The PL analysis showed no significant group differences for phosphatidyl inositol (PI) levels; however, significant racial differences were observed in phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC). The W showed higher PE levels than BL and lower PC. The RBC rheological (PE / PS) properties (deformability) was lower in diabetics and AC than CC. The saturated nature of RBC [(SPH+PC) / (PE+PS)] was the lowest in CC (P< 0.056).

The combination of increased S / P, and increased saturated nature, decreased PE / PS, increased CH / PL indicate decreased membrane fluidity and decreased RBC

deformability, which might contribute to decreased IB in DMII. Differences in PE and PC levels between BL and W possibly indicate a racial difference in cause of insulin resistance. The racial differences in developing DMII need to be recognized so the therapeutic agents can target the exact problem in the metabolic pathway to correct the insulin resistance.

Role Of Membrane Lipids In Developing Insulin Resistant Diabetes Mellitus Type II In Caucasians And African Americans

BY

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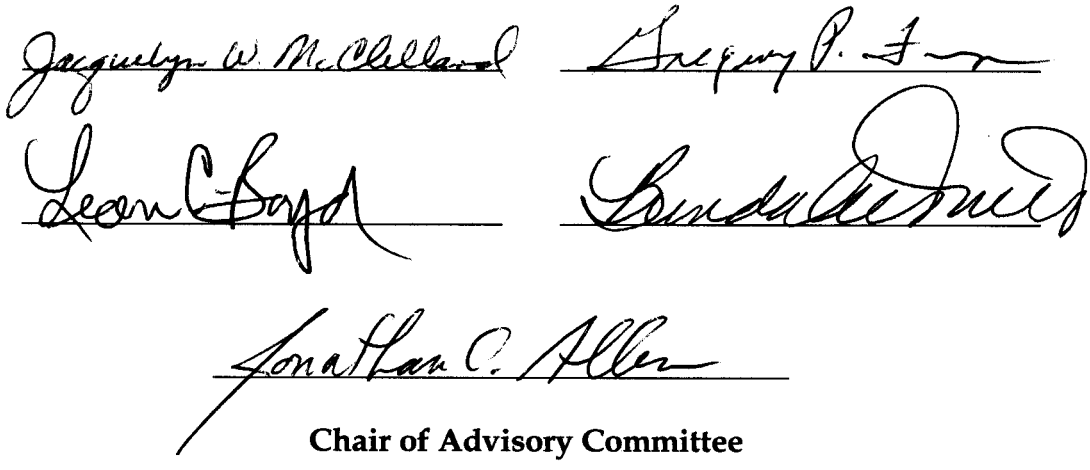

Chair of Advisory Committee

“In The Name Of God all mighty and merciful”

I thank God for his mercy, love and being the light of knowledge that shines in the darkness of all ignorance.

DEDICATION

I dedicate this dissertation to my beautiful mother Parrie whose love has kept me determined in finding a way to battle diabetes that has taken so much from her, to my loving husband Dr. Cyril A. Allen whose continuous encouragement and support has turned my dreams into reality, to my beautiful son Tony for all the joy he brings into my heart and soul, to my father Reza for his love and kindness, to my sister & my best friend Parvin for making me an aunt for a second time, to my brother Ali for his kindness, and last but not least to my second mother Attorney Sylvia X. Allen for her love and continuous encouragements.

Biography

Hengameh G. Allen was born on December 22nd 1962 to father Reza Ghaderkhani and mother Parvaneh Miralinaghi in Tehran, Iran. She lived in Iran for the first 15 years of her life and because of the revolution in Iran she had to leave her home to pursue her education. She then attended Massanutten Military Academy in Woodstock Virginia.

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She is dedicated to understanding the role that nutrition plays in pathophysiology of diabetes and how the burden of this disease can be reduced in communities with an emphasis on minority health.

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CHAPTER I

A Review of Diabetes & The Role Of Membrane Lipids

INTRODUCTION

An Overview of Diabetes Mellitus

Diabetes is a serious, costly and complicated disease and is rapidly rising in incidence. Diabetes mellitus is a disorder of most notably glucose metabolism, accompanied by long-term vascular and neurologic complications. Diabetes has a distinct etiology, clinical presentation, and course. There are two types of diabetes: 1) diabetes type I also known as Insulin Dependent Diabetes Mellitus (DMI) and 2) diabetes type II known as Non-Insulin Dependent Diabetes Mellitus (DMII).

The burden of this disease is greater among the minority populations and the elderly. The African Americans and Native Americans have greater incidence of diabetes than the Caucasians. Diabetes poses a great public health concern. The incidence of diabetes during 1980-1994 has increased by 39% (CDC, 1994). The incidence has been the highest among black females. The rate of increase between 1980-1994 in age-adjusted prevalence was highest among black males (29% increase). In 1994 all diabetes-related deaths from cardiovascular complications were 44%, and the lower extremity disease was more common among diabetics.

The discovery of insulin from the islets, and its isolation as the active glucose-lowering agent, and its use in diabetic animals by Banting and Best in Toronto and by Paulesco in Romania resulted in the successful treatment of diabetic patients on January 11, 1922 (Banting and Best, 1922; Burrow et al., 1982).

Insulin provided hope for controlling a previously fatal disease. The use of insulin also resulted in dramatically lowering the mortality associated with metabolic acidosis. However insulin-treated patients developed seemingly relentless long-term complications leading to blindness, kidney failure, and peripheral and cardiovascular disease. A fatal disease transformed into a chronic disease revealing the related complications and premature mortality associated with this disease.

Modifications of insulin have provided insulin formulations that will achieve the targeted glucose levels throughout the day (Zinman, 1989). Insulin analogues, created by the substitution of specific amino acids and other routes of administration (e.g. nasal and rectal) in treating diabetes (Brange, et al., 1990 and Selam, et al., 1990) are being investigated. Pancreas transplantation and encapsulated islet cell transplantation have become successful methods of treatment in recent years, (Nathan et al., 1991, Robertson, 1992 and Lanza et al., 1992).

Diabetes Mellitus Type II (DMII)

Non-insulin dependent diabetes mellitus accounts for 95% of the diagnosed diabetes cases. The data from the National Health and Nutrition Examination Survey confirmed an overall prevalence of 6.6 percent in the United States (Harris, 1987). There are more than 500,000 new cases reported each year, and the projected prevalence for the next decade is 10 percent. Approximately

one half of the DMII patients are unaware of their disorder. The risk factors for DMII include older age, obesity, and a history of gestational diabetes. The increase in the prevalence of DMII in the United States is commonly believed to be due to an aging population that is also increasingly obese and sedentary. About 90 percent of DMII patients are obese with mostly central obesity, which is reflected by an increased waist-to-hip ratio that may be an even more important risk factor for DMII than obesity, defined as excess body fat (Hartz et al., 1984). Also, certain isolated ethnic populations, such as the Pima Indians of Gila River, Arizona, and the Nauruan islanders of Nauru, Micronesia, have DMII prevalence as high as 50 percent in the adult population (Savage et al., 1979; Zimmet et al., 1977). The suspected contributory factor in these populations seems to be the dietary changes leading to obesity, and a sedentary life-style.

Genetic predisposition is also a major factor in developing DMII . A glucokinase with an abnormally high Km (Michaelis constant) has been identified in some, but not all, families with maturity-onset diabetes in the young (MODY) and may be the cause of decreased insulin secretion (Froguel, 1992). The individuals with MODY tend to be thin individuals. A variant of MODY in African Americans has been described in Florida (Winter et al., 1987).

PATHOGENESIS

Insulin Resistance in Diabetes Mellitus Type II

Although the major factor in Diabetes Mellitus Type I (IDDM) is low or total absence of insulin secretion, there are multiple factors leading to DMII. Insulin resistance (i.e., decreased sensitivity to insulin action) seems to be the major contributory factor in these patients. This lack of adequate insulin action could be caused at the pre-receptor, at the receptor, or at the post-receptor level. The presence of neutralizing insulin antibodies in plasma that can bind insulin, or a defective insulin molecule (very rare) can be the cause of pre-receptor malfunction. The receptor problem can be due to inability of insulin to optimally bind its receptor (e.g. defective receptor or presence of other interfering substances) or the presence of insulin receptor antibody.

The post-receptor problems can result from inadequate molecular activation of their signal transduction pathways. Abnormalities are apparent either in the structure of the insulin receptor, in the phosphorylation of the tyrosine residue by the enzyme tyrosine kinase that is integral to receptor function or impaired membrane lipid action (improper membrane fluidity). A defective insulin-mediated stimulation of tyrosine kinase and autophosphorylation has been demonstrated to be impaired in DMII (Moller and Flier, 1991).

The previously mentioned receptor and post-receptor defects may also exist as a result of inappropriate membrane fluidity. Phospholipids (PL) and the fatty acid species attached to PL play a major role in hormonal signal transduction (e.g. phosphatidylinosital and phosphatidylserine) and not having the appropriate amounts of these molecules may lead to either decreased, or increased membrane fluidity. Cholesterol also plays an important role in determining the membrane fluidity and contributes to rigidity of the membrane. These effects are reversible with improved diet, glycemia and probably do not account entirely for insulin resistance.

The metabolic abnormalities resulting from insulin resistance are: persistent hyperglycemia, especially postprandial hyperglycemia (Mitrakou et al., 1990), elevated levels of VLDL and triglycerides and decreased levels of high-density lipoprotein (HDL) cholesterol (Walden et al., 1984).

Complications

Diabetes complications include a spectrum of microvascular and neurologic disorders. Retinopathy and nephropathy are common with diabetes. Macrovascular complications, including cardiac, peripheral and cerebrovascular disease, occur in greater numbers in diabetics than the nondiabetic population.

It is possible that hyperglycemia causes many of the complications, perhaps through glycosylation of lysine residue within membrane proteins or through other unknown mechanisms. In certain studies it has been shown that

restoring normal glucose levels prevented or ameliorated diabetic retinopathy and nephropathy (Engerman and Kern, 1987; Mauer et al., 1975; Gray and Watkins, 1976).

Biomembranes

Biological membranes are organized in sheet-like assemblies composed mostly of proteins and lipids. They have an important function in metabolic regulation of cells. They are highly selective permeability barriers and contain specialized pumps and channels that mediate transport of molecules across the membrane. The biomembrane mediates communication between the internal cellular environment and the external cellular environment via interactions of protein molecules called receptors with other stimulatory molecules. In certain cells the membrane is capable of generating chemical or electrical impulses, which serve as signals.

The membrane molecules are associated by non-covalent interactions, which are cooperative. The lipids are arranged in asymmetric bilayers with the phosphate groups facing the outside and the inside of the membrane. The phosphate head groups contribute to the negative charge on the cell surface. The three major kinds of membrane lipids are phospholipids, glycolipids and cholesterol with the phospholipids being the major class of membrane lipids. The fatty acids bound to the phospholipids and glycolipids usually contain even numbered carbon chains (typically 14-24) with the palmitate and 18-carbon fatty

acids being the most common ones. The fatty acids found in animal tissues are not branched and may be unsaturated containing cis bonds. Phosphatidate is the major substrate for synthesis of major phosphoglycerides present in the membrane. The common alcohol moieties of phosphoglycerides are serine, choline, glycerol, ethanolamine and inositol. The membrane phospholipids have a hydrophobic (non-polar) tail and a hydrophilic (polar) head. Phospholipids and glycolipids cluster via van der Waals attractive forces to minimize the number of exposed hydrocarbon chains.

The cis configuration contributes to a greater fluidity of the membrane. The kink created by the cis double bond interferes with a highly ordered packing of fatty acyl chains hence increasing membrane fluidity.

Cholesterol makes up a large proportion (20-25% by mass) of the mammalian plasma membrane lipid and plays a major role regulating membrane fluidity. The intercalation of the cholesterol molecules between the hydrocarbon tails of the lipids in the bilayer greatly broadens the phase-transition temperature range. The rigid ring structure of cholesterol restricts the mobility of fatty acyl chains of lipids in the liquid-crystalline state and thus decreases fluidity.

Membrane lipids contribute greatly to hormonal signal transduction. The amounts of cholesterol and phospholipids in the membrane have dramatic effects on the membrane fluidity. The increased membrane fluidity will allow for better transverse movement of the membrane proteins (i.e. hormone

receptors, ion channels, etc.) involved in many metabolic functions of the cell.

Membrane Fluidity & Diabetes

Membrane fluidity plays a very important role in proper hormonal action especially when peptide hormones are concerned. There is adequate evidence of impaired membrane fluidity in diabetics (Garnier et al., 1990; Keelan, 1990; Mooradian et al., 1990; Kantar et al., 1991).

Decreased plasma membrane fluidity either due to increased CH to PL or increased saturated (SAT) to polyunsaturated (PUFA) fatty acids is considered an important predisposing factor for increased exposure of platelets to the external environment and in turn can result in increased sensitivity in diabetic patients (Watala, 1991). In DMII platelet membrane fluidity abnormality contributes to platelet function alterations (Caimi, et al., 1995). The membrane lipid fluidity tends to decline with age and shows an age-dependent increase in CH / PL and a decrease in lipid / protein ratio (Carmen, 1997)

There is altered phosphatidylinositol (PI) and phosphatidic acid (PA) changes in the RBC membrane of diabetic subjects due to a decreased metabolic pool size of phosphatidates (Kamada, 1992). Horrobin (1995) has linked the presence of abnormal concentrations of 20 and 22-carbon essential fatty acids (EFA)to coronary and peripheral vascular disease. That study showed that even though elevated CH levels is associated with coronary artery disease, the hyperlipidemia cannot solely explain the pathophysiology of this disease.

Insulin Resistance & Diabetes Mellitus Type II

Insulin resistance (IR) and lipid metabolism seem to be closely related. Hyperinsulinemia, central obesity and dyslipidemias are typically accompanied by insulin resistance and are thought to lead to overproduction of VLDL (Modan, et al., 1988). Insulin resistance in diabetics can result from membrane lipid changes (a post-receptor defect) (Defronzo et al., 1989; Shulman et al., 1990). Dietary habits such as increased fat (e.g. saturated and cholesterol) intake can lead to hyperlipidemia hence increasing the cellular LDL. The number of insulin receptors and the amount of insulin bound in the tested subjects with increased LDL were correspondingly low (Sanghvi et al., 1983).

Hyperinsulinemia occurs in cases of insulin resistance since glucose cannot be cleared as rapidly (especially post-prandially) in the absence of adequate insulin binding, so the hyperglycemia provokes further insulin secretion in response to plasma glucose levels.

Obesity is an important risk factor in alterations in membrane lipids and eventually development of insulin resistance. Membrane lipid alterations in the past have been detected by the CH / PL ratio but also changes in the types of fatty acids (i.e. PUFA, MUFA and TFA's) that comprise the PL.

For example obese children exhibit significantly higher values of arachidonic acid (AA) and the AA / linoleic acid (LA) ratios were greater than normal children. The plasma glucose was inversely related to AA in TG, and plasma TG level was inversely related to AA in PL (Tamas, 1996). The delta-6

desaturase enzyme activity was higher in obese children possibly due to higher fasting insulin levels.

Dietary fats can influence insulin action. Both insulin binding and insulin responsiveness are altered by dietary fat-induced changes in the fatty acid composition of the PL of adipocyte plasma membranes (Clandinin, et al., 1993).

Insulin Resistance & Membrane Lipids

The IR problem may also exist as a result of inappropriate membrane fluidity. Phospholipids play a major role in hormonal signal transduction and not having the appropriate amounts of such molecules may lead to either decreased, or increased membrane fluidity. Insulin resistance in skeletal muscle is thought to be caused by membrane lipid changes (Defronzo et al., 1989; Shulman, et al., 1990).

Cholesterol also plays an important role in determining the membrane fluidity and contributes to rigidity of the membrane. It has also been demonstrated that the amount of insulin specifically bound and the number of insulin receptors per cell were inversely correlated with LDL level. The number of insulin receptors and the amount of insulin bound in the tested subjects with increased LDL were correspondingly low (Sanghvi et al., 1983). These effects are reversible with improved glycemia and probably do not entirely account for insulin resistance.

Racial Differences in DMII & Its Complications

The disparity in the prevalence of DMII and cardiovascular disease between African Americans and Caucasians has been well established. Ethnic

differences in several risk factors for these diseases are evident even in childhood. In one study the African American children seem to show a greater disease risk than Caucasian children, even after adjusting for body composition, social class background, and dietary patterns (Lindquist and Gower, 2000).

Insulin resistance has been associated with increased cardiovascular risk factors, dyslipidemia, hypertension, impaired fibrinolysis, and coagulation even among non-diabetic individuals. The relationship between insulin resistance and cardiovascular risk factors is not fully understood in DMII. Haffner and co-workers concluded that insulin-resistance in DMII subjects were associated with more atherogenic cardiovascular risk factor profiles than insulin-sensitive DMII subjects and that this was only partially related to increased obesity and an adverse body fat distribution (Haffner et al, 1999). The metabolic abnormalities resulting from insulin resistance are: persistent hyperglycemia, especially post-prandial hyperglycemia (Mitrakou et al., 1990), elevated levels of VLDL and triglycerides and decreased levels of high-density lipoprotein (HDL) cholesterol (Walden et al., 1984).

Prolonged hyperglycemia and IR leads to complications such as: blindness, kidney disease, amputations, heart attack and stroke. However, AFA experience higher rates of at least three of the serious complications of diabetes: blindness, amputation and end stage renal disease (kidney failure). African Americans with diabetes are 2.6 to 5.6 times more likely to suffer from kidney disease, with more than 4,000 new cases of end stage renal disease each year. African

Americans are twice as likely to suffer from diabetes-related blindness and are 1.5 to 2.5 times more likely to suffer from lower limb amputations (American Diabetes Association, 2000).

Insulin resistance seems to be related to more diabetes complications than the hyperglycemia by itself. In one study it was shown that hyperglycemia alone fails to cause nephropathy in patients with DMII because sub-optimal diabetic control is present in DMII patients without end-stage renal disease (Freedman et al., 1995). Freedman and co-workers also demonstrated that differences in family size and degree of diabetic control are unlikely to account for the differences observed between families in the minority groups studied. Hyperlipidemia has been considered as an etiology since it results in decreased insulin receptor numbers, but the type of hyperlipidemia (e.g. type II mostly in AA vs. mostly type IV in Caucasian) shows a racially different effect on hypertension among patients with DMII (Werk, 1993).

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CHAPTER II

Can Anthropometric Measurements and Diet Analysis Serve As Useful Tools In Determining Risk Factors for Insulin Resistant Diabetes Type II Among Caucasians and African Americans?

INTRODUCTION

Obesity is a metabolic disturbance mostly occurring in societies where energy intake exceeds energy expenditure. Thirty million adult Americans weigh more than 26% (10-40% of the population in various age groups) above their desirable weight (Kuczmarski, 1994). Prevelance of obesity is increasing in all major race / sex groups including younger adults age 25 to 44. Obesity has been recognized as a major cause of DMII with insulin resistance (Barrett-Conner et al., 1982) as well as hypertension, coronary artery disease, arthritis and pulmonary diseases.

The distribution of fat in the central or upper body regions is more likely to be associated with hypertension than lower body fat (in the gluteal or thigh region) (Ohlson et al., 1985; Lapidus et al., 1984; Larsson, et al. 1984). The central obesity fat can result in the release of non-esterified free fatty acids (NEFA) into the portal vein. The increased plasma NEFA will in turn cause an excess hepatic synthesis of triglycerides leading to insulin resistance and hyperinsulinemia.

Case-control comparisons of 356 diabetics matched for age and obesity with 356 non-diabetics confirmed the significantly higher triglyceride levels in diabetes. Conversely, hypertriglyceridemia was associated with diabetes in 29 per cent of non-obese men and 25 per cent of obese men, and in 10 per cent of non-obese women and 21 per cent of obese women (Barrett-Connor et al. 1982).

Obesity is also considered a major factor in causing insulin receptor dysfunction. This dysfunction is thought to be related to the increased

expression of tumor necrosis factor alpha (TNF-α) and abnormality in protein tyrosine phosphatase in skeletal muscle (Nippon, 2000). Therefore, the enhancement of insulin sensitivity by reducing weight, lowering plasma lipid levels and modulating factors affecting insulin receptors has been a recommended treatment for insulin resistance.

Central obesity is implicated in the development of insulin resistance by increasing insulin demand and eventually leading to hyperinsulinemia. Prolonged hyperinsulinemia (possibly via receptor down-regulation) can lead to insulin resistance at the hepatic and muscle tissues. Ironically the adipose tissue sensitivity to insulin remains high. The adipose tissue insulin sensitivity is partially responsible for the hypertriglyceridemia via hormone sensitive lipase stimulation by insulin in diabetic individuals. Some of the insulin resistance in obesity is attributed to a decrease in insulin receptors, but post-receptor defects are also present (Skrha, 1996). Weight reduction in overweight diabetic individuals leads to an improvement in glycemic control and reduction of plasma lipids in most patients (Purnell, 2000).

Anthropometric measurements have been helpful in determining the risk factors in developing DMII. The body mass index (BMI) is used as a tool to determine whether an individual is over weight or obese medically. It is clear that the body fat distribution correlates closely to developing complications among the overweight individuals. The abdominal visceral adiposity has also been shown to be associated closely with the development of more health

problems than individuals with a lower central obesity (Després, 1989 and 1990). Individuals with altered metabolic profiles such as the lipids and the fasting insulin levels seem to have a more substantial risk of ischemic heart disease [Lamarche et al., 1996) as seen in DMII.

The anthropometric measurements seem to be a fairly accurate method of predicting risk factors for developing DMII (Ohlson et al., 1985; Lapidus et al., 1984; Larsson et al., 1984). Determination of waist to hip ratio (W / H) is also helpful in predicting abdominal visceral adiposity in men and pre-menopausal women (Pouliot et al., 1994). Increased W / H is associated with a greater possibility of finding metabolic abnormalities such as insulin resistance and glucose intolerance (Després et al., 1993; Lemieux et al., 1996). It is reasonable to assume that an increased W / H is secondary to increased visceral adiposity (if no other medical conditions exist such as ascites) and can be used as a useful tool for DMII risk assessment. It is important to note that such an increase in W / H can be observed even in the absence of any change in the patient's BMI.

In this study we investigate if anthropometric measurements differ among diabetics of different races. We also investigated whether nutrient intake of these individuals relates to anthropometric measurements.

METHODOLOGY

Subjects

The subjects were recruited from 4 groups 1) Caucasian Control (CC) (n=10), 2) African American Control (AC) (n=10), 3) Caucasian Diabetics (CD) (n=5), 4) African American Diabetics (AD) (n=10). The diabetic subjects were type II diabetics with insulin resistance (they take insulin injections daily). The subjects were age and sex-matched in both racial groups. The subject ages ranged from 23-60 years and equal number of males and females in each group except in CD group (3 males and 2 females). The control subjects were recruited based on the following eligibility requirements: 1) must have no health problems, 2) must not be taking any medications, 3) female subjects must not be taking estrogen (the blood was collected on the last week of menstrual cycle). The diabetic subjects were on insulin injections and were not excluded based on any other illnesses.

Diet Analysis

The subjects were asked to record a three-day diet record consisting of two week days and one weekend day. The subjects were instructed on how to determine serving sizes of dietary intake. They were instructed to be as accurate and precise as possible while recording their diet. The diet record was to reflect an average nutrient consumption (including nutritional supplements) by each

individual. The collected diet records were analyzed by utilizing Nutritionist V computer program.

Anthropometric Determination

BMI (kg / m2) was computed from measurements of weight to the nearest pound and height to the nearest centimeter (BMI= W / h^2 X 2.2 kg / lb X 10^{-4} m^2 / cm^2). The ratio of waist (umbilical level) and hip (maximum buttocks) circumference was calculated.

Body Fat Percent

The body fat percent was obtained by measuring skin folds on different parts of the body. In males the chest, mid-thigh and triceps, abdominal skin folds were measured and in females the suprailium, triceps, mid-thigh skin folds were measured. The body fat % was obtained by reading the chart provided with the caliper (Fat-O-Meter, Novel Products, Inc. Rockton, IL). The same individual determined the measurements to avoid variability in measurement technique.

Statistics

Statistical analysis was performed using SAS computer software (SAS, Cary NC). Data were analyzed by 2-way-ANOVA using General Linear Model and the Pearson Correlation procedure. The data was split by health status

(diabetic vs. non-diabetic) and race (Caucasian vs. African American). Also the age was used as a covariant in analysis of all dependent variables. Group means were compared by least significant difference at $p<0.05$.

RESULTS

Total Macronutrient Consumption (Table 1)

The average Calorie consumption of CC group was 1958 kilocalories (Kcal) and the consumption was approximately 100 Kcal more than the AC group (Table 1). Although the CD group's average Calorie consumption (2383 Kcal.) was 769 Calories greater than the AD group, statistical analysis did not show significant differences.

The average protein intakes were 84, 74, 100 and 83 and 94 g in CC, AC, CD and AD groups respectively. There were no significant differences when compared racially or by health status (control vs. diabetic).

The average carbohydrate (CHO) consumption also did not show a statistical significance among different groups when compared racially and by health status.

The average fat intake was significantly lower in the control (CC and AC) than in CD group, which had the greatest average fat consumption (P <0.05). Racial comparisons indicated a higher fat intake by the Caucasian subjects than

the African American subjects (P <0.05). The fat intake was also categorized as the amount of saturated fat (Sat), monounsaturated fat (MUFA), polyunsaturated fat (PUFA), linoleic acid (LA), linolenic acid (GLA), docosohexaenoic acid (DHA) and eicosopentaenoic acid (EPA). The percentage of dietary fat from SAT, MUFA and PUFA categories were similar among the groups (Figure 1). The CC group had the lowest Sat% consumption (Table 1) and it is shown that the diabetics had a lower PUFA% consumption than the control subjects. The diabetics also consumed larger amounts of Chol than the control groups (P <0.05) but no statistical significance was seen between CD and AD groups. The consumption of Sat (P <0.05), MUFA (P <0.01) and PUFA (P= 0.06) among CD subjects was greater than AD group. The MUFA consumption was lower among the control subjects (CC and AC) groups when compared with the diabetics (CD and AD) (P <0.05). In all four groups a much greater Omega 6 fatty acid consumption than Omega 3 fatty acids was apparent.

Total Micronutrient Intake (Table 2)

The average micronutrient intake was calculated for all four groups. Vitamin A intake was significantly greater in the CC group than in the other three groups. The greatest consumption was by the CC group (P <0.05) and the lowest consumption by the AC group (P <0.05). When the groups were racially compared the Caucasians consumed more vitamin A than the African Americans

(P <0.05). The control subjects consumed more vitamin A than their diabetic counterparts.

The average intake of antioxidant nutrients (e.g. vitamin E, beta-carotene) was greater among the CC group than the rest of the groups. The total mineral intake among all four groups was mostly below the RDA (with the exception of iron in the CD group) and other micronutrient intakes did not differ among groups.

Anthropometric Measurements

Body Fat Percent

The body fat percent (BF%) was the lowest in CC group and the highest in the AD group (Figure 2). The CC had a lower BF% (17.2%) in comparison to the AC group (21.2%) (P <0.01). The same pattern was seen in the diabetic groups with the CD having a lower BF% (25.2%) than the AD group (31.7%) (P <0.05).

BMI (Figure 3a & 3b)

The diabetic male and female groups had a greater BMI than the control subjects. The female AD had the greatest average BMI (29.0) among the female subjects, but the CD males had the greatest BMI (41.6) among the other male subjects (P <0.05). The data also showed a difference between the control and the diabetics (P <0.05) in both male and female subjects.

The waist to hip ratio is shown in Figure 4 and waist to thigh ratio is shown in Figure 5. The control subjects had similar measurements but the diabetics (CD and AD) showed a higher W / H (1.0 and 0.9 respectively) indicating central obesity. The W / H value is slightly lower in the AC and the AD group possibly due to a wider hip measurement seen in African American individuals in comparison to the Caucasians. The average W / T value was also greater in the diabetic groups in comparison to their respective control groups. The data showed the greatest ratios in the CD group.

DISCUSSION

One of the objectives of this study was to examine whether anthropometric measurements vary with the individual 's health state and whether there are racial differences among diabetics. We also investigated whether nutrient intake of these individuals relates to anthropometric differences.

The micronutrient consumption was fairly similar in all groups with the exception of vitamin A. The greatest consumption was by the CC group (P <0.05) and the lowest consumption by the AC group (P <0.05). Vitamin A's antioxidant properties and its role in night vision are well established. Poor dietary habits as well as pathological changes caused by DMII (and increased oxidative stress) may partially be responsible for development of complications

in some diabetics. The antioxidants can protect membrane lipids and proteins from oxidative stress and can be beneficial, if consumed in adequate amounts.

The data also suggest that central obesity (higher W / H) was present in the individuals with DMII. The higher total fat and cholesterol intake (total, Sat, MUFA, PUFA and chol) in the diabetic groups is consistent with the abdominal adiposity present in DMII subjects.

The higher total fat intake by CD group may not be the only factor responsible for insulin resistance because the AD group consumed the lowest total fat among all four groups. The Chol intake data showed both diabetic groups had a high Chol consumption. Higher Chol intake has been known to increase plasma cholesterol levels as well as increased cell membrane cholesterol, possibly resulting in fewer insulin receptors on the cells (Sanghvi et al., 1983).

It is reasonable to assume that the type of lipid consumed is a more important parameter to consider than the total quantity of lipid by itself. It is obvious from previous clinical literature that increased fat intake is associated with weight gain, worsening of diabetes and increased the risk of developing macrovascular complications. Suggested mechanisms for insulin resistance are insulin-binding problems, a post receptor defect, or possibly a combination of the two (Skrha, 1996). It is not clear whether genetic, dietary or both factors are responsible for the higher rate of DMII in African Americans.

Also based on the results of this study we cannot conclude that high fat intake is responsible for increased BF%. The AD did not consume a higher total

fat but had the highest BF%, so other metabolic factors need to be considered. Based on the data obtained in this study, the dietary factors do not seem be the sole contributory factor and other cellular defects may be responsible. We will make an attempt to answer some of the cellular defect concerns in the other parts of this research project by analyzing at the membrane lipids and describing how they may affect insulin binding.

The anthropometric measurements were different in diabetic individuals but no racial differences were obvious. Also diet analysis serves as a more useful tool to detect whether metabolic alterations are related to the dietary habits. The dietary analysis also can be helpful in risk evaluation in individuals with altered anthropometric (e.g. BMI and BF%) measurements. The W/H and W/T may not be as accurate as the BMI and BF% since body shape differences (e.g. hip sizes) among different racial/sex groups exist.

TABLE 1. The average macronutrient consumption among different groups.
Note: All values show mean +/_ Standard Deviation

	Daily Value	CC	AC	CD	AD
Calorie *(Kcal)	2000-2500	1958+/− 429	1865 +/− 876	2383 +/− 1210	1614 +/− 464
Protein* (g)	50-65 (g)	84 +/− 84	74 +/− 40	100 +/− 51	83 + / - 22
CHO* (g)	300-375 (g)	251 + / - 42	230 + / - 99	229 + / - 76	175 + / - 58
Fat* (g)	65-80 (g)	66 + / - 22	67 + / - 28	120 + / - 80**e**	65 + / - 27
Chol* (mg)	300 (mg)	268 + / - 141	237 + / - 112	471 + / - 286**e1**	390 + / - 217**e1**
Sat * (g)	20-25 (g)	22 + / - 9	23 + / - 11	43 + / - 28**e2**	23 + / - 12
MUFA* (g)	20-25 (g)	23 + / - 8**e1**	21 + / - 8 **e1**	40 + / - 24**e3**	23 + / - 12
PUFA* (g)	25-30 (g)	11 + / - 4	10 + / - 3	16 + / - 11**e4**	8 + / - 4
C18:2* (g)	None Established	7 + / - 3	5 + / - 2	8 + / - 7	6 + / - 4
C18:3* (g)	None Established	0.69 + / - .4	0.51 + / - 0.2	0.74 + / - .5	1 + / - .5

* CHO=carbohydrate; Chol= Cholesterol; Sat= saturated Fat;

MUFA= Monounsaturated Fat; PUFA= Polyunsaturated Fat;

C18:2= Linoleic Acid; C18:3= Linolenic Acid; EPA= Eicosopentaenoic Acid;

DHA= Docosohexaenoic Acid;

CC= Caucasian Control (n=10); AC= African American Control (n=10);

CD= Caucasian Diabetic (n=5); AD= African American Diabetic (n=10);

e: *P* <0.05 when compared to the other three groups

e1: *P*<0.05 Control vs. Diabetics

e2: *P* <0.05 CD vs. AD

e3: *P* <0.01 CD vs. AD

e4: *P* <0.06 CD vs. AD

Table 2. The average macronutrient consumption among different groups

	RDIs	CC	AC	CD	AD
Sodium (g)	2.4 (g)	2.8	2.4	3.9	2.7
Potassium (g)	3.5 (g)	2.8	2	2	2.1
Vitamin A (RE)	800 (RE)	1415	574	726	674
Beta-Carotene (μg)	None Established	498	53.7	44.7	60.3
Vitamin C (mg)	RDIs	135	147	43.7	86.4
Calcium (mg)	60 (mg)	658	601	830	566
Iron (mg)	18 (mg)	14	14	15	13
Vitamin D (μg)	5 (mg)	5	1.7	2	8.9
Vitamin E (ATE)	8 (ATE)	16.1	3.9	6.4	7
Thiamine (mg)	1.5 (mg)	1.4	1.4	1.6	1.6
Riboflavin (mg)	1.7 (mg)	2	1.3	1.8	1.7
Niacin (mg)	20 (mg)	21.8	17.5	20.5	21.7
Vitamin B6 (mg)	2 (mg)	1.8	1.3	1.3	1.7
Folic Acid (μg)	0.4 (mg)	337.6	259.3	176.9	264.4
Vitamin B12 (μg)	6 (mg)	4.5	3.7	4.5	4.6
Biotin (μg)	30 (mg)	25.7	8.7	11.1	13.8
Pantothenoic Acid (mg)	10 (mg)	4.6	2.4	2.6	4.1
Vitamin K (μg)	65 (mg)	71.9	53	35.5	35.1
Phosphorous (mg)	1000 (mg)	1221	968	1258	1030
Magnesium (mg)	400 (mg)	274.4	193	171	193
Zinc (mg)	15 (mg)	11.5	7.7	11.8	12.2
Copper (mg)	2 (mg)	1.2	0.8	0.9	0.8
Manganese (mg)	3.5 (mg)	2.3	1.1	0.9	1.9
Selenium (mg)	0.055 (mg)	0.1	0.1	0.1	0.1
Fluoride (μg)	2750 (mg)	684	241	401	349
Chromium (mg)	0.125 (mg)	0.03	0.03	0.02	0.05
Molybdenum (μg)	162.5 (mg)	25.7	9.5	23.4	39.6

NOTE: CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic; RDI= Recommended Dietary Intake.

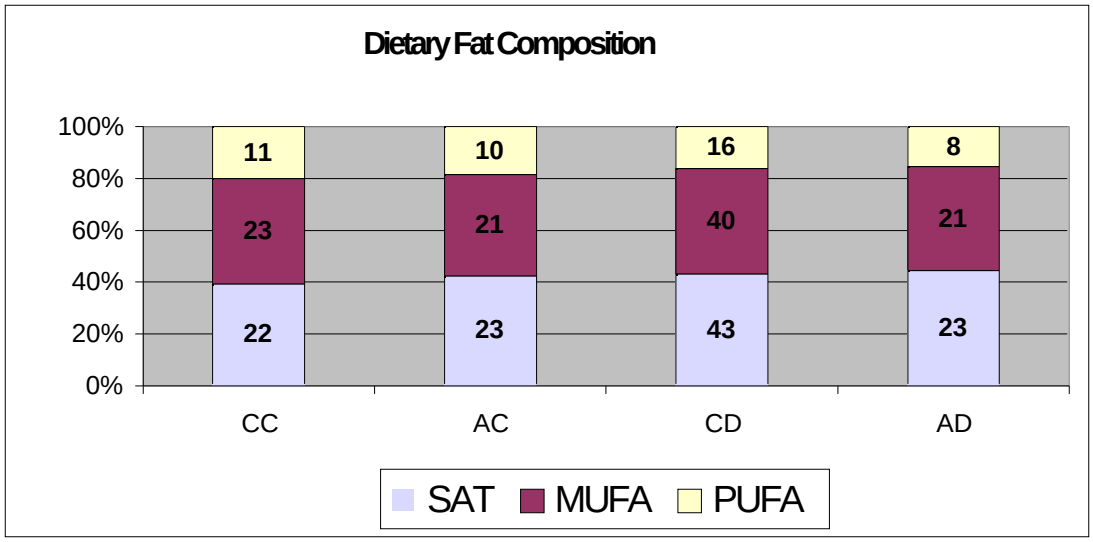

Figure 1. A comparison of average of different classes of dietary fatty acids, CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic; SAT= Saturated Fat; MUFA= Monounsaturated Fat; PUFA= Polyunsaturated Fat.
Note: The numbers on the data series indicates the actual grams of fat intake in each class.

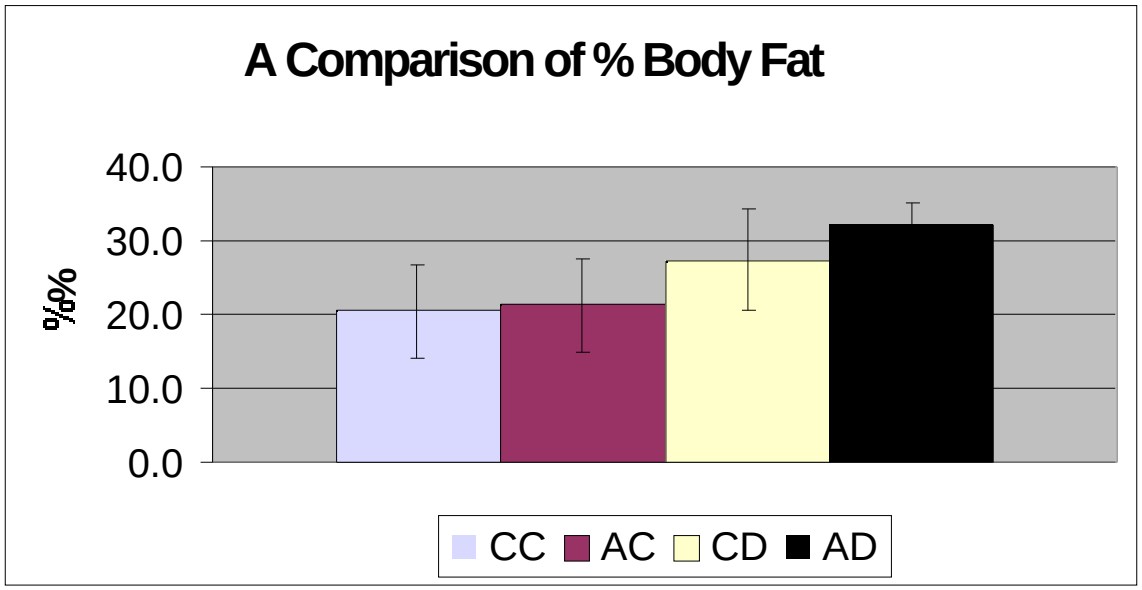

Figure 2. A comparison of body fat percentage. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic.
Note: The bars show mean +/− standard deviation.

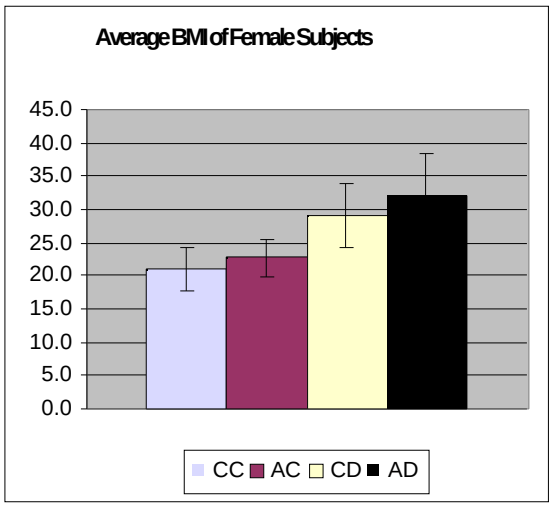

Figure 3a. An average BMI for female subjects
CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic.

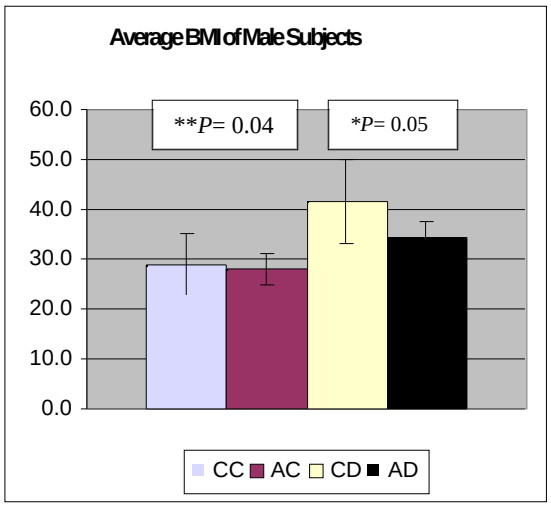

Figure 3b. An average BMI for male subjects
CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic,
* Comparison among diabetics,
** Control vs. Diabetic.

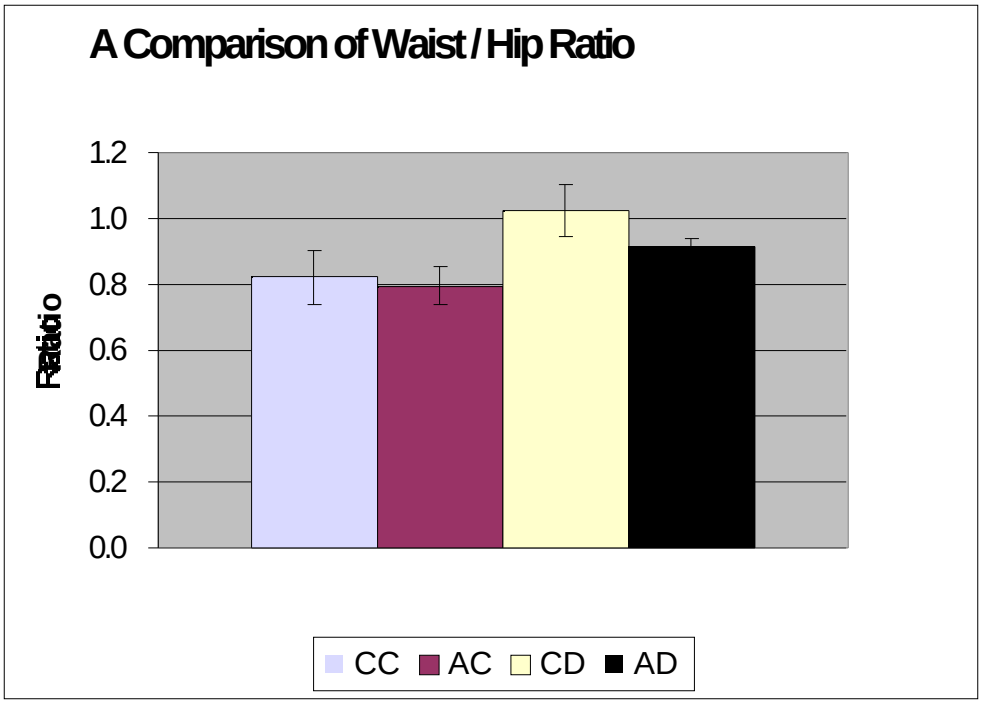

Figure 4. A comparison of waist to hip ratio. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic.

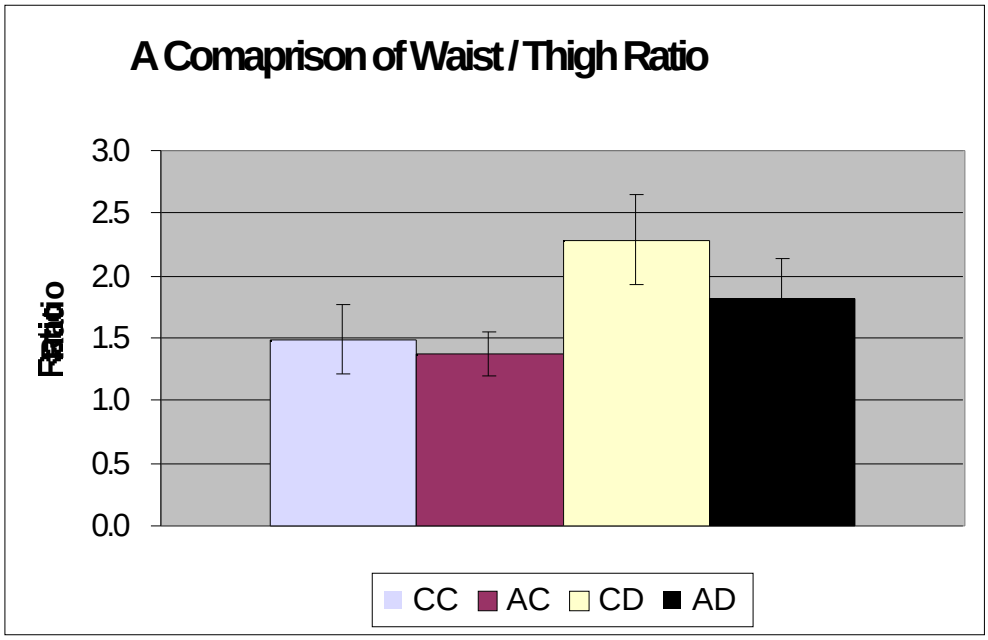

Figure 5. A comparison of waist to thigh ratio. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic. Note: The bars show mean +/− standard deviation.

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Chapter III

White Blood Cell Delta-6 Desaturase Enzyme Activity In Insulin Resistant Diabetic Individuals

INTRODUCTION

Diabetes results in several severe complications such as diabetic neuropathy, retinopathy, nephropathy and microvascular diseases. It is important to understand the role of lipids in the development of the insulin resistant state and diabetes complications. Lipids contribute greatly to the cell membrane fluidity, production of prostaglandins and the hormonal signal transduction pathway.

The lipid composition of cellular membranes is regulated to maintain membrane fluidity. It is speculated that lack of enzyme transcription due to inadequate insulin action may be the contributing factor for plasma membrane (PM) fluidity alterations in diabetes. Alterations in saturated / unsaturated PM fatty acids (FA) have been implicated in various disease states including cardiovascular disease, obesity, non-insulin-dependent diabetes mellitus, hypertension, neurological diseases, immune disorders, and cancer (Siguel, 1983). Activity of desaturase enzyme is sensitive to dietary changes (Giron et al., 1996), hormonal imbalance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds (Ntambi, 1999).

The first step in the conversion of the alpha linoleic acid (C18:2), an essential fatty acid (EFA) to omega 6 (n-6) gamma-linolenic acid (GLA) is impaired in diabetes (Faas and Carter, 1980). This impairment is due to a lack of delta-6-desaturase (D6D) enzyme, which is an insulin-dependent enzyme

(Medeiros et al., 1995). In more severe cases of EFA metabolism impairment, the enzymatic actions are affected in two places, which is caused by a production deficit of the delta-5-desaturase enzyme (D5D) (also an insulin dependent enzyme), further down the conversion chain.

The enzyme inadequacy creates a shortage of GLA and its metabolites prostacyclin and prostaglandins (Horrbin, 1993). Some diabetic complications are possibly related to chronic deficiency of prostacyclin and prostaglandins (Ramsammy et al., 1993 and Clark, 1985), and this is central to the pathogenesis of diabetes.

Investigations have demonstrated alterations of PM FA composition in cells from diabetic individuals (Prisco et al., 1989; Garnier et al., 1990 and Kantar, 1991). The alterations are associated with lack of enzyme activity (Eck et al., 1977), decreased enzyme transcription due to lack of insulin action (Faas and Carter, 1980) and improper dietary fat intake or a combination of all the mentioned etiologies. Viruses also have been known to block D6D enzyme, which is necessary for prostaglandin E_1 synthesis (Das, 1981) and the enzyme impairment possibly can lead to alteration in membrane fluidity and FA composition (as a secondary effect) of the infected cells (Williams et al., 1994).

Delta-6-desaturase enzyme has been thoroughly studied because of its importance in the enzymatic conversion of EFA in the metabolic pathway leading to the formation of prostaglandins. Delta-6-desaturase enzyme, a microsomal protein, is present in all nucleated cells capable of synthesizing their

own unsaturated fatty acids. In most of the studies rat liver tissue has been used to study the activity of this enzyme (De Gomez et al., 1983). We have selected white blood cell (WBC) microsomal proteins to study the activity of this enzyme since collection of hepatic tissue is an invasive procedure accompanied by great risks to human subjects. The microsomes are the site where desaturation of fatty acids occur in the cells.

Red blood cells are a suitable specimen and its PM FA composition is representative of muscle PM FA (Baur, 2000). Since D6D activity is absent in RBC's (Alberts, 1994) they acquire their membrane FA from the plasma and the plasma lipids in a fasting state is representative of hepatic FA synthesis activity.

METHODOLOGY

Subjects

The subjects were recruited from 4 groups 1) Caucasian Control (CC) (n=10), 2) African American Control (AC) (n=10), 3) Caucasian Diabetics (CD) (n=5), 4) African American Diabetics (AD) (n=10). The diabetic subjects were type II diabetics with insulin resistance (they take insulin injections daily). The subjects were age and sex-matched in both racial groups. The subject ages ranged from 23-60 years and equal number of males and females in each group except in CD group (3 males and 2 females). The control subjects were recruited based on the following eligibility requirements: 1) must have no health problems, 2) must not be taking any medications, 3) female subjects must not be taking estrogen (the blood was collected on the last week of menstrual cycle). The diabetic subjects were on insulin injections and were not excluded based on any other illnesses.

Preparation of WBC microsomal proteins

Several blood samples were collected by venipuncture after an overnight fast using 22 Gauge vacutainer needles (Beckton Dickenson, Franklin Lakes, NJ) in heparnized vacutainer tubes (Beckton Dickenson, Franklin Lakes, NJ). The samples were kept on ice until arrival at the laboratory. The samples were then centrifuged for 10 minutes in a clinical centrifuge and the plasma was aspirated

and stored in –20°C freezer. The buffy coat was then collected carefully to reduce RBC contamination. The samples were then washed twice with a saline solution (1:2 dilution) and the visible buffy coat was removed each time. The collected WBC's (approximately 0.5 ml) were then mixed with a saline solution (1:2 dilution) and were carefully placed on 2 ml of 1-step human reagent purchased from Accurate Chemicals and Scientific Corporation (Westbury, NY) (Bayum, 1983). The tubes were centrifuged at
room temperature for 20 minutes. The upper bands of mononuclear and polymorphonuclear cells were removed and saved. This process was repeated two times to assure removal of all remaining RBC from the samples. The collected cells were washed with physiological saline solution twice and then centrifuged. The saline was aspirated and discarded and the cells were mixed with 4 ml of 0.25 M sucrose solution and homogenized for 3 minutes. The microsomal proteins were recovered based on the instructions described by Shin et al. (1995).

Desaturase Enzyme Assay

Based on the amount of microsomal proteins (mg / ml) varying nmoles of methyl ester of linoleic acid (Nu Check Prep, Inc. Elysian, MN) (LA) was used to keep a ratio of 1 mg / ml of microsomal protein to 10 nmoles of LA. The microsomal proteins and LA were mixed and incubated at 37°C for 20 minutes while shaking. The lipids were then extracted according to Folch et al., (1956).

Extracted samples were prepared for analysis by filtering through florosil columns. The filtered samples were dried under nitrogen and reconstituted in 25 μL of iso-octane and then analyzed on Hewlett Packard 5890 GC for LA and GLA detection. The enzyme activity was calculated by product (GLA) to substrate (LA) ratio and reported as pmoles of GLA per minute per mg of microsomal protein.

Calculation Of Other Desaturase Enzyme Activity

Product / substrate ratio of RBC membrane FA (the membrane FA analysis as described in chapter IV) was used for calculating an approximate enzyme activity. The following RBC membrane FA ratios were used to calculate enzyme activity values: 1) arachidonic acid n-6 to linoleic acid ratio for D5D enzyme activity, 2) linolenic acid to linoleic acid for D6D n-6 enzyme activity, 3) alpha-linolnic acid to linoleic acid for D5D omega 3 (n-3), 4) linoleic acid to oleic acid ratio for D6D n-3.

RESULTS

The D6D enzyme activity (Figure 1) was measured by utilizing a conversion assay but the D5D enzyme activity was estimated by taking the arachidonic acid to linoleic acid ratio (product to substrate ratio) available from the RBC membrane fatty acid data (Figure 2) (see chapter IV for RBC membrane

lipids).

There was a clear difference between the control and the diabetics' D6D enzyme activity ($P < 0.05$). The racial comparison among the subjects revealed no significant difference in either control or diabetic groups. In the statistical analysis age was included as a covariant but did not show any significance in all four groups. The results are consistent with the published literature confirming impaired D6D activity (Clark, Queener, 1985 and Eck et al., 1979) in diabetics.

The calculated D5D enzyme activity (based on RBC PM FA) was the greatest in the AD group ($P < 0.01$) and the lowest in the CC group (Figure 2). The diabetic groups CD and AD were almost statistically different ($P = 0.06$), but there were no statistical differences between the control subjects.

The D6D n-3 (Figure 4) and D5D n-3 (Figure 5) were also calculated by using the RBC data (Chapter IV). The D6D n-3 were in the order CC>AC>CD>AD in enzyme activity and the opposite order was seen in D5D n-3 (CC<AC<CD<AD). The calculated enzyme activity for the n-3 pathway (Figures 4 and 5) showed a similar pattern as seen in the n-6 pathway (Figures 2 and 3).

The D6D activity was lower in diabetics than in controls in both racial groups, but the D5D activity was higher in diabetics in both the n-3 and n-6 metabolic pathways.

DISCUSSION

The diabetic subjects and the AC group showed a much lower D6D enzyme activity than the CC group based on the LA conversion assay (Figure 1). The direct assay is a more sensitive method for detecting enzyme because enzyme activity was detected in the diabetic subject cells, whereas evidence of D6D was not seen in those groups by the calculation method (Figure 3) that used RBC FA data listed in Chapter IV.

The D6D enzyme is very important in EFA metabolism and membrane fluidity. Decreased activity is associated with decreased hormonal regulation, decreased membrane fluidity and decreased synthesis of long chain polyunsaturated fatty acids. Membrane integrity is an important factor in regulation of cellular metabolism and passage of hormones into the target cell. The greater the FA saturation in the membrane, the faster steroid hormone passage will be. But the opposite (decreased saturation) applies to insulin binding. Delta-6-desaturase enzyme is the rate-limiting enzyme in the desaturation pathway and is tightly regulated. Many different factors such as diet, hormones, fats and temperature can influence its activity. On the other hand D5D is less tightly regulated and is activated to prevent substrate (MUFA) accumulation.

Insulin hormone stimulates D6D and D5D transcription and their activation leads to increased desaturation and membrane fluidity. It has been demonstrated that in a drug induced diabetes model (e.g. using alloxan), absence

of insulin resulted in a lower rate of desaturation (Mercuri et al., 1967). In another study, Blond et al., (1989) demonstrated that in old obese Zucker rats, insulin did not show the same regulatory effect as shown by Mideiros et al., (1995). Mideiros et al. demonstrated that serum insulin levels showed a positive correlation with higher PUFA to MUFA ratios that represented D5D and D6D pathway activity in serum. Medeiros et al. also demonstrated that a relationship between insulin and D6D enzyme activity was not found. Since D6D is the rate limiting enzyme, elevated insulin levels would affect D5D more than D6D enzyme activity so that C18:2 and C18:3 (products of D6D) would not accumulate in the cells (Mideiros et al., 1995). The enzyme activity calculation method used by Medeiros et al., (1995) fails to demonstrate the β−oxidation and diet-induced effects on desaturase enzymes. In a direct method such as the one used by this study one can obtain a better estimate of enzyme activity. However if a calculation method is to be used, RBC membrane FA is a suitable specimen for estimating of the hepatic FA synthesis activity because the RBC's lack desaturase activity.

The data from the direct method showed a dramatically lower D6D activity in diabetics and the AC group than in the CC group. This could be as a result of lower insulin stimulation, presence of inhibitory compounds, viral interference with D6D or dietary factors. The calculated D5D showed much higher activity (CC<AC<CD<AD) in the AD, possibly due to hyperinsulinemia usually seen in such individuals.

The enzyme activity should be correlated with the plasma membrane fluidity measures and it may explain some of the pathologic changes leading to insulin resistance and greater risk of developing DMII in African Americans. The low D6D enzyme activity reduces the rate of long chain-PUFA synthesis and incorporation into cell membrane, which can eventually lead to decreased membrane fluidity and insulin resistance.

This study cannot determine whether insulin resistance is the cause of reduced D6D activity or reduced D6D activity causes membrane alterations leading to insulin resistance. One can speculate that a decline in D6D is associated with presence of insulin resistance.

Another factor influencing insulin resistance and D6D enzyme activity is dietary trans fatty acids (TFA). The TFA have been implicated in competitive inhibition of D6D (Booyens, 1992). Another study showed that the dietary trans fatty acids are preferentially incorporated into the liver microsomal lipids and act as inhibitors for delta-9 and delta-6 desaturases. This indicates that the presence of trans fatty acids in the diet may alter EFA metabolism and membrane composition through their action on the desaturases (Mahfouz, 1981).

Therefore, for clinical management of diabetes evaluation of the desaturase activity should be added to knowledge of the diabetic individual's hormonal status, dietary habits and insulin sensitivity, so preventive measures can be taken. Enzyme activity can be increased by improving diet, increasing omega-3 PUFA consumption and reducing cholesterol and TFA intake (Keelan et

al., 1994) with a possible benefit of improving insulin sensitivity.

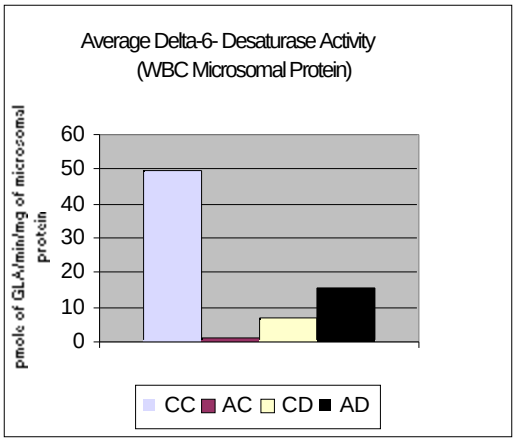

Figure 1. Delta-6-Desaturase enzyme activity CC (Caucasian Control), AC(African American Control), CD (Caucasian Diabetics) and AD (African American Diabetics)
NOTE: The WBC microsomal protein was used for obtaining the enzyme activity.

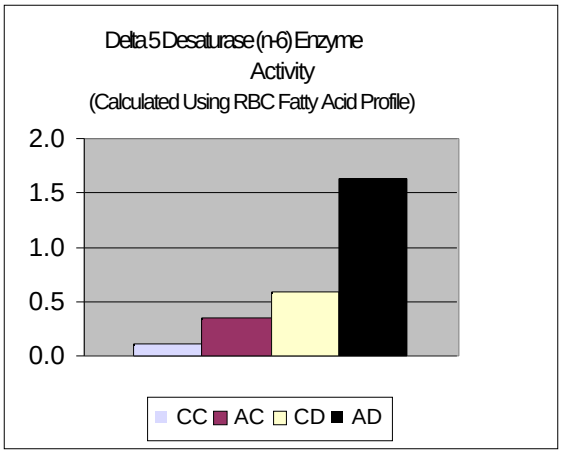

Figure 2. Delta-5-desaturase enzyme activity in CC (Caucasian Control), AC (African American Control), CD (Caucasian Diabetics) and AD (African American Diabetics).
NOTE: RBC membrane fatty acid was used for calculation of the approximate hepatic microsomal enzyme activity (substrate / product)

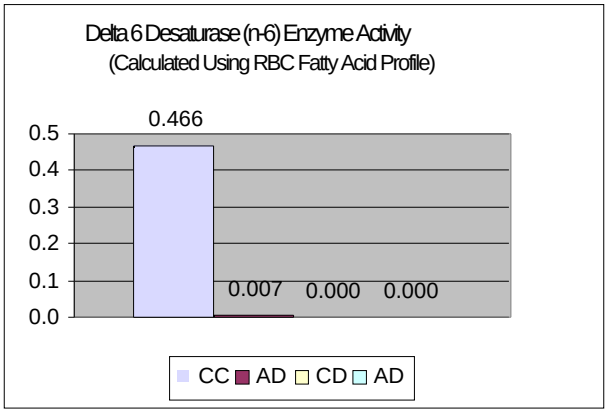

Figure 3. Delta-6-Desaturase enzyme activity in CC (Caucasian Control), AC(African American Control), CD (Caucasian Diabetics) and AD (African American Diabetics).
NOTE: The RBC fatty acid values were used for calculating the approximate hepatic enzyme activity (product / substrate).

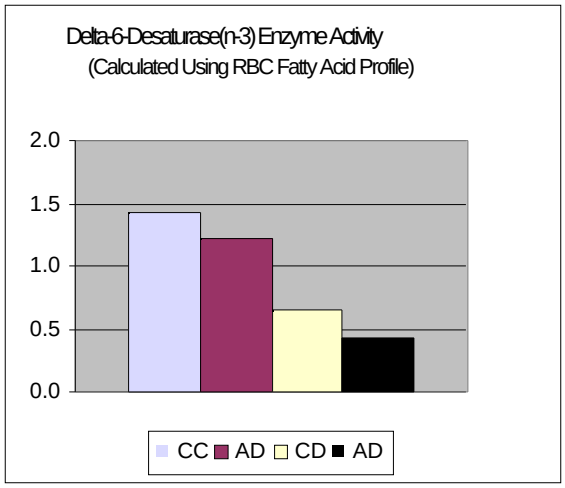

Figure 4. Delta-6-Desaturase (n-3) enzyme activity in CC (Caucasian Control), AC(African American Control), CD (Caucasian Diabetics) and AD (African American Diabetics).
NOTE: The RBC fatty acid values were used for calculating approximate hepatic enzyme activity (product / substrate).

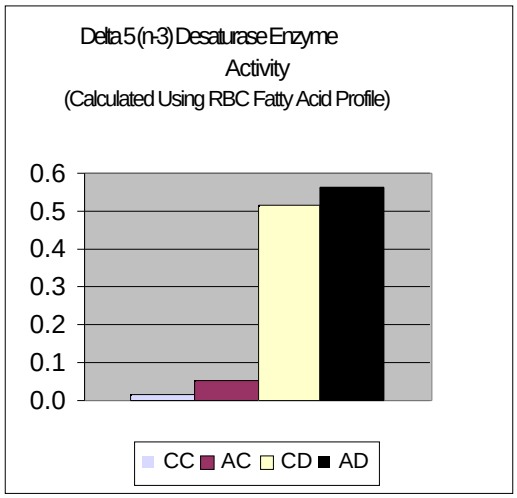

Figure 5. Delta-5-Desaturase enzyme activity in CC (Caucasian Control), AC(African American Control), CD (Caucasian Diabetics) and AD (African American Diabetics).
NOTE: The RBC fatty acid values were used for calculating approximate hepatic enzyme activity (product / substrate).

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CHAPTER IV

Erythrocyte Membrane Lipids & Blood Constituents In Insulin Resistant Caucasian & African American Diabetics

INTRODUCTION

Membrane Lipids

In animal cells membrane lipids are usually fatty acid (FA) chains of about 14-22 carbons and up to six unsaturated (double) bonds. The unsaturated bonds cause a kink in the lipid that prevents the close packing of molecules that normally occurs with saturated fatty acids. The membrane lipids determine the membrane fluidity.

Membrane lipids are important in controlling membrane-bound enzyme function. The cells maintain homeostasis by modulating several cellular functions such as changing membrane fluidity to accommodate cellular metabolic needs. One example, the saturation and desaturation of membrane lipids, changes with temperature fluctuations in cells (Cossins and Bowler, 1987). The colder temperatures cause the saturated membrane lipids to solidify into viscous crystalline gels. The desaturase enzymes become activated as a result of cooling to maintain membrane fluidity. They become activated to increase the polyunsaturated FA (PUFA) incorporation into the membrane, hence, increasing the membrane fluidity. This process is called Homeoviscous adaptation.

The viscosity of the fluid mosaic membrane directly affects enzyme function. The membrane-bound enzymes seem to undergo flexing movements during catalysis, and they only work properly when the viscosity of the surrounding membrane remains constant.

Diabetes & Membrane Lipids

Etiology of diabetes type II (DMII) is different among the diagnosed cases and this makes finding the cause more difficult. However, in an insulin resistant state a common etiology exists and that is impaired insulin signal transduction. Hence, lack of insulin related cellular actions leads to many known diabetic complications.

Alterations in membrane fatty acids have been demonstrated in various tissues in diabetic animal models (Huang et al., 1984 and Holman, 1983). The lipid alterations are thought to be responsible for prolonged receptor exposure to the external environment and increased sensitivity of cells such as platelets in diabetics (Urano et al., 1991).

Fatty acid desaturation has an important function in changing membrane fluidity. The delta-6-desaturase and delta-5-desaturase are insulin dependent enzymes (Medeiros et al., 1995) and in diabetes decreased insulin action may be responsible for decreased enzyme activity (Cameron et al., 1997). Activity of desaturase enzymes are also sensitive to dietary changes (Giron et al., 1996), hormonal imbalance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds (Ntambi, 1999).

Arachidonic acid concentration is an important indicator of desaturase enzyme activity in cells. The first step in the conversion of the alpha linoleic acid (C18:2 n-6), an essential fatty acid (EFA) to omega 6 (D6) gamma-linolenic

acid (GLA) (18:3 n-6) is impaired in diabetes (Faas and Carter, 1980). This impairment is due to lack of delta-6-desaturase (D6D) enzyme, which is an insulin dependent enzyme (Medeiros et al., 1995). In more severe cases of EFA metabolism impairment, the enzymatic actions are affected in two places, which is caused by a production deficit of the delta-5-desaturase enzyme (D5D) (also an insulin dependent enzyme), further down the conversion chain.

Erythrocytes (RBC) in diabetics are known to be less flexible due to membrane rigidity and this is considered to be part of the pathology seen in diabetic microvascular complications (Garnier et al., 1990). The metabolically labile pool of phosphatidate is decreased and phosphatidyl inositol levels are altered in RBC membranes from diabetic patients (Kamada et al., 1992).

Plasma lipids in a fasting state are helpful in determining hepatic lipid synthesis and can be used in correlation with RBC membrane lipids. The RBC is suitable for studying membrane lipids in diabetics and can be helpful in evaluating alterations in a disease state (Holman et al., 1983).

Insulin Resistance & Insulin Action

Insulin is a peptide hormone and its action begins by binding of the hormone to the cellular receptor. The insulin receptor is a dimer made of 2 α and 2 β chains linked by disulfide bonds forming βααβ structure. The binding of the hormone is very specific resulting in a sequence of events involving the membrane lipids and several enzymes. Upon transmitting the signal,

endocytosis of the ligand-receptor complex occurs and lysosomal proteolysis will recycle the subunits to the membrane.

The beta-subunits contain tyrosine residues that are phosphorylated by tyrosine kinase enzyme. The phosphorylation invoking a conformational change of beta-subunit and phospholipase C_γ (PLC_γ) is then activated. The enzyme PLC_γ will act upon phosphoinositol diphosphate (PIP_2) resulting in inositol triphosphate (IP_3) and diacylglycerol (DAG). The IP_3 will bind to the calcium channels on the endoplasmic reticulum membrane and liberate calcium ions into the cytoplasm. The DAG in the presence of phosphatidyl serine (PS) and calcium ions will activate the protein kinase C (PKC). The PKC will then phosphorylate certain proteins and induce a cellular response such as increased transcription of glucose transporters. The glucose transporters will migrate to the cell membrane and pick up the glucose molecules and transport them into the cytoplasm so that they can be utilized for energy.

In an insulin-resistant state the cell may have one or more defects in the signal transduction pathway. The problem may originate from impaired receptor phosphorylation by tyrosine kinase (TK), low PLCγ activity, low PIP_2 or PS levels, inadequate intracellular calcium, inadequate activation of PKC or other hormones antagonizing the action of insulin. Insulin resistance will lead to low cellular glucose uptake and eventually development of diabetes type II.

Insulin resistance (i.e., decreased sensitivity to insulin action) seems to be the major contributory factor in patients with diabetes mellitus type II. This lack

of adequate insulin action could be caused at the pre-receptor, at the receptor, or at the post-receptor level. The presence of neutralizing insulin antibodies in plasma that can bind insulin, or a defective insulin molecule (very rare) can be the cause of pre-receptor malfunction. The receptor problem can be due to inability of insulin to optimally bind its receptor (e.g. defective receptor or presence of other interfering substances) or the presence of insulin receptor antibody. The post-receptor problems can lie within the signal transduction pathway. Abnormalities are apparent either in the structure of the insulin receptor, in the phosphorylation of the tyrosine residue by the enzyme tyrosine kinase that is integral to its function or impaired membrane lipid action (improper membrane fluidity). A defective insulin-mediated stimulation of tyrosine kinase and autophosphorylation has been demonstrated to be impaired in DMII (Moller and Flier, 1991).

In the present study RBC membrane lipid composition was determined in both healthy participants and insulin resistant diabetics from two racial groups (African Americans and Caucasians). The membrane lipids evaluated were: total cholesterol (TC), phospholipids (PL) and fatty acid (FA) compositions. Also plasma constituents such as; glucose (Glu), cholesterol (Chol), TRG (TRG), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), hemoglobin A_1C (Hgb A_1C) and insulin level were measured for evaluation purposes. Because of the increased risk of diabetes in African Americans, we sought to determine whether differences in membrane lipids

could be considered as a risk factor in developing insulin resistant diabetes.

METHODOLOGY

Subjects

The subjects were recruited from 4 groups 1) Caucasian Control (CC) (n=10), 2) African American Control (AC) (n=10), 3) Caucasian Diabetics (CD) (n=5), 4) African American Diabetics (AD) (n=10). The diabetic subjects were type II diabetics with insulin resistance (they take insulin injections daily). The subjects were age and sex-matched in both racial groups. The subject ages ranged from 23-60 years and equal number of males and females in each group except in CD group (3 males and 2 females). The control subjects were recruited based on the following eligibility requirements: 1) must have no health problems, 2) must not be taking any medications, 3) female subjects must not be taking estrogen (the blood was collected on the last week of menstrual cycle). The diabetic subjects were on insulin injections and were not excluded based on any other illnesses.

Blood Collection

Several blood samples were collected by venipuncture after an overnight fast with a 22 Gauge vacutainer needles (Beckton Dickenson, Franklin Lakes, NJ) in ETDA and serum separator vacutainer tubes (Beckton Dickenson, Franklin

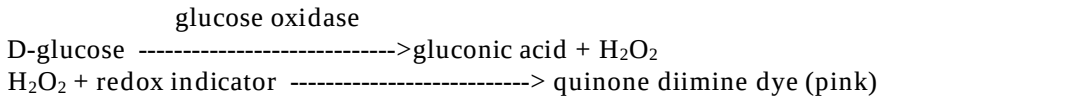
Lakes, NJ). The samples were kept cool until arrival at the laboratory. The samples were then centrifuged within one hour of collection time for 10 minutes in a bench-top clinical centrifuge and the serum sample was aspirated and analyzed immediately and the unused portions were stored in –20°C freezer for future use. The EDTA tubes were used for the collection of RBC's for membrane lipid analysis. The tubes were spun down as described before. The buffy coat was carefully removed, washed and stored. The RBC's were then washed with 2 ml of normal physiological saline twice and spun. The RBC pellets were then reconstituted with equal amounts of normal saline (50% hematocrit) and stored in –20°C freezer until further analysis.

Serum Analysis

The serum samples were analyzed for total serum GLU, TRG, TC, VLDL, LDL, HDL and insulin concentrations.

Glucose Analysis

The testing principle for GLU analysis was based on the glucose oxidase enzymatic, colorimetric, endpoint method (CDC, 1976). The sample size for analysis is 10 mL.



The color intensity read spectrophotometrically at the wavelength of 620-650nm correlates with GLU levels.

Lipid Profile

The serum sample was applied to the Cholestech, which contains pads containing the reagents necessary for the lipid analysis. The LDL and VLDL were precipitated with dextran sulfate and magnesium acetate precipitating reagent. The HDL cholesterol was then transferred to the HDL reaction pad. The TC was measured by an enzymatic end point method (Allain et al., 1974). The HDL was also measured by the method formulation of Allain et al., and Roeschlau et al., (1974). The total TRG levels were determined by an enzymatic, colorimetric, endpoint method (Fossati and Prencipe, 1982). The following principle applied:

$$\text{TRG} \xrightarrow{\text{Lipoprotein Lipase}} \text{Glycerol + Fatty Acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-1-phosphate + ADP}$$

$$\text{Glycerol-1-phosphate} \xrightarrow{\text{Glycerophosphate Oxidase}} \text{Dihydroacetone Phosphate + } H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine (AAP) + Sodium N-Ethyl-(3-Sulfopropyl)m-Anisidine} \xrightarrow{\text{Peroxidase}} \text{Quinoneminie Dye + } H_2O$$

The VLDL was calculated by dividing the TRG level by five.

Insulin Concentration

Insulin levels were measured by utilizing an Insulin RIA kit (Diagnostic Products Corporation, Los Angeles, CA). A 200 µL portion of the patient serum was utilized and the standards were handled according to the written procedure provided by the manufacturer. The 200 µL of sample was pipetted directly to the

bottom of the insulin antibody coated tubes. Then 1.0 ml of [^{125}I] insulin was added to each tube including the standards, which then was vortexed. The samples were incubated for 24 hours at room temperature. After incubation the tubes were decanted thoroughly (removing any visible moisture to avoid discrepancies in the procedure). The samples were counted for 1 minute by a gamma counter and the results were recorded.

Glycated Hemoglobin Protocol

Five ml of blood were collected in vacutainers containing EDTA (Becton Dickinson, Franklin Lakes, NJ). All reagents and columns were brought to RT^{0}C. The ambient temperature was recorded before analysis by placing a thermometer in an unused Hb A_{1C} tube (The temperature must be between 18-28^{0}C). The columns were protected from draft during use.

One tube was labeled for each control and unknown being assayed. 200 µL of hemolyzing reagent was pipetted into each labeled tube. The whole blood tubes were mixed well. 50 µL of whole blood was pipetted into each tube containing hemolyzing reagent. The excess blood was wiped from the outside of the pipette tip before addition to hemolyzing reagent. The mixture was then mixed well by aspiration and dispensing with the same pipette. The mixture was gently mixed and left at RT^{o}C for 10 minutes with occasional shaking. A fresh column was prepared for each test by standing the column above the test tube.

The column cap was removed before the bottom closure to avoid air bubbles. After the column started to drain, the top disc was pushed down until it contacted the top of the resin bed.

The column was allowed to drain until the liquid level reached the top disc. 50 µL of hemolysate was pipetted directly onto the top. The column was allowed to drain until the liquid level was absorbed completely into the top disc. The sides of the columns were rinsed with 200µL of Hgb A_{1C} Wash Buffer and were allowed to drain until the liquid level was absorbed completely into the top disc. After the flow stopped, the solutions that were drained from the column were discarded. A clean test tube was placed beneath the columns and 4.0 ml of Hb A_{1C} Wash Buffer were pipetted to the column and the entire volume was collected. The flow stopped automatically when the liquid level reached the top disc. The "wash" fraction took less than 40 minutes.

The spectrophotometer wavelength was set to 415 nm and the absorbance of each fraction was measured within an hour of collection. The following calculation was used to determine the Hb A_1 in samples analyzed:

Hb A_{1C} [%] = W / (W+ 5 E) X 100%

Where: W = absorbance of "Wash" fraction and E = absorbance of "Elusion" fraction.

The following temperature correction protocol was used for optimal accuracy:

T°C at which test was performed	T°C Correction Factor (TCF)	y-intercept
18	1.46	1.81
19	1.34	1.32
20	1.24	0.89
21	1.15	0.55
22	1.07	0.26
23	1.00	0
24	0.94	-0.22
25	0.89	-0.42
26	0.84	-0.60
27	0.80	-0.76
28	0.76	-0.87

Y = Adjusted % Hb A_1 value
X = Observed value (at ToC other than 23°C)
m = TCF for specified temperature
b = y-intercept value for specified T°C
To adjust the following linear equation was used: $Y = mX + b$

RBC Lipid Extraction and Analysis

Folch lipid extraction method (Folch et al., 1956**)** was used. For each sample type three preparations were made. One ml of RBC was homogenized after addition of 1 ml of BHT (0.05%) chloroform and 9 ml of methanol. An additional 20 ml of chloroform was added to the sample, which was then homogenized for another 2 minutes. The sample was filtered into a graduated cylinder. The solid residue on the filter paper was re-suspended with chloroform:methanol [(C:M) (2:1)] and was further homogenized for an additional 2 min. The mixture was filtered into the cylinder with the original extraction and was washed with an additional 30 ml of C:M (2:1).

The total extraction volume was measured and 0.88% potassium chloride (1/4 of the total volume) was added. The mixture was shaken and allowed to

separate for 1.5 hours. The supernatant was aspirated and discarded. The lower lipid layer containing the chloroform : methanol : water (C:M:W) was poured into a round bottom glass flask. The flask was placed on a rotovapor instrument until the lipid samples were dried.

The dried lipids were re-dissolved in 4-8 ml of chloroform and were dried again. This step was repeated 2 more times. The final re-suspensions in chloroform were transferred to a clean pre-weighed test tube.

Separation Into Polar vs. Non-Polar Fractions

Solid phase extraction tubes containing silica were used. The SPE columns were conditioned with 2 ml of hexane. Two ml of crude lipid extract was poured onto the column followed by an additional 2 ml of hexane. The first extract was collected in a test tube and the non-polar fraction continued to be eluted by an additional 5 ml of acetone. The polar fraction was eluted into a second test tube by using 10 ml of methanol.

The polar samples were dried under the nitrogen probe and were re-dissolved in 1 ml of chloroform.

Phospholipid Quantitation

The polar extractions contained the cellular phospholipids and were quantitated by a high performance liquid chromatography (HPLC) method. The HPLC unit was thoroughly cleaned and fresh reagents were prepared prior to

analysis. The mobile phase consisted of methyltertiarybutylether : methanol : aqueous ammonium acetate (pH of 8.6) (5:8:2). The ammonium acetate with a pH of 8.6 was prepared by addition of 2 volumes of 0.001 M ammonium acetate to 1 volume of 0.001 M acetic acid (dilution factor of 2:1) and the pH was adjusted to 8.6. The reagents were filtered using nylon filters with pore size of 0.45 microns. All the polar samples were also filtered utilizing gas tight syringe #1001 (1 ml Hamilton) with a 3N syringe filter (0.45 micron nylon).

The column used was a pre-packed microporasil column (30cm X 3.9 mm), the flow rate was 2.0 ml / minute and the evaporative light scattering detector was used for detection. The samples were then reconstituted in 50µL of the mobile phase. A 30 µL sample size was injected and each sample was repeated twice and an average quantitative value for total phospholipid level was recorded. The samples were then reported in percent total phospholipid concentration.

Sterol Extraction

The non-polar samples were dried under nitrogen in a water bath at 38^0C and 5 mL of the saponification mix was added to the dried sample. The tubes were shaken and then the boiling beads were added to each tube. All the tubes were placed into the water bath at 98^0C and refluxed for 90 minutes. Upon cooling 5 ml of ether was added to the saponified samples slowly, and the tubes were capped and moderately hand shaken to mix the phases (the cap was

loosened under the hood to ventilate the tubes). The samples were centrifuged for 5 min. The upper layer was removed and an additional 5 ml of ether was added and then the two ether layers were pooled. The samples were dried, reconstituted in 30 µL of iso-octane and analyzed by a gas liquid chromatography (GLC) method.

Preparation of RBC for Binding Studies (Gambhir et al., 1978)

Ten ml of blood were collected in heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) from each subject. The tubes were then centrifuged for 10 min. at 400 X g at RT^{0}C. The plasma was aspirated and the cell pellet was mixed with one part of physiologic saline and then layered on 3 ml of 1-step polymorphs solution (Accurate Chemical and Scientific Corporation Westbury, NY) in a glass tube. The mixture was then centrifuged for 20 min at RT^{0}C. The saline, monocyte, 1-step polymorphs solution, granulocyte phases, and the upper layer was removed and saved for further processing. The cell pellet was again re-suspended on one part of saline and separation of white blood cells was repeated. The resulting cell pellet was then re-suspended in 2 parts buffer G prepared as described by Gambhir et al., (1977) to equilibrate the cells. The previous step was repeated twice to assure removal of all non-RBC cells. The RBC pellet was re-suspended in buffer G to contain a hematocrit of 50%. The RBC viability was checked by the Trypan blue dye exclusion technique.

RBC Binding Study

Four hundred µL of the 50% hematocrit cell suspension was mixed with 80 pg of [^{125}I] Insulin (in 25 µL of buffer) , and concentrations of unlabeled insulin (0 - 0.5 X 10^5 ng) and buffer G was added to constitute a volume of 0.5 ml (Gambhir et al., 1977). The mixture was then incubated at 30^0C for 3.5 hrs.

200 µL of the incubated suspension was then removed and pipetted into pre-chilled microtainer tubes containing 200 µL of dibutyl phthalate and 300µL of buffer G. The microtainer tubes were then centrifuged at 4^0C for 4 minutes. The cells were trapped under the gel in the microtainer tubes. The buffer and dibutyl phthalate layer were then aspirated via a suction system. The tubes (the top) were rinsed twice with deionized water and the excess water was again removed by suction. The microtainer tubes were then dropped in glass tubes and were read for radioactivity on a gamma counter. The percent binding was determined by using the following formula:

The radioactivity bound = RBC pellet radioactivity / total radioactivity (in a 200 µL portion of the incubated cell suspension).

Statistics

Statistical analysis was performed using SAS computer software (SAS Cary NC). Data were analyzed by 2-way-ANOVA using General Linear Model and the Pearson Correlation procedure. The data was split by health status

(diabetic vs. non-diabetic) and race (Caucasian vs. African American). Also the age was used as a covariant in analysis of all dependent variables. Analysis means were compared by least significant difference at $p<0.05$.

RESULTS

The Blood Constituents

The serum evaluation (Table 1) of GLU levels showed that the diabetic subjects had higher fasting GLU levels (P <0.01) as expected. There were no racial differences seen in the GLU levels (Figure 1) in both groups. The Hgb A_1C levels were significantly different between the AD and the rest of the subjects (P<0.01). Among the diabetic groups the Hgb A_1C levels were statistically different (P <0.05) indicating a better glycemic control in the CD group. There was no significant difference seen in the TC levels any of the groups. The triglyceride levels were higher levels in diabetic individuals (P<0.01). The lipoprotein evaluation indicated significant differences in VLDL (P<0.01), LDL (P<0.05) and HDL (P<0.0001) levels between control and diabetic subjects. There were no racial differences seen among any of the groups.

The RBC Membrane Fatty Acid Composition

The RBC membrane FA (Figure 2) were analyzed (Table 2) for total saturated (SAT), total monounsaturated (MUFA), total polyunsaturated (PUFA), saturate / polyunsaturated ratio (S / P) (Figure 3), total fatty acids co-eluting with odd chain fatty acids (OCFA), and total trans fatty acids (TFA). There were no significance differences in SAT levels. The highest MUFA levels were present in the CD group (P <0.01). The PUFA levels were the lowest in CD group (P <0.05)

and there were no significant racial differences seen. The S / P (Figure 3) is used as an indicator for membrane fluidity and this evaluation showed higher ratios in the diabetic subjects ($P < 0.05$). The analysis of the membrane FA showed the presence of certain fatty acids, which co-elute at the same retention time as the odd chain fatty acid standard (OCFA). The highest level of OCFA was detected in the CD group ($P < 0.05$). There were no racial differences in OCFA levels among the groups.

The RBC Membrane Total Cholesterol

The analysis of RBC (Table 2) total membrane cholesterol levels (CH) showed (Figure 4) that the AC group possessed the lowest average membrane CH levels and the CD had the highest CH levels, but the results were not statistically significant.

The RBC Membrane Phospholipid

The RBC membrane (Figure 5) was analyzed for phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), sphingomyelin (SPH) and lysophosphatidyl choline (LPC). The only significant differences were observed in the PE and PC levels. The Caucasian subjects had higher PE levels ($P = 0.01$) than the African American subjects. The highest PE levels were observed in the CD group and the lowest in the AD group. The PC level was the highest in the AD ($P < 0.05$) group and the

lowest in the CD group. The racial comparison of PE showed a statistically significant difference between the groups (P= 0.01). The PC evaluation showed racial differences among diabetics (P <0.05).

The saturated nature (Figure 6) of the RBC was calculated by using the following formula: (SPH+PC) / (PE+PS). The calculated results (Table 3) indicate racial differences in saturated nature of RBC (P <0.0001). The decreased membrane fluidity is also evaluated by obtaining the membrane CH / PL ratio (Figure 7). Diabetic subjects had higher CH / PL ratio (P <0.0001) and there were no racial differences seen.

The calculated rheological (PE / PS) parameters (Table 3) of RBC were higher in the Caucasian subjects (p= 0.05), indicating better deformability of RBC (Figure 8).

RBC Insulin Binding

The insulin binding was higher in the AC (P <0.01) and AD (P <0.05) than the CC and CD (Figure 9). Comparison of control subjects as a group with the diabetics as one group did not show any statistical significance even though the binding averages were lower. However, the racial comparison was significant (P <0.01).

DISCUSSION

African Americans have 2 times greater risk of developing diabetes than Caucasians and are more likely to develop diabetes-related complications (American Diabetes Association, 2000). Therefore, we determined how membrane fluidity and might affect insulin binding.

There have been several studies done on the membrane lipids in RBC's of diabetic patients (Garnier et al., 1990 and Tilivis, 1985). The arachidonic acid level has been used to evaluate EFA metabolism in diabetics, but there have been conflicting reports. One possibility for varying levels of arachidonic acid reported among diabetics is the existence of different types of DMII. It is important to know whether the individual suffers from lipid metabolism disorders, decreased insulin level, insulin resistance or other autoimmune related DMII. In most of the published studies the diabetic patients were not categorized (e.g. DMII vs. insulin resistant DMII).

In this study insulin resistant DMII subjects from two racial groups were selected with the hope of limiting any variations in our patient type. We determined if membrane alterations are a possible reason for insulin resistance, and whether membrane lipid alterations are responsible for racial differences in higher risks for developing diabetes complications.

The data are consistent with the published data regarding higher GLU, Hgb A_{1C} and lipid levels in diabetics (Table 1). No racial differences were detected in plasma constituents. The glucose to insulin ratios (Figure 1) were the

same for the Caucasian subjects (ratio=3), but it was lower in the African American subjects (AC=1 and AD=2). We do not have the data for the amount of insulin injected by diabetic subjects.

The results indicated racial differences in Hgb A_{1C} (P <0.05) levels. The AD group had an average Hgb A_{1C} of 10 (Table 1), indicative of a poorly controlled diabetic state. The elevated Hgb A_{1C} is not only detrimental to the proteins, but it also has been associated with higher oxygen affinity of glycated hemoglobin and a decreased velocity of oxygen release (Marschner et al., 1995). The decreased velocity of oxygen release can deprive the tissue of oxygen and create tissue hypoxia. The hypoxia can therefore trigger production of growth factors such as vascular endothelial growth factor (VEGF) (Mukhopadhyay, 1995 and Shweiki et al., 1992), which then will activate protein tyrosine kinase enzyme (PTK) and PI signal transduction pathway. The elevated growth factors have been associated with some diabetes related complications (Ferrara, 1999).

The RBC membrane evaluation (Figure 2) was done to determine membrane properties. The SAT level in the RBC membrane was higher in the diabetic subjects (P <0.05) accompanied by lower levels of PUFA (P <0.01) indicating lower membrane fluidity estimated by evaluating the S/P (Figure 3). The MUFA level was much higher in the CD group (P <0.01) while having lower PUFA possibly due to EFA metabolism defects. The desaturation of MUFA requires the action of desaturase enzymes, which are insulin-dependent enzymes. The desaturase enzyme defect is a well-established fact (Holman, et

al., 1983 and Faas et al., 1980). The TFA analysis showed an elevation in the AD group and not the others (P <0.01). The exact role of TFA has not been established but they behave similarly to SAT. Therefore, they may contribute to the increased rigidity of the membrane. The presence of OCFA in the CD clearly exceeds the values seen in the other groups (P <0.05). The presence of OCFA in the membranes has been associated with carboxylase enzyme problems either due to biotin deficiency or decreased enzyme activity. The OCFA are present in children with an inborn error of organic acid metabolism and lack of carboxylase enzyme leads to increased circulation of propionic acid causing propionic acidemia. There have been mentions of adult onset of a biotinidase enzyme decreased activity in some adults, but it rarely is reported. However, presence of OCFA can contribute to gluconeogenesis in the liver. If the propionic acid cannot be utilized in the TCA cycle (conversion to succinyl-CoA via propionyl-CoA carboxylase enzyme) then they will be utilized in FA synthesis or will be used as substrates for gluconeogenesis. The origin of the eluted OCFA needs further investigation.

The CH / PL (Figure 7) ratios were also calculated to further evaluate the membrane fluidity. The diabetic subjects had higher RBC CH (Figure 4) levels that increased the CH / PL ratio and indicate increased membrane rigidity. Membrane S / P ratios have been routinely used to determine membrane fluidity in cells (Berlin et al., 1989; Jones et al., 1998; Candiloros et al., 1998). Berlin et al (1989) showed that there is direct correlation between membrane fluidity and red

cell membrane insulin binding and demonstrated a relationship between receptor binding and cell membrane fluidity in human females.

In the current study the S/P ratio in the diabetic individuals was higher than in the control subjects indicating decreased membrane fluidity (increased saturation). Several factors can influence this ratio such as: diet, hepatic fatty acid synthesis, desaturase enzymes, hormones and other chemicals. It is suspected that the S/P ratio may be induced by high fat consumption in diabetics. Desaturase enzymes play an important role in determining saturation level in the membrane. Studies have shown that decreased desaturation in diabetics could be due to lack of insulin sensitivity (Faas et al., 1980).

The RBC phospholipid analysis (Table 3) showed lower levels in diabetics but did not show any statistical significance as seen in other studies (Kamada et al., 1992) possibly due to a lower number of subjects per study group. Phosphatidyl inositol is an integral part of cellular and sub-cellular membranes, and it exerts its effect through membrane-mediated events. It also modulates the activity of several important membrane-bound enzymes (e.g. Na^{+}-K^{+} ATPase, alkaline phosphatase, acetyl-CoA carboxylase and tyrosine hydroxylase) as well as having a role in the insulin signal transduction pathway. Phosphatidyl inositol is also rich in arachidonic acid and serves as a significant source of eicosanoids (Needleman et al., 1986). The RBC membrane analysis did not show any changes in arachidonic acid levels consistent with the PI results.

However; there were significant racial differences (Table 3) observed in PE

and PC. The AD showed lower levels of PE and higher levels of PC. This phenomenon can be due to a higher rate of conversion of PE to PC in the synthetic pathway. The CD group showed higher levels of PE and lower levels PC possibly due to a lower conversion level or possible down regulation of CTP:choline-phosphate cytidylyltransferase enzyme (CT) (the regulatory enzyme in PC synthesis) by inhibitory compounds. In work done by Miami Heart Research Institute the differential effects of inhibitors of protein kinase (PK) or tyrosine kinase (TK) on PC biosynthesis in monocytes were studied in the absence or presence of kinase inhibitors. It was shown that protein kinase A (PKA) inhibitors decreased choline incorporation into PC, while PKA activator had no effect. Also protein kinase C (PKC) inhibitors inhibited PC biosynthesis; on the other hand, PKC activator was stimulatory. The study also showed that the inhibition of PC biosynthesis was accompanied by the inactivation of CT. Interestingly, TK inhibitor (genistein) markedly stimulated CT and PC biosynthesis (Miami Heart Research Institute, 1994). Hormones such as glucocorticoids may increase the amount of CT enzyme activity via increased synthesis of lipid co-factors (Sharma et al., 1993). Certain FA, which serves as substrates for PC synthesis are considered stimulatory to PC synthesis. The FA's effect could also be due to compensation for a fatty acid deficiency, which is consistent with an activation of CT enzyme (Burkhardt et al., 1988). Based on our study the only fatty acid showing a direct correlation with PC was an eicosatrienoic acid (C20:3, an Omega 3 FA) ($r=0.48$, $P<0.01$). The PE level in

diabetics has been shown to be increased in all cell types studied, whereas SPH and PC showed a decrease in platelets and PMN (Labrouche et al., 1996). The Caucasian diabetic results are consistent with the literature findings, but the African American diabetics did not show an increase in PE (accompanied by decreased PC). The higher levels of PC in the African American diabetics can be due to any of the above-mentioned mechanisms. The TK inhibition seems to be a reasonable explanation for the increased PC, since the insulin levels in African Americans (as a group) are higher.

Phosphatidyl ethanolamine in relation to PS (PE / PS ratio) has also been correlated with the rheological properties and is used to predict the behavior of RBC (Labrouche et al., 1996). The stiffening of RBC as has been considered as a cause of nephropathy and microangiopathies (Simpson, 1985). The rheological property of RBC is important when they pass through the capillaries. Decreases in PE / PS may be part of the pathology involved in the increased amputation rate in the African American diabetics.

The African Americans had higher saturated nature values then the Caucasians, which means there is a tendency to incorporate more saturated fatty acid into membranes. But no significant differences between diabetic groups were detected. These results are further indication that membrane fluidity changes in diabetics possibly is a risk factor in non-diabetic African Americans. The increased saturation over time can lead to insulin resistance and, eventually, development of DMII.

Based on the results obtained, the AD group seems to have the least stable membrane (low PE/PS) and the African American subjects have higher membrane saturated nature (P <0.01). It can be speculated that the membrane properties of diabetic individuals (especially the African American diabetics) may contribute to the increased risk of insulin resistance and higher risks of developing diabetic complications, especially higher rates of amputations. The combination of increased S/P, saturate nature, increased LPC and decreased rheological properties of RBC membrane lipids have severe consequences and can contribute to the severity of diabetes among diabetics (especially African American diabetics).

The scientific community must attempt to improve membrane fluidity in diabetic individuals so that insulin sensitivity can be restored. Improved insulin sensitivity will result in lowering of the blood glucose levels, reduction in glycated hemoglobin and an improved lipid profile. Also improving RBC rheological properties in diabetics along with improved glycemic control may help prevent development of diabetic complications. The membrane property differences in the RBCs of African Americans may be due to genetic reasons or poor dietary habits. Better dietary habits need to be promoted among individuals with higher risks. Increasing PUFA intake has been proven beneficial for diabetic individuals in activating the desaturase enzyme and improving the membrane fluidity.

The diabetic subjects did show lower insulin binding when compared to their respective control groups. However, the AD group had higher insulin binding in comparison to CD, as did the AC Group when compared with CC. Decreased receptor concentration and β-cell dysfunction has been noted in diabetics (Olefsky et al., 1982 and Naidoo et al., 1987) and this may be responsible for lower insulin binding. Also, based on the membrane lipid results, insulin binding is greater in membranes containing less cholesterol (as seen in AC and AD). The results also suggest that increased cellular cholesterol may result in lower insulin receptor numbers on the cells.

Because the cells were washed thoroughly so all the interfering substances were removed, the binding data by itself does not account for other metabolic factors that are involved in a diabetic state such as, circulating insulin antibodies, presence of antagonizing hormones, post-receptor defects, and membrane fluidity changes. However, the higher insulin binding in African Americans indicates absence of receptor binding defects and may indicate higher receptor numbers.

The current therapeutic agents available in the market mostly promote insulin secretion, increased glucose transporters, block glucose absorption and peroxisome proliferative receptor-γ activation, or provide exogenous insulin administration. None of current therapies used for diabetes treatment promotes membrane fluidity by manipulations of enzymes involved in PL and EFA metabolic pathways except insulin hormone injection. But if the patient is

already suffering from severe insulin resistance, exogenous insulin does not seem to be very effective. Also, taking insulin injections while being insulin resistant can increase the risk of developing antibodies to the insulin hormone and also create hyperinsulinemia, which can increase the cellular pool of farnesylated p21Ras and thereby augment cellular mitogenic response to a variety of growth factors (Leitner et al., 1997). At this point promoting good nutritional habits, increasing physical activity and stimulating the insulin signal transduction pathway in cells seems to be the only option for treatment of DMII.

Table 1. Blood constituents in healthy (control) & diabetic subjects

	Control Subjects		*Diabetic Subjects*	
Blood Constituents	**CC**	**AC**	**CD**	**AD**
Glucose	84.15 +/- 15	81.65 +/- 13	145.7 +/- 41	244.625 +/- 143**
Hgb A1C	5.4 +/- 0.8	6.0 +/- 1.3	6.7 +/- 0.5	10 +/- 3*
T. Cholesterol	185 +/- 17	185 +/- 17	207 +/- 41	222 +/- 74
TRG	91 +/- 42	84 +/- 34	154 +/- 36	176 +/- 95**
VLDL	18 +/- 8.4	17 +/- 8	31 +/- 7.5	35 +/- 19**
LDL	104 +/- 17	108 +/- 13	135 +/- 36	143 +/- 68*
HDL	62 +/- 14	62 +/- 10.5	42 +/- 11	45 +/- 4**
Insulin	32 +/- 25	59 +/- 41	39 +/- 23	43 +/- 15

NOTE: CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic

* Control vs. Diabetic *P* <0.05
** Control vs. Diabetic *P* <0.001;

Table 2. Fatty Acid Composition of RBC membrane in healthy and Diabetic

	Control Subjects		*Diabetic Subjects*	
Lipids	**CC**	**AC**	**CD**	**AD**
SAT (% total FA including OCFA)	30.8	30.7	31.2	36.8*
MUFA (% total FA including OCFA)	19.3	35.9	49.2**	24.6
PUFA (% total FA including OCFA)	43.0	33.0	18.5*	37.9
S/P	0.7	0.9	1.7*	1.0
OCFA (% total FA)	12.4	25.8	32.9*	9.9
TFA (% total FA)	2.3	3.0	2.9	5.9*
Cholesterol (mcg/cell)	4.07E-08	2.09E-08	5.76E-08	4.26E-08

NOTE: CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic. SAT= total saturates; MUFA= total monounsaturated; PUFA= total polyunsaturated; S/P= saturate/polyunsaturated; OCFA= total fatty acids co-eluting with odd chain fatty acids; TFA= total trans fatty acids

* Control vs. Diabetic *P* <0.05
** Control vs. Diabetic *P* <0.01

Table 3. Phospholipid composition of RBC membrane in healthy and diabetic individuals

	Control Subjects		*Diabetic Subjects*	
Phospholipid	**CC**	**AC**	**CD**	**AD**
PI%	30.2	33.1	23.3	28.7
PS%	18.6	23.0	21.1	25.5
PE%	32.5	22.3	37.9[a]	12.6
PC%	14.7	14.3	4.4	25.2[b]
SPH%	2.1	5.3	7	4.3
LPC%	1.7	2.1	2.1	3.7
PE/PS[c]	1.7	1.0	1.7[d]	0.5
Saturated Nature[e]	21.0	29.0[e1]	29	34[e2]

NOTE: CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic; PI%= % total phosphatidyl inositol; PS%= %total phosphatidyl serine; PE%= % total phosphatidyl ethanolamine; PC%= Phosphatidyl Choline; SPH%= % total sphingomyelin; LPC%= % total lysophosphatidyl choline.

a: indicates racial difference between Caucasians & African Americans (p= 0.01)
b: indicates racial differences among Caucasians & African American diabetics (P <0.05)
c: Indicates rheological properties of RBC
d: Indicates racial difference between Caucasians & African Americans p=0.056
e: obtained by (SPH+PC)/(PE+PS);
e1: indicates racial differences among control subjects (P <0.001);
e2: indicates racial difference among Caucasians & African Americans (P <0.0001).

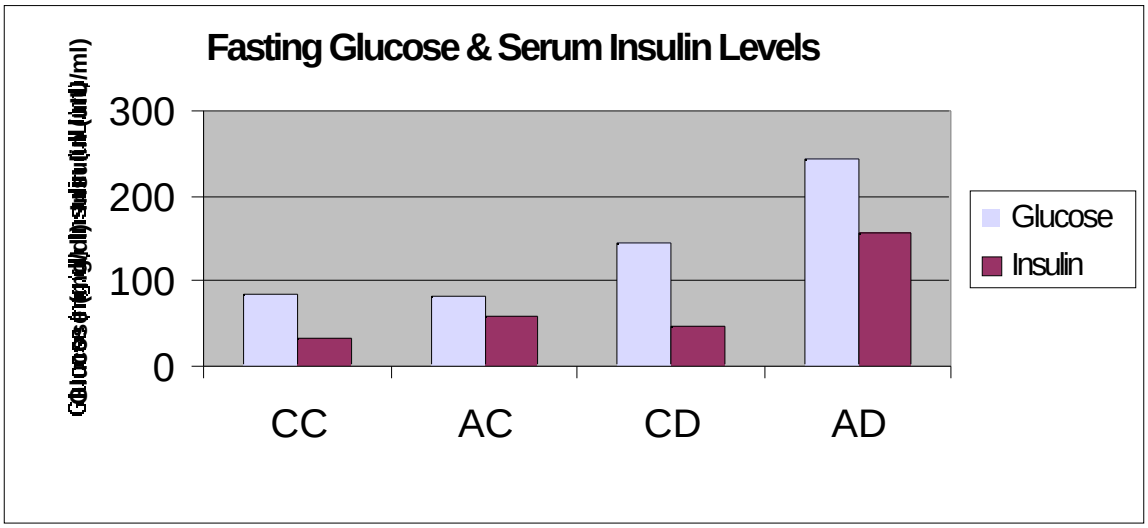


Figure 1. Fasting glucose & serum insulin levels. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic.

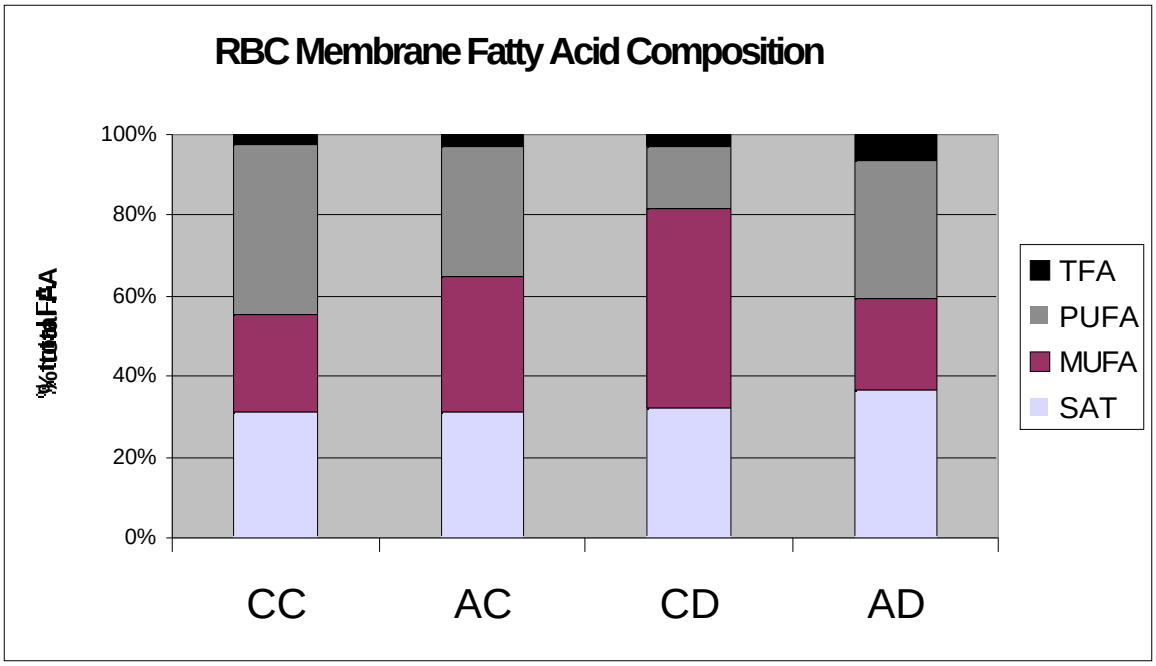


Figure 2. RBC membrane fatty acid composition. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, SAT= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, TFA= trans fatty acids.

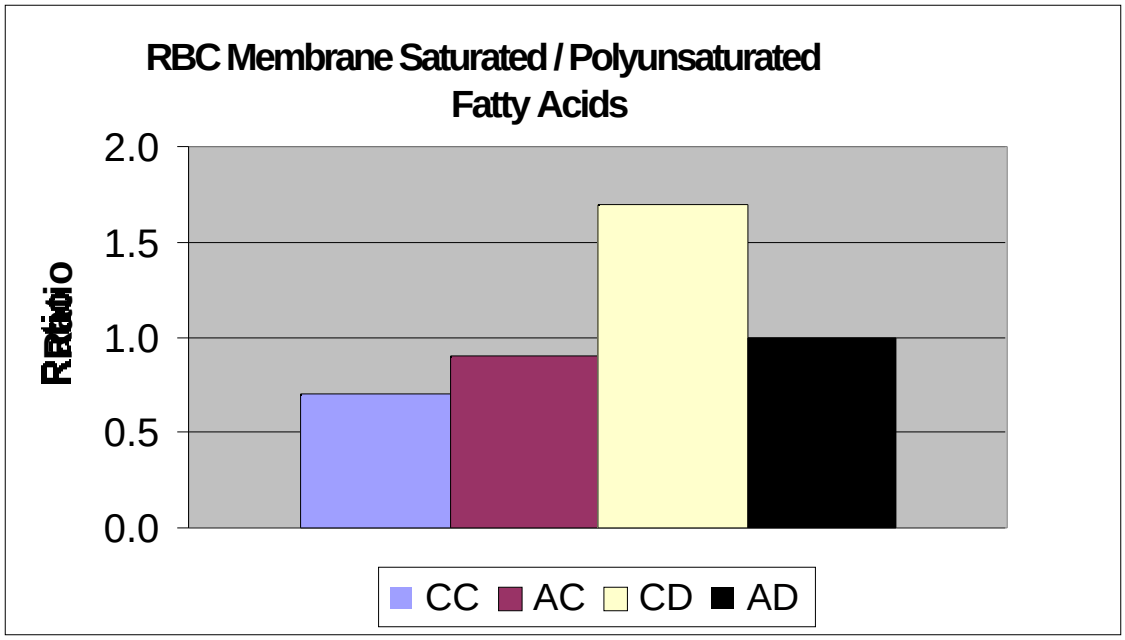


Figure 3. RBC membrane saturated to polyunsaturated fatty acid ratio. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic; SAT= Total saturated fatty acids; PUFA= Total polyunsaturated fatty acids, S / P = saturated / polyunsaturated ratio.

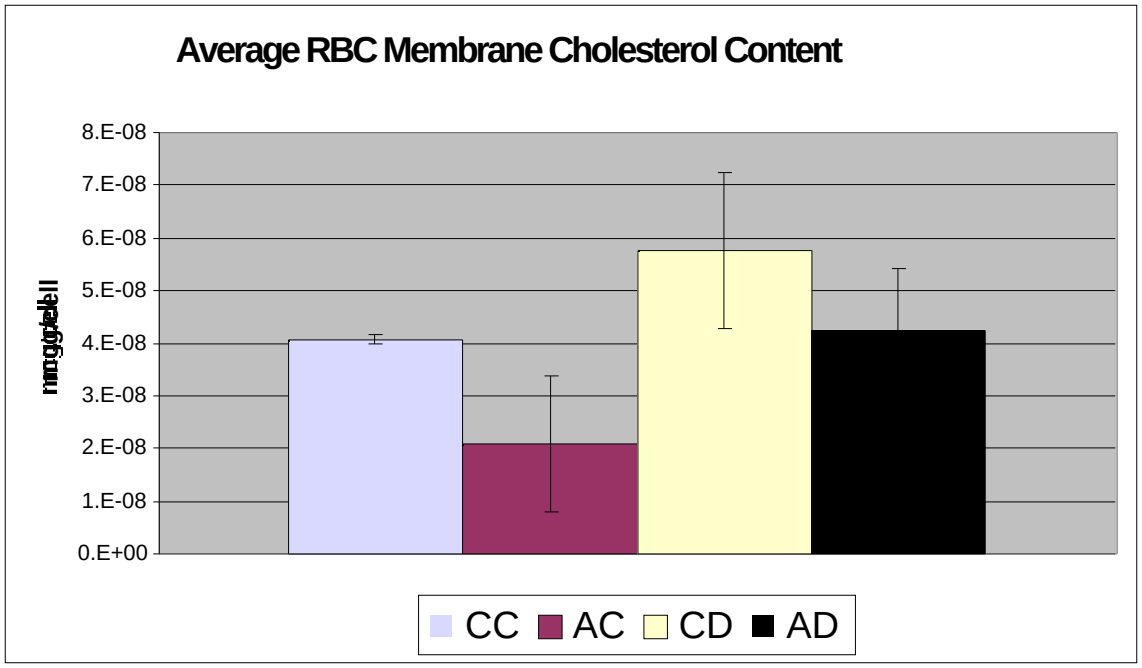


Figure 4. Average RBC membrane cholesterol content. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, RBC Chol= red blood cell membrane cholesterol.

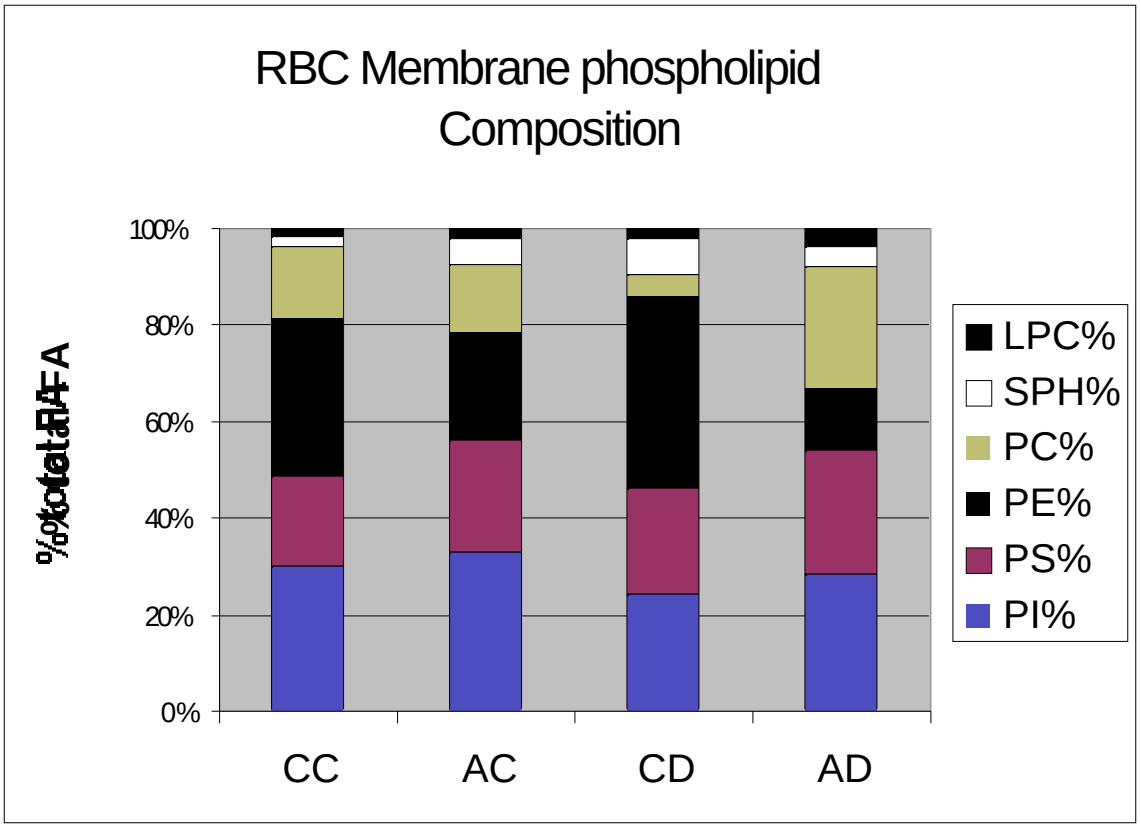


Figure 5. RBC membrane phospholipid composition. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, PI%= phosphatidyl inositol%, PS%= phosphatidyl serine%, PE%= phosphatidyl ethanolamine%, PC%= phosphatidyl choline%, SPH= sphingomyelin, LPC%= lysophosphatidyl choline.

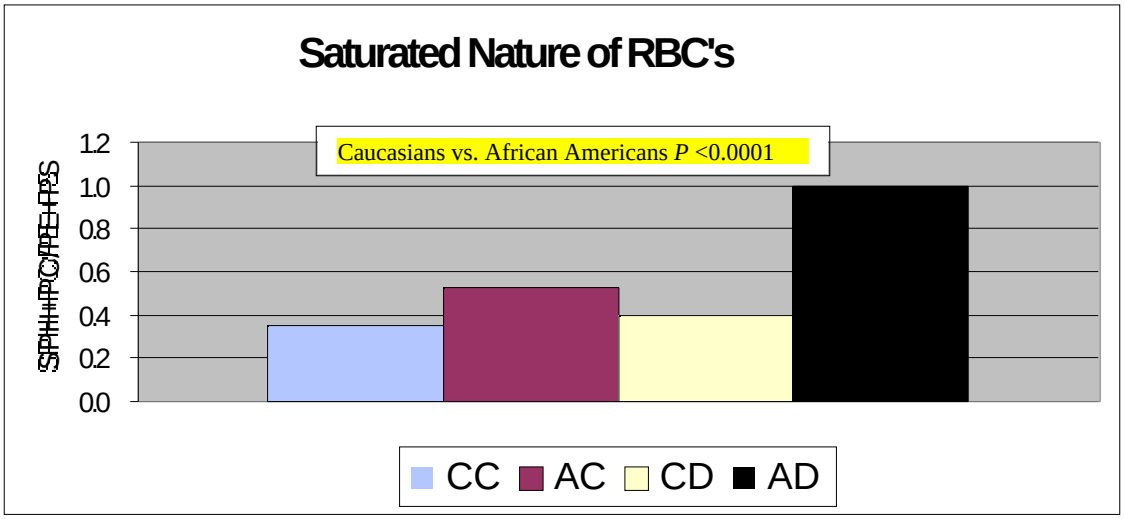


Figure 6. Saturated nature of RBC membrane lipids, calculated by (SPH+PC)/(PE+PS). CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, PS= phosphatidyl serine, PE= phosphatidyl ethanolamine, PC= phosphatidyl choline, SPH= sphingomyelin, LPC= lysophosphatidyl choline.

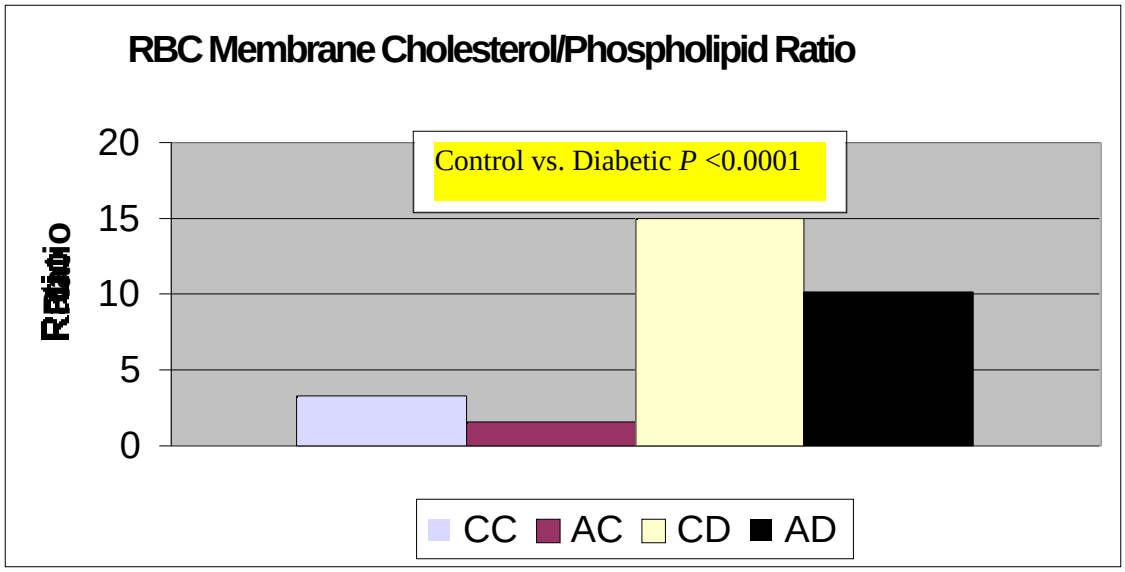


Figure 7. RBC membrane cholesterol to phospholipid ratio. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, Chol / PL= cholesterol / phospholipid ratio.

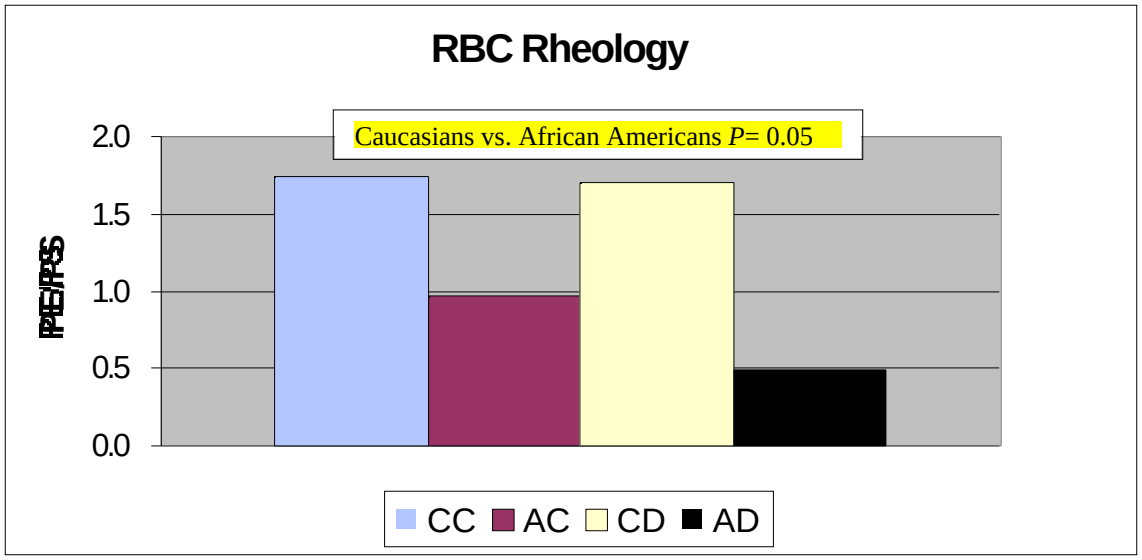


Figure 8. RBC rheology (measure of RBC deformability). CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, RBC Rheology= PE / PS (RBC deformability).

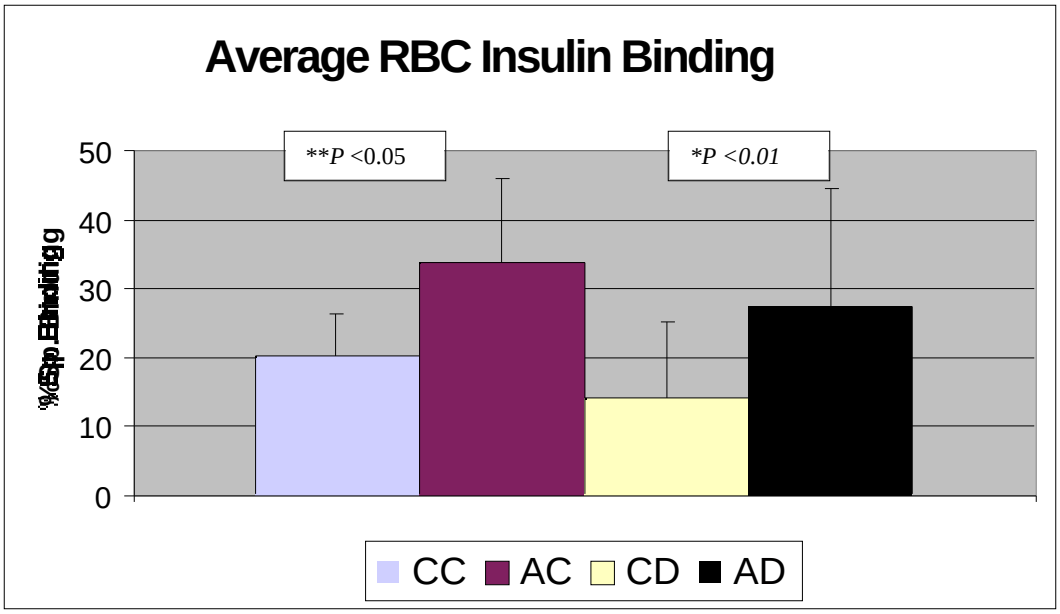


Figure 9. Percent specific insulin binding to isolated RBC membrane. The %specific binding was obtained by the following formula: The radioactivity bound = RBC pellet radioactivity / total radioactivity (in a 200 µL portion of the incubated cell suspension). CC= Caucasian Control, AC= African American Control, CD= Caucasian Diabetics and AD= African American Diabetics.
* CD vs. AD
** CC vs. AC

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CHAPTER V

Is There A Relationship Between Membrane Fluidity and Diet Intake with Insulin Binding & Etiology of Insulin Resistance in Caucasians and African Americans?

Objectives

This study was designed to answer the following questions:
1) Are there membrane lipid differences (i.e. dietary or genetic) between the African Americans and Caucasians and if yes, is this difference contributing to higher risk of diabetes among African Americans? 2) Does membrane lipid composition affect the insulin binding (plasma glucose clearance) in diabetic individuals?

Subjects

The subjects were recruited from 4 groups 1) Caucasian Control (CC) (n=10), 2) African American Control (AC) (n=10), 3) Caucasian Diabetics (CD) (n=5), 4) African American Diabetics (AD) (n=10). The diabetic subjects were type II diabetics with insulin resistance (they take insulin injections daily). The subjects were age and sex-matched in both racial groups. The subject ages ranged from 23-60 years and equal number of males and females in each group except in CD group (3 males and 2 females). The control subjects were recruited based on the following eligibility requirements: 1) must have no health problems, 2) must not be taking any medications, 3) female subjects must not be taking estrogen (the blood was collected on the last week of menstrual cycle). The diabetic subjects were on insulin injections and were not excluded based on any other illnesses.

Statistics

Statistical analysis was performed using SAS computer software (SAS, Cary NC). Data were analyzed by 2-way-ANOVA using General Linear Model and the Pearson Correlation procedure. The data were split by health status (diabetic vs. non-diabetic) and race (Caucasian vs. African American). Also the age was used as a covariant in analysis of all dependent variables. Analysis means were compared by least significant difference at $p<0.05$.

Effects of dietary Intake

In order to answer these questions a series of experiments were planned and several parameters were measured to assess any metabolic changes. The anthropometric measurements were made to assess visceral adiposity, which is a indication of insulin resistance (Després, 1990 and Larsson, 1984).

The diabetic individuals showed central obesity, which is thought to be consistent with insulin resistance (Figure 1). The dietary intake evaluations (Table 1) indicated cholesterol was the only macronutrient showing positive correlation with only BMI ($r= 0.48$, $P <0.01$) and not any other anthropometric measurements.

Cholesterol intake did not show any correlations with any blood constituents (Table 2), membrane lipids or membrane properties. But certain fatty acids such as linolenic acid intake showed positive correlation with serum

glucose level (r=0.45, P <0.01), Hemoglobin A_{1C} (r=0.035, P <0.05) and delta-6-desaturase enzyme (D6D) (r=0.55, P <0.01). Intake of this nutrient tended to be higher in diabetic than control subjects.

Linolenic acid is considered an essential fatty acid and is present in great amounts in fish (mostly ice-water fish). In this study subjects with high linolenic acid intake ate more omega-3 oils, commonly as a fish oil supplement. Linolenic acid serves as a substrate for D6D. The enzyme activity is sensitive to dietary changes (Giron et al. 1996), hormonal imbalance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds (Ntambi, 1999). Dietary linolenic acid consumption has been shown to reduce the level of hepatic D6D mRNA by 50% and completely prevent the increase in fatty acid synthase mRNA (Nakamura et al., 2000), but in our study linolenic acid consumption was correlated with WBC D6D activity (r= 0.55, P <0.01).

The dietary intakes were also evaluated by looking at how they may affect membrane properties (Table 3) and insulin binding. Oleic acid showed positive correlation with saturate nature of RBC membranes (r=0.44, P < 0.01), but not with D6D. Linolenic acid was positively correlated with RBC membrane total cholesterol, but further controlled studies are needed to determine any link between increased membrane cholesterol and linolenic acid intake level. Omega-3 fatty acids were beneficial for patients with heart disease (Leng et al., 1999), but

linolenic acid intake showed no correlations with blood lipid profile in that study.

In summary the macronutrient intake did not show a significant correlation with blood lipid levels, membrane properties and the anthropometric measurements as expected. Dietary intake was not closely associated with either membrane composition or low insulin binding. Genetic and hormonal influences may be more closely associated with the etiology of diabetes. However, all diabetic subjects in this study had received prior dietary counseling. A prospective study may show different results.

RBC Membrane fluidity, Insulin Binding and Glycemic Control

The results of the study showed poorer diabetes control in African American diabetics (AD) as indicated by their higher glucose (P <0.05) and Hgb A_{1C} (P 0.04) levels (Figure 2). The importance of Hgb A_{1C} is evidenced by the fact that glycosylation of body proteins occur at high serum glucose levels and this process is detrimental to the proteins. Another problem is higher oxygen affinity of glycated hemoglobin and a decreased velocity of oxygen release (Marschner et al, 1995). The decreased velocity of oxygen release can deprive the tissue of oxygen and create tissue hypoxia. The hypoxia can therefore trigger production of growth factors such as vascular endothelial growth factor (VEGF) (Mukhopadhyay, 1995 and Shweiki et al, 1992), which then may activate protein tyrosine kinase enzyme (PTK) and the PI signal transduction pathway. The

elevated growth factors have been associated with some diabetes related complications (Ferrara, 1999).

Increased RBC cholesterol was possibly associated with increased glucose and glycated hemoglobin levels (r= 0.65, P <0.05) and negatively associated with insulin binding. This relationship suggests that poor blood glucose homeostasis may be due to lower insulin binding (r= -0.61, P <0.05). The added membrane cholesterol might compensate for glycation of membrane proteins and the resultant in decline in membrane fluidity. The CH / PL is a parameter used to determine membrane fluidity and it seems to affect insulin binding. Altered membrane fluidity has been seen in individuals with hyperinsulinemia (Candiloros et al, 1996) and in diabetic individuals, which can indicate lower insulin binding. Lower insulin binding can result in increased circulating insulin due to low glucose clearance.

The RBC membrane cholesterol / phospholipid (CH / PL) ratio was correlated with LDL positively (r=0.42, P <0.01), and HDL negatively (r=-0.56, P <0.05). Since LDL is the major cholesterol donor and HDL is the major cholesterol scavenger, this association is consistent with the increased cholesterol uptake by cells. Figure 3 shows CH / PL in diabetics, whose cells apparently have greater cholesterol uptake.

The TFA analysis showed an elevation in the AD group relative to the others (P <0.01) but no correlations were seen with insulin binding and glucose levels (not shown). The exact role of TFA has not been established but they

behave similar to SAT. Therefore, they may contribute to the increased rigidity of the membrane. The presence of OCFA (fatty acids eluting at the same retention times as the odd chain fatty acid standard) in the CD clearly exceeds the values seen in the other groups (P <0.05). The presence of OCFA in the membranes has been associated with carboxylase enzyme defects either associated with biotin deficiency or an inborn error of organic acid metabolism in children lacking carboxylase enzymes, which lead to increased circulation of propionic acid causing propionic academia. Adult onset of decreased biotinidase enzyme activity in some adults has been reported rarely. However, presence of OCFA can contribute to gluconeogenesis in the liver. If the propionic acid cannot be utilized in the TCA cycle (conversion to succinyl-CoA by the action of propionyl-CoA carboxylase enzyme) then it will be utilized in FA synthesis or will be used as substrates for gluconeogenesis. The elevated OCFA in these subjects needs further investigation.

The S/P ratio (Figure 5) in the diabetic individuals was higher (P <0.05) than the control subjects indicating decreased membrane fluidity (increased saturation). Factors that can influence this ratio include diet, hepatic fatty acid synthesis, desaturase enzymes, hormones and other chemicals. Desaturase enzymes have been known to play an important role in determining saturation level in the membrane. In this study, S/P was not correlated with the D6D activity measured. Studies have shown that decreased desaturation in diabetics could be due to lack of insulin sensitivity (Faas et al, 1980). We did see

a negative correlation between S / P and insulin binding (r= -0.45, $P < 0.01$) and a positive correlation with insulin levels (r= 0.42, $P < 0.01$).

Membrane S / P ratios have also been routinely used to determine membrane fluidity in cells (Berlin et al, 1989; Jones et al, 1998; Candiloros et al, 1998). Berlin et al., (1989) showed that there was direct correlation between membrane fluidity and red cell membrane insulin binding and demonstrated a relation between receptor binding and cell membrane fluidity in human females.

The RBC phospholipid analysis (Figure 6) did not show any significant differences in PI concentrations as seen in other studies (Kamada et al, 1992). Phosphatidyl inositol is an integral part of cellular and sub-cellular membranes, and its role in insulin signal transduction pathway is well established.

However, significant racial differences (Figure 7) were observed in PE and PC. The African Americans (AC and AD) showed lower levels of PE and higher levels of PC. This phenomenon can be due to a higher rate of conversion of PE to PC in the synthetic pathway. The Caucasian subjects showed higher levels of PE and lower levels of PC, possibly due to a lower conversion level or down regulation of CTP:choline-phosphate cytidylyltransferase enzyme (CT) (the regulatory enzyme in PC synthesis) by other inhibitory factors. In the Miami Heart Research Institute study differential effects of inhibitors of protein kinase (PK) or tyrosine kinase (TK) on PC biosynthesis in monocytes were studied in the absence or presence of kinase inhibitors. It was shown that protein kinase A (PKA) inhibitors decreased choline incorporation into PC, while PKA activator

had no effect. Also protein kinase C (PKC) inhibitors inhibited PC biosynthesis. On the other hand, PKC activator was stimulatory. The study also showed that the inhibition of PC biosynthesis was accompanied by the inactivation of CT. Interestingly, a TK inhibitor (genistein) markedly stimulated CT and PC biosynthesis (Miami Heart Research Institute, 1994). Tyrosine kinase is responsible for phosphorylation of tyrosine residues on the beta sub-unit of the insulin receptor and some other signal transduction molecules. So, inhibition of TK can result in halted signal transduction and relaying insulin effect down to the DNA. Therefore, no PKC activation or cellular response can occur.

Certain fatty acids serve as substrates for PC synthesis and are considered stimulatory to PC synthesis (Sharma et al, 1993). The FA's effect could also be due to compensation for a fatty acid deficiency, which is consistent with an activation of CT enzyme (Burkhardt et al, 1988). Based on our study the only fatty acid showing a positive correlation with PC was an eicosatrienoic acid (C20:3, an Omega-3 FA) ($r=0.48$, $P<0.01$). The PE level in diabetics has been shown to increase in all cell types studied, whereas SPH and PC decreased in platelets and PMN (Labrouche et al, 1996). The Caucasian diabetic results are consistent with the literature findings, but the African American diabetics did not show an increase in PE (accompanied by decreased PC). The higher levels of PC in the African Americans can be due to any of the above-mentioned mechanisms. The decreased TK activity seems to be a reasonable explanation for presence of insulin resistance and the increased PC. The insulin levels in African

Americans (as a group) were slightly higher but the insulin binding was greater. The greater insulin binding, higher glucose levels, higher glycated hemoglobin and lower membrane fluidity suggest a TK defect. If insulin binds and no hypoglycemic effect is observed then one can assume post-receptor defects (e.g. TK defect).

Phosphatidyl ethanolamine in relation to PS (PE / PS ratios) has also been correlated with the rheological alteration and is used to predict the behavior of RBC (Labrouche et al, 1996). The stiffening of RBC has been considered as a cause of nephropathy and microangiopathies (Simpson, 1985). The African Americans had higher saturate nature values than the Caucasians (P= 0.056). But no significant differences between diabetic groups were detected. These results are further indication of membrane fluidity problems in diabetics and may be a risk factor in non-diabetic African Americans. The decreased fluidity over time can lead to insulin resistance and eventually development of NIDDM.

Our data showed the AD group seems to have the least stable membrane (low PE / PS) and the African American subjects to have higher membrane saturated nature (Figure 7) (P= 0.0001).

The results also indicate (Table 4) that increased RBC CH was associated with hyperglycemia (r= 0.65, P <0.05) due to decreased insulin binding (r= 0.63, P <0.05). Also decreased fluidity as indicated by CH / PL correlated with LDL (r=0.42, P <0.01) and HDL (r= -0.56, P <0.05). Decreased membrane fluidity was also indicated by increased S / P, which correlated with insulin level (r= 0.42,

P <0.01) and insulin binding (r= -0.45, *P* < 0.05).

It can be speculated that the membrane properties of diabetic individuals (especially the African American diabetics) may contribute to the increased risk of insulin resistance and higher risks of developing diabetic complications, especially higher rates of amputations. The combination of increased S/P, high saturate nature, increased LPC and decreased rheological properties of RBC membrane lipids have severe consequences and can contribute to the severity of diabetes (especially in African American diabetics).

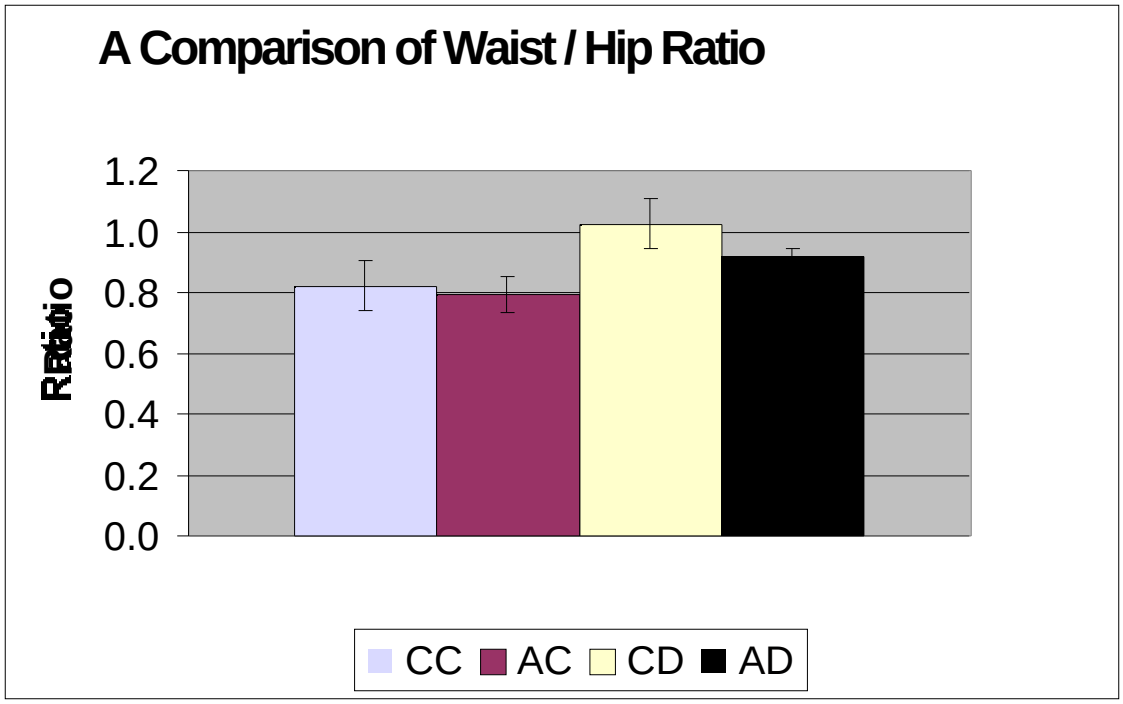

Figure 1. A comparison of waist to hip ratio. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic.

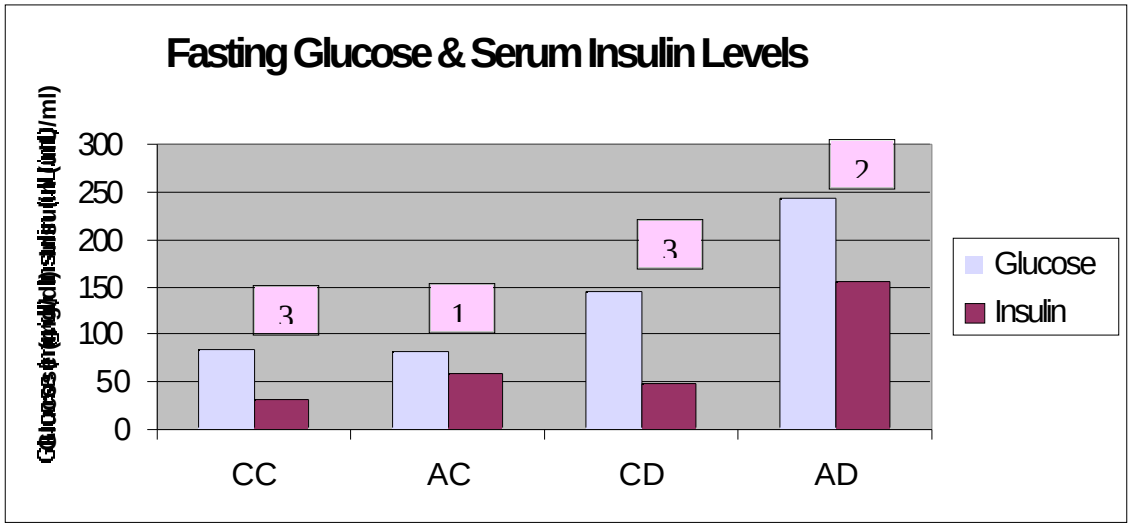

Figure 2. Fasting Glucose & Serum Insulin Levels. The glucose to insulin ratio is indicated in the text boxes. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic.

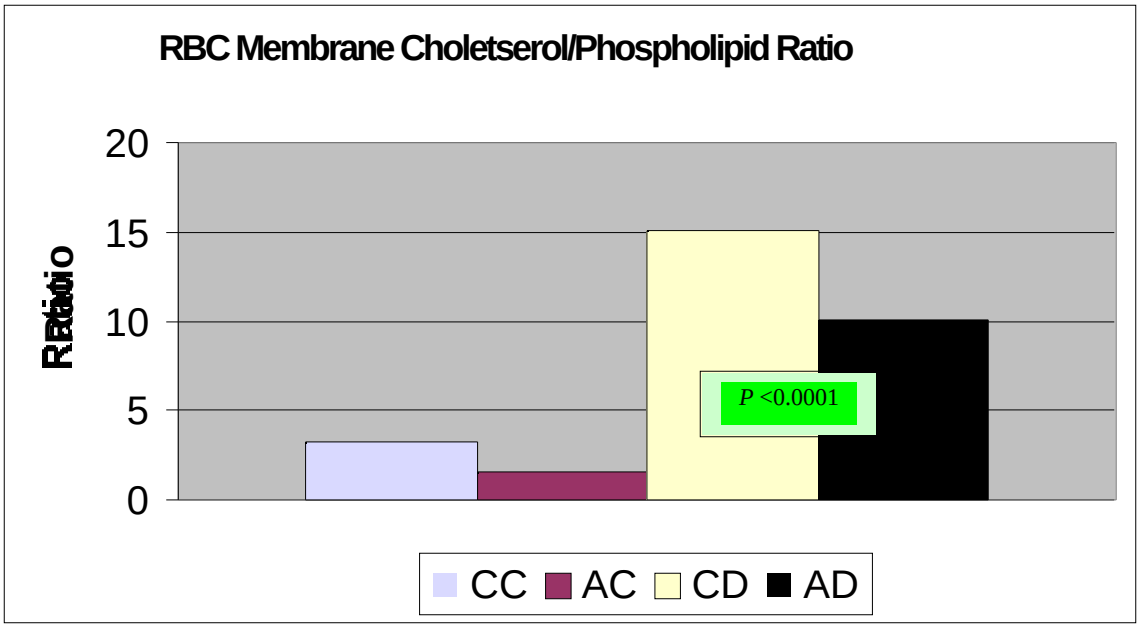

Figure 3. RBC Membrane Cholesterol / Phospholipid Ratio. The *P* value indicates a significant difference between control and diabetic subjects. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, Chol / PL= cholesterol / phospholipid ratio.

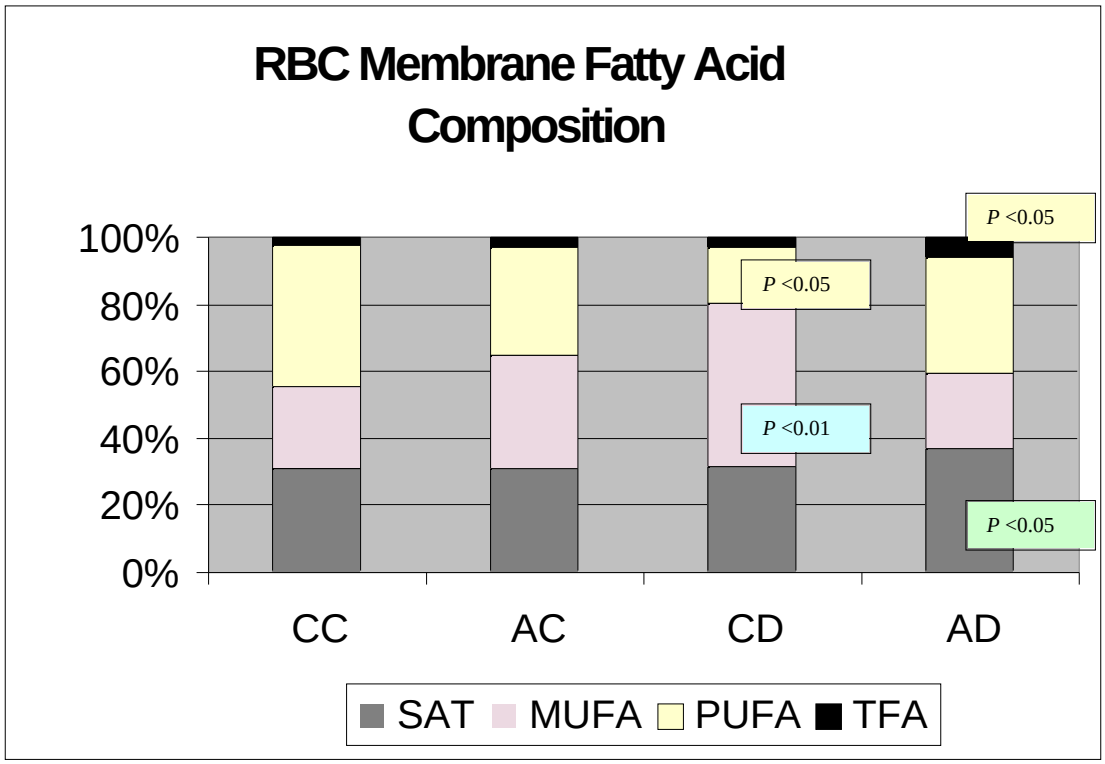

Figure 4. RBC Membrane Fatty Acid Composition. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, SAT= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, TFA= trans fatty acids. The statistical significance is listed in the text boxes based on the comparison of tagged group with the other groups.

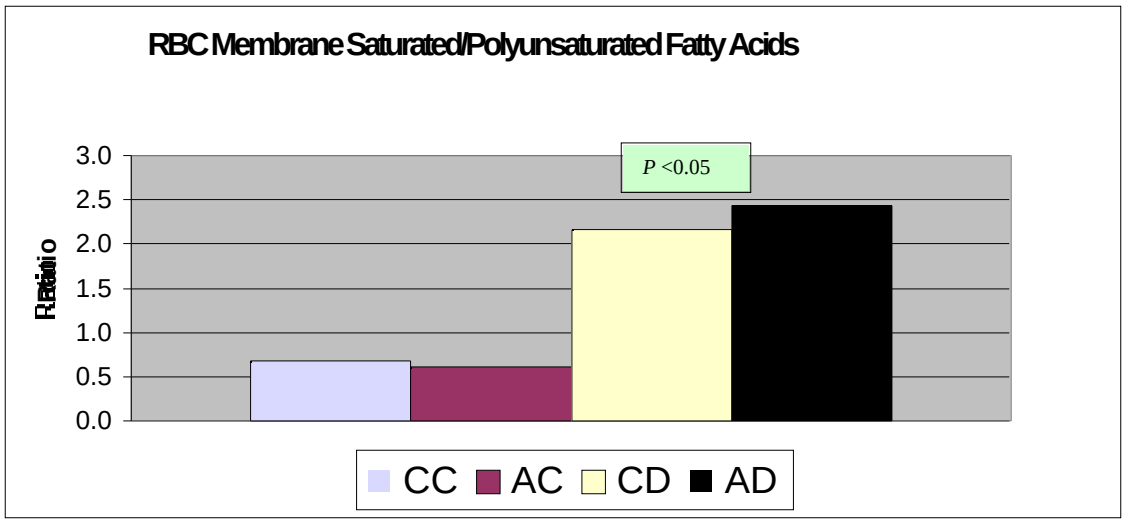

Figure 5. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic; SAT= Total saturated fatty acids; PUFA= Total Polyunsaturated fatty acids, S/P = saturated/polyunsaturated ratio. NOTE: The *P* value indicates significant difference between Control and Diabetics.

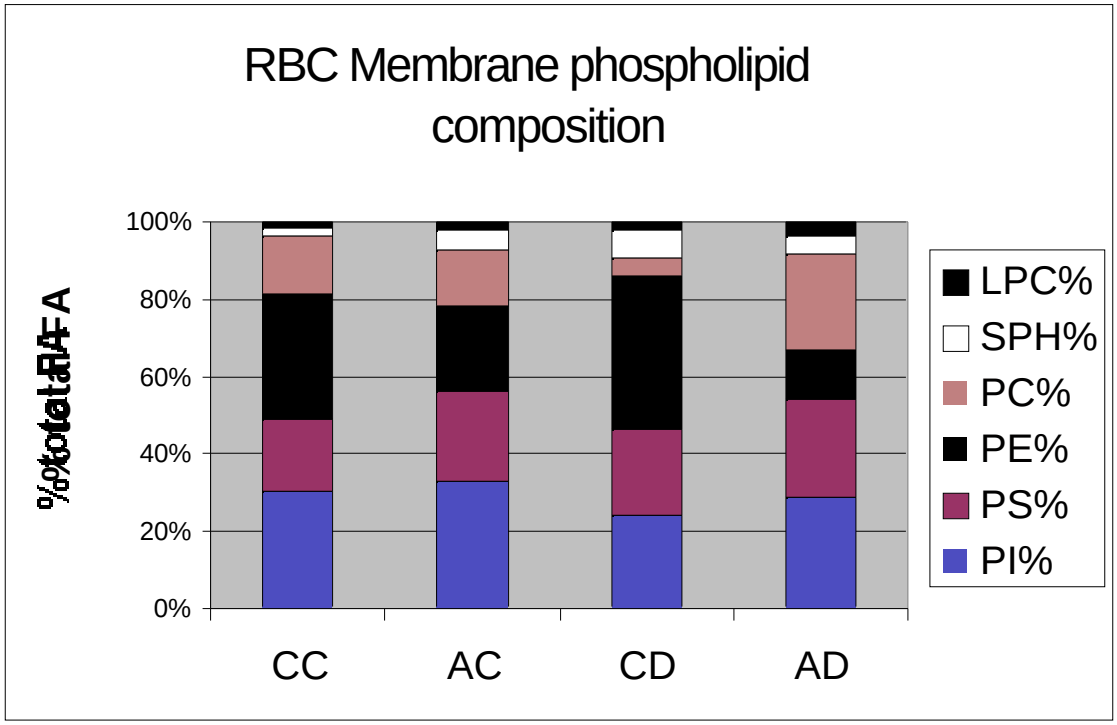

Figure 6. Speciation of RBC membrane phospholipids. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, PI%= phosphatidyl inositol%, PS%= phosphatidyl serine%, PE%= phosphatidyl ethanolamine%, PC%= phosphatidyl choline%, SPH= sphingomyelin, LPC%= lysophosphatidyl choline.
NOTE: The PE value of CD group is significantly different from CC group and AD (*P*= 0.01). PC value of AD is significantly different from AC and CD (*P* <0.05).

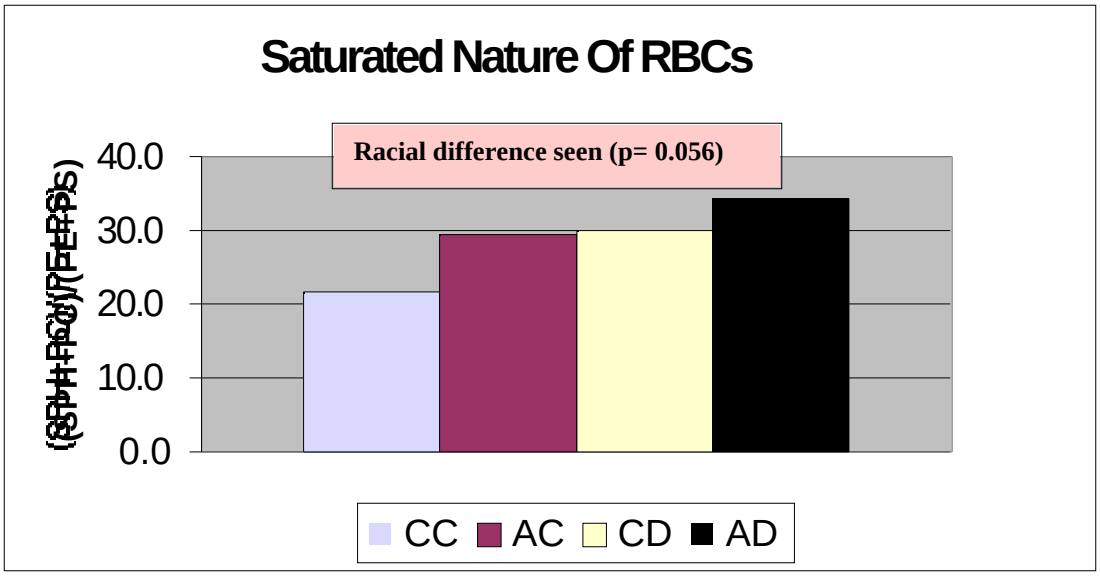

Figure 7. Saturated Nature Of RBCs. CC= Caucasian Control; AC= African American Control; CD= Caucasian Diabetic; AD= African American Diabetic, PS= phosphatidyl serine, PE= phosphatidyl ethanolamine, PC= phosphatidyl choline, SPH= sphingomyelin, LPC= lysophosphatidyl choline.

Table 1. Correlation Coefficient Between Diet & Anthropometric measures

	W/H	W/T	Body Fat%	BMI
Nutrients				
Kilocalories (kcal)	0.09	0.14	-0.09	0.05
Carbohydrate (gm)	-0.12	0.26	0.03	0.21
Protein (gm)	0.23	-0.11	-0.32	-0.17
Fat, total (gm)	0.21	0.29	0.51	0.21
Cholesterol (mg)	0.3	0.51	0.34	***.48**
Saturated Fat (gm)	0.22	0.34	0.19	0.25
Monounsaturated Fat (gm)	0.25	0.34	0.15	0.28
Polyunsaturated Fat (gm)	0.21	0.23	0.04	0.22
Oleic Acid (gm)	-0.02	0.19	-0.05	0.05
Linoleic Acid (gm)	0.11	0.22	-0.06	0.22
Linolenic Acid (gm)	0.1	0.17	-0.05	0.16

Note: W/H= Waist to hip ratio; W/T= Waist to thigh ratio; BMI= Body mass index (kg/m^2)

*** p= <0.05**

Table 2. Correlation Coefficient Between Blood Constituents & Nutrient Intake

	Glucose	T. Cholesterol	Triglyceride	VLDL	LDL	HDL	Hgb A_{1C}
Nutrients							
Kilocalories (kcal)	-0.24	-0.29	-0.13	-0.13	-0.33	0.17	-0.11
Carbohydrate (gm)	-0.31	***-0.34**	-0.23	-0.22	***-0.39**	0.29	-0.16
Protein (gm)	-0.1	-0.21	0.01	0.01	-0.25	0.02	0.001
Fat, total (gm)	-0.15	-0.18	-0.04	-0.03	-0.21	0.05	-0.05
Cholesterol (mg)	-0.02	-0.01	0.17	0.17	-0.03	-0.16	0.07
Saturated Fat (gm)	-0.15	-0.14	0.009	0.01	-0.18	0.04	-0.07
Monounsaturated Fat (gm)	-0.13	-0.19	-0.03	-0.02	-0.19	-0.01	-0.05
Polyunsaturated Fat (gm)	-0.17	-0.19	-0.15	-0.14	-0.15	0.01	-0.13
Oleic Acid (gm)	-0.09	-0.05	-0.05	-0.04	-0.1	0.15	-0.01
Linoleic Acid (gm)	0.13	0.19	-0.04	-0.06	-0.06	-0.04	0.16
Linolenic Acid (gm)	****0.45**	-0.05	-0.09	-0.09	-0.04	0.02	***0.35**

*** p= <0.05**

**** p= <0.01**

Table 3. Correlation Coefficient Between RBC Membrane Fluidity, D-6-Desaturase Enzyme Activity & Nutrient Intake

	RBC Chol	Saturate Nature	Chol / PL	Rheology	S/P	D-6-Desat
Nutrients						
Kilocalories (kcal)	0.17	-0.19	0.09	0.17	0.1	0.2
Carbohydrate (gm)	0.07	-0.1	-0.19	0.12	-0.003	0.17
Protein (gm)	0.2	-0.1	0.16	0.17	0.3	0.25
Fat, total (gm)	0.19	0.1	0.29	0.21	0.12	0.16
Cholesterol (mg)	-0.04	-0.09	0.23	-0.01	0.25	0.12
Saturated Fat (gm)	0.17	-0.15	0.29	0.13	0.16	0.14
Monounsaturated Fat (gm)	0.18	-0.16	0.26	0.21	0.13	0.23
Polyunsaturated Fat (gm)	0.11	-0.32	0.26	0.34	0.03	0.05
Oleic Acid (gm)	0.26	***-0.44**	0.15	0.32	-0.04	-0.1
Linoleic Acid (gm)	0.35	-0.33	0.18	0.21	-0.06	0.25
Linolenic Acid (gm)	***0.49**	-0.02	0.005	-0.06	-0.04	****0.55**

Note: RBC Chol= red blood cell membrane cholesterol; Saturate Nature is a calculated by sphingomyelin + phosphatidyl choline / phosphatidyl ethanolamine + phosphatidyl serine; Rheology = phosphatidyl Ethanolamine / phosphatidyl serine

*** p= <0.05**

**** p= <0.01**

Table 4. Correlation Coefficient of RBC Membrane Fluidity, Blood Constituents, Insulin Binding & Delta-6-desaturase Enzyme Activity

	Gluc	Chol	Trig	VLDL	LDL	HDL	Hgb A1C	D-6-Desat	Insulin	Ins Bind
RBC Chol (mcg/cell)	**0.65	0.1	0.16	0.17	0.09	-0.06	**0.63	0.2	0.4	**-0.61
Cholesterol / Phospholipid	0.37	0.34	0.34	0.34	*0.42	**-0.56	0.21	-0.09	0.07	-0.42
Saturate Nature	0.06	0.06	0.16	0.15	-0.01	0.02	0.1	**0.45	0.12	0.16
Rheology	-0.1	-0.12	-0.26	-0.25	0.01	-0.003	0.008	-0.2	-0.13	0.16
SAT / PUFA	0.13	-0.11	0.42	0.42	-0.18	-0.33	0.13	0.03	*0.42	*-0.45

Note: RBC Chol= red blood cell membrane cholesterol; Saturate Nature is a calculated by sphingomyelin + phosphatidyl choline / phosphatidyl ethanolamine + phosphatidyl serine; Rheology = phosphatidyl ethanolamine / phosphatidyl serine; SAT / PUFA= saturated fatty acids to polyunsaturated fatty acid ratio; Gluc= glucose; Chol= total cholesterol; TRG= triglyceride; Hgb A_1c= glycated hemoglobin; Ins Bind= insulin binding.

* *P* <0.01

** *P* <0.05

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