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OBESITY, NON-INSULIN-DEPENDENT DIABETES MELLITUS AND AGE, BUT NOT ESTROGEN, WERE ASSOCIATED WITH CHANGES OF Δ6 AND 
Δ5 DESATURASE ENZYME ACTIVITY IN FEMALE 
SHHF/MCC-FA<sup>CP</sup> RATS AND HUMANS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for 
the Degree Doctor of Philosophy in the Graduate 
School of The Ohio State University

By

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The Ohio State University
1996

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ABSTRACT

The impact of insulin and age on Δ6 and Δ5 desaturase (Δ6D and Δ5D) activities has received much attention by investigators. However, data from female humans and animals were insufficient. Two studies using SHHF/Mcc-faCP female rats (Study I) and pre- and post-menopausal women with non-insulin-dependent diabetes mellitus (NIDDM) (Study II) are conducted to investigate the effects of insulin and estrogen, with aging, on Δ6D and Δ5D enzyme activities and to seek a link between age, hormonal status, desaturase activity and arachidonic acid metabolism.

Fatty acids (FA) of different lipid fractions were extracted from tissues and measured using gas chromatography. Hormone levels were analyzed using radioimmunoassay (RIA) or enzyme immunoassay (EIA). Desaturase enzyme activities were determined either directly by using 14C-labeled substrates in the rat study or indirectly by calculating the ratios of product FA and substrate FA in the human study. Platelet aggregation as the functional impact of alteration of desaturase enzymes was measured using a whole blood aggregometer.

In the Study I, a significant genotype effect for both Δ6D and total Δ5D enzyme activities of female rats was observed, but age did not have a
significant influence on Δ6D enzyme activity. Only the activity of Δ5D consistently decreased with age. Product fatty acids of desaturase enzymes in liver, serum, and platelet phospholipids (PL) correlated with changes of desaturase activities, but not with platelet aggregation. Platelet aggregation did not differ with age or genotype. No correlations were found between platelet aggregation and desaturase activities.

In the Study II, diabetic condition had a significant positive effect on arachidonic acid from serum PL and overall indices of desaturase activity. There were significant positive correlations between overall desaturase activity and insulin or c-peptide. However, the effect of diabetes on platelet aggregation was not significant. Production of thromboxane (TXB2) in diabetic subjects was not different with controls, but a correlation was found between insulin and TXB2. Estrogen did not have a significant effect on any of the measures in this study.

Both studies indicated the significant diabetic and/or genotype effect on Δ6D and Δ5D enzyme activities and fatty acid compositions of tissues, but the effect was not as consistent on platelet aggregation. Estrogen and age did not have a strong influence on Δ6D enzyme activity as did insulin, only Δ5D activity decreased with age in the rat study. There was a link between insulin, desaturase activity and fatty acid compositions in tissue fractions, but the link did not extend to platelet aggregation as a functional indicator of desaturase activity and pathology.
To my dear parents

with love and gratitude
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ABBREVIATION

AA = arachidonic acid
ACAT = acyl-CoA : cholesterol acyltransferase
Apo = apolipoprotein
ASCVD = Atherosclerotic cardiovascular disease
BSA = bovine serum albumin
CA = coagulant activity
cAMP = cyclic adenosine monophosphate
CE = cholesteryl ester
CHD = coronary heart disease
CO = cyclooxygenase
Δ5 D = Δ5 desaturase
Δ6 D = Δ6 desaturase
DDW = deionized distilled water
DG = 1,2-diacylglycerol; diglyceride
DGLA = dihomo-γ-linolenic acid
DHA = docosapentaenoic acid
DM = Diabetes Mellitus
EFA = essential fatty acid
EIA = emzyme immunoassay
EPA = eicosapentaenoic acid
FAME = fatty acid methyl ester
FAO = Food and Agricultural Organization
FFA = free fatty acid
G6P = glucose-6-phosphate
GI protein = inhibitory G protein
GLA = y-linolenic acid
G protein = stimulatory G protein
HbA1c = glycosylated hemoglobin
HDL = high-density lipoprotein
HMG CoA = 3-hydroxyl-3-methyl glutaryl CoA
IDDM = insulin-dependent diabetes mellitus
IDL = intermediate density lipoprotein
IGF-I = insulin-like growth factor I
IGT = impaired glucose tolerance
IP3 = inositol 1,4,5-triphosphate
LA = linoleic acid
LCAT = lecithin : cholesterol acyltransferase
LDL = low-density lipoprotein
LNA = \( \alpha \)-linolenic acid
LP = lipoprotein
LPL = lipoprotein lipase
LSO = linseed oil
LT = leukotriene
MG = monoglyceride
mRNA = messenger RNA
MUFA = monounsaturated fatty acid
NIDDM = non-insulin-dependent diabetes mellitus
NSAID = non-steroidal anti-inflammatory drugs
NSB = non-specific binding
OA = oleic acid
OC = oral contraceptives
OGTT = oral glucose tolerance test
PAEF = platelet aggregation enhancing factor
PAF = platelet activating factor
PC = phosphatidylcholine
PDGF = platelet derived growth factor
PFK = phosphofructokinase
PG = prostaglandin
PGH2 = prostaglandin H2
PGI2 = prostacyclin
PGI3 = prostacyclin I3
PI = phosphatidylinositol
PL = phospholipid
PLO = palm oil
PS = phosphatidylserine
PUFA = polyunsaturated fatty acid
RIA = radioimmunoassay
SFA = saturated fatty acid
SFO = safflower oil
TG = triacylglycerol; triglyceride
TX = thromboxane
TXA2 = thromboxane A2
TXS = thromboxane synthetase
UDPG = uridine diphosphoglucose
VLDL = very-low-density lipoprotein
vWF = von Willebrand Factor
WHO = World Health Organization
Background and Justification

The prevalence of non-insulin dependent diabetes mellitus (NIDDM), hypertension, dyslipidemia, and atherosclerosis increases with progressive obesity and advancing age (Debry, 1987; Kannel, 1988; Reaven, 1988; DeFronzo and Ferrannini, 1991; Simonson). In the United States, 6 to 7 million people have diabetes and 90% of them are NIDDM (American Diabetes Association, 1993). By age 70, 45 to 50% of the elderly are obese and hypertensive, 10 to 12% have NIDDM, and over 50% have evidence of atherosclerotic cardiovascular disease (ASCAD) (DeFronzo and Ferrannini, 1991). Obesity, NIDDM, hypertension, dyslipidemia, and atherosclerosis are closely related and usually referred to as insulin-resistance syndrome or syndrome X. Hyperinsulinemia resulting from insulin resistance, which is commonly observed in obese individuals, has a significant role in the association of these disorders. To understand why older, and especially obese, adults are prone to such disorders, it is desirable to understand the biochemical and physiological events of aging and obesity.

The major cause of death in both type I and type II diabetes is ASCAD such as myocardial infarction due to coronary artery atheroma (Jialal and Chait, 1989;
Kristensen et al., 1989; Turner and Neil, 1992). Diabetic patients also have an increased risk of developing peripheral vascular disease, which may lead to stroke. Even the protective effect against heart disease observed in normal pre-menopausal women is lost in diabetes (Jialal and Chait, 1989; Barrett-Connor et al., 1991). According to the report of Panzram (1987), type II diabetic men and women in their 40s or 50s have about a 2-fold and 3.5-fold increased risk of cardiovascular death, respectively, compared to non-diabetic people. Diabetes occurring at an earlier age presents a greater health risk, especially in females. Diabetic pre-menopausal women have a 16-fold increased risk of heart disease compared to the healthy population. Overall, mortality in early-onset patients is increased 5-fold in men, but 11-fold in women (Dorman, 1984). The mechanism of increased risk for vascular disease and stroke in diabetic patients, especially in women, is not understood. Further studies have to be conducted.

Thrombosis plays an important role in the pathogenesis of atherosclerosis (Crowley, 1983; Sussman, 1985; Mustard et al., 1986; Monocada et al., 1986; Ross, 1986; Jialal and Chait, 1989; Notarbartolo et al., 1992). Vessel endothelial injury and platelet-vessel wall interaction are the initiating events of atherosclerosis. When the arterial endothelial wall is injured, platelets adhere to the area of damaged endothelium and release platelet-derived growth factor (PDGF), which stimulates smooth muscle cell proliferation. Thromboxane A2 (TXA2), and ADP are secreted from platelet granules enhancing aggregation and causing the formation of a platelet plug. Smooth muscle cells and macrophages incorporate cholesterol to form foam cells which are deposited on arterial walls as atheromatous plaques (atheroma). Advanced atherosclerosis leads to narrow vessels and roughened surface that predisposes to thrombus formation around the atheroma.
Both TXA₂, a strong aggregator produced by platelets, and prostacyclin (PGI₂), an equally potent antiaggregator synthesized by vascular endothelial cells, affect thrombosis formation from platelets (Dillon, 1987; Notarbartolo et al., 1992). The balance of platelet TXA₂ and vascular PGI₂ may affect atherogenesis (Notarbartolo et al., 1992). Hornstra et al. (1983) suggested that platelet TXA₂ formation is more important than vascular PGI₂ production in regulating arterial thrombosis. PGI₂ is thought to be simply a local regulator of platelet function because of its very low level in circulation (Hornstra et al., 1983; Longenecker, 1985; Notarbartolo et al., 1992), unlike TXA₂. Abnormalities in platelet functions, platelet aggregation, and fibrinolysis are seen in experimental animals (Ruf et al., 1991; Iida et al., 1993) and human diabetics (Winocour, 1985; Davis et al., 1985; Mandal et al., 1993). Diabetic patients synthesize more TXA₂ than healthy individuals (Lagarde, 1980; Halushka, 1981; Davi et al., 1982), but vascular biosynthesis of PGI₂ decreases in both IDDM and NIDDM patients (Winocour et al., 1985).

Age is also associated with abnormal TXA₂ and PGI₂ production. With advancing age, vascular PGI₂ production decreases but platelet formation of TXA₂ increases (Chang et al., 1980; Kent et al., 1981; Murota, 1983; Vericel et al., 1985; Moncada, 1986; Dillon, 1987; Meydani et al., 1992). This may account, in part, for the higher incidence of coronary artery disease in old and NIDDM patients compared to young and healthy individuals. The balance of TXA₂ and PGI₂ is also involved in blood pressure regulation since TXA₂ is a vasoconstrictor and PGI₂ is a vasodilator (Dillon, 1987). Diabetic patients tend to be hypertensive, which may be related to the increased TXA₂/PGI₂ ratio.
Arachidonic acid (AA; C20:4ω6) is the metabolic precursor for both TXA2 and PGI2 (Mayes, 1990a). It is released from platelet membrane phospholipids (PL) by phospholipase A2 and converted into TXA2 by sequential actions of cyclooxygenase, endoperoxidase and thromboxane synthase (Longenecker, 1985; Mayes, 1990b). The content of AA in platelet membrane may play an important role in platelet function. AA originates from either the diet or endogenous synthesis via microsomal desaturation and elongation (Dillon, 1987; Mayes, 1990a). Fatty acids in the ω3, ω6 and ω9 families compete as substrates for the desaturase enzymes (Mayes, 1990a). The composition of fatty acids in tissues, e.g., serum, platelet, and hepatocytes, may be affected by the dietary sources (Hornstra et al., 1983; Socini et al., 1983; Weaver et al., 1985; Vericel et al., 1987; Lee et al., 1988; McDonald et al., 1989; Piche and Mahadevappa, 1990; Phinney et al., 1990; Agren et al., 1990; Agren et al., 1991; Kwon et al, 1991; Hansen et al., 1993) and desaturase activities (Wahle, 1983; Mead et al., 1986; Marra and Alaniz, 1990).

Δ6 desaturase (Δ6D) is the rate-limiting enzyme in the synthesis of AA from linoleic acid (C18:2ω6; LA) (Brenner, 1981; Dillon, 1987; Mayes, 1990a; Yamazaki, 1992). Activity of Δ6D may decrease with age in humans and rats (Peluffo, 1974; Horrobin, 1981; Brenner, 1981; Bordoni, 1988; Blond, 1989; Hrelia, 1989; Kalen, 1989; Hrelia, 1990; Bourre, 1990; Hrelia, 1991; Ullmann, 1991a; Ullmann, 1991b). However, these studies were done with either male subjects only or mixed genders. A previous study using female subjects did not find evidence of decreased Δ6D activity in older women (Liu and Medeiros, 1995).
Estrogen suppresses Δ6D and Δ5D activity (Brenner, 1981; Gonzalez et al., 1986), but a correlation between estrogen level and indices of Δ6D and Δ5D activity in aging women was not observed (Liu and Medeiros, 1995). Since loss of estrogen is one manifestation of aging, estrogen loss in women may effect Δ6D activity differently. Further investigation is needed to understand the effect of reduced Δ6D activity on risk factors for cardiovascular disease related to platelet aggregation.

Insulin increases Δ6D and Δ5D activity (El Boustani et al., 1989; Brenner, 1990; Mimouni and Poisson, 1992). Hyperinsulinemia is commonly seen in obese people (Reaven, 1988; DeFronzo and Ferrannini, 1991) and rats (McCune et al., 1990; Radin et al., 1993) and is often associated with insulin resistance. Enhanced activity of Δ6D observed in obese humans and animals may be in part due to hyperinsulinemia. In our previous studies, obese women (BMI > 30) had significantly higher (p<.05) insulin levels than lean women (Medeiros et al., 1995). Insulin level was positively correlated with serum 20:4ω6/18:3ω6 ratio, an index of Δ5D activity. However, no correlation was observed between insulin and serum and platelet 18:3ω6/18:2ω6 index of Δ6D activity. Obese women also had significantly greater platelet aggregation and shorter lag time compared to lean women (P<.05). A possible explanation for the altered platelet function in obese women could be increased AA formation (Δ6D and Δ5D activity) and metabolism (production of PGI2 and TXA2). Both desaturase activity and eicosanoid formation can be affected by hormonal activity, specifically insulin and estrogen.
Why do diabetics, especially pre-menopausal diabetic women, and older individuals have greater risk for developing atherosclerotic cardiovascular diseases such as coronary artery disease than non-diabetics? Since thrombosis is strongly associated with atherogenesis, platelet aggregation and related lipid metabolism may play an important role in the mechanism. Insulin and estrogen are two major hormones that may also be involved.

Two studies were conducted. In the first study, using a spontaneous hypertensive, heart failure rat model (SHHF/Mcc-fSP), microsomal Δ6D and Δ5D activities were measured directly. These rats are genetically homogeneous with known genotypes. In this study, microsomal Δ6D and Δ5D activities could be directly measured. Dietary influence was also minimized by providing the same commercial diet. However, insulin and estrogen effects were not able to be determined in the first study, and the link between AA content in tissue lipid fractions and platelet aggregation was not clearly established. Also whether or not the relationship between hormonal status and desaturase activities can exist in humans was not determined. The second study using human females applied and compared the findings observed from the animal study to humans. The association between insulin, estrogen, fatty acid composition, prostaglandin production, and platelet aggregation were determined in the second study.

The objective of these studies is to establish a link between age, hormonal status, desaturase activity and arachidonic acid metabolism. The primary questions to be answered in these studies are: Does desaturase activity (either Δ6D or Δ5D) differ with age in females? Numerous researchers evaluating males or mixed gender populations suggest that desaturase enzyme activity is decreased with aging. The previous study suggested a different story for
females (Liu and Medeiros, 1995). Would hyperinsulinemia resulting from insulin resistance in obese rats and in obese, diabetic women exacerbate the changes of desaturase activity? Also, would loss of estrogen in post-menopausal women change desaturase activity after the known inhibitory influence of estrogen on desaturase activity is lost?

**Questions and hypotheses**

These two studies were designed to answer the following questions:

**Question 1:** Did Δ6D and Δ5D activities (direct measures or indices) vary with phenotype (obese and lean) and/or age in female humans and rats?

**Hypothesis:** Old and obese females would have higher Δ6D and Δ5D activities.

**Question 2:** Did measures of platelet aggregation change in obese and/or old females?

**Hypothesis:** Platelet aggregation would be greater and clotting would commence in less time in obese females (both humans and rats) than in lean females. Age would exacerbate the effect of obesity on platelet aggregation.

**Question 3:** Did measures of platelet aggregation and Δ6D and Δ5D activity correlate with serum insulin, estrogen, and/or fasting glucose levels in females?

**Hypothesis:** Measures of platelet aggregation and Δ6D and Δ5D activity indices would be positively correlated with serum insulin and fasting glucose levels because both factors represent the severity of insulin resistance. There would be a negative correlation between platelet aggregation, Δ6D, Δ5D activity and serum estrogen concentrations.
**Question 4:** Did measures of platelet aggregation and Δ6D and Δ5D activity correlate with the fatty acid compositions, especially arachidonic acid, in different lipid fractions from different tissues?

**Hypothesis:** There would be a positive correlation between the measures of platelet aggregation and AA levels in serum and platelets phospholipids.

**Question 5:** Did thromboxane A₂ (TXA₂) production correlate with platelet aggregation, ATP release, platelet phospholipid AA content, indices of Δ6D and Δ5D activity, serum insulin and estrogen levels in females?

**Hypothesis:** TXA₂ level would be positively correlated with platelet aggregation, ATP release, platelet phospholipid AA content, indices of Δ6D and Δ5D activity, serum insulin and estrogen levels.
CHAPTER 2

LITERATURE REVIEW

NON-INSULIN-DEPENDENT DIABETES MELLITUS
AND OBESITY

Diabetes Mellitus

Diabetes mellitus is a chronic disorder resulting from an disorder of insulin secretion and/or insulin response. It is the fourth leading cause of death by disease in America (American Diabetes Association, 1993). In the United States, 6.5 million people (about 3% of the population) are diagnosed with diabetes. Overall, there are about 14 million (5% to 6% of the population) diagnosed and undiagnosed cases of diabetes mellitus (ADA, 1993).

Two major types of diabetes have been extensively studied: insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). This classification is based on the therapeutic requirement for insulin supplementation. If classified according to pathology, the former is type I diabetes and the latter is type II diabetes. There are different clinical characteristics between these two types of diabetes. Briefly, IDDM (type I) generally presents in non-obese children or young adults within a short period...
of time (2-4 weeks). The onset age varies between 0 and 30 years. These patients exhibit typical symptoms: hyperglycemia, polyphagia, polyuria, polydipsia. They are likely to develop ketoacidosis if untreated. They require insulin therapy and respond to insulin sensitively. The β-cells of the pancreas may be destroyed by immune or inflammatory cells. Antibodies directed against the islet cells can be detected in these patients suggesting that IDDM may be an autoimmune disease.

Compared to IDDM, NIDDM usually appears later in life (35 years or later) in obese people. Symptoms often last for years. Hyperglycemia in NIDDM is more moderate compared to IDDM patients. Ketoacidosis rarely occurs in these patients unless a major infection appears. They can be treated with diet and/or oral hypoglycemic agents. Insulin is usually not required to keep these patients alive, since insulin can still be secreted from the pancreas. However, they are usually insulin resistant and a higher insulin concentration may be required for proper action. Islet cell antibodies are rarely present in this type of diabetes (Turner and Nell, 1992).

The criteria for diabetes and impaired glucose tolerance (IGT) have been established by World Health Organization using a 75 g oral glucose tolerance test (WHO, 1985) (Table 1). The fasting plasma glucose concentration is stable from day to day in normal and NIDDM patients with a proper state of nutrition and in good health. The fasting plasma glucose concentration can be used as an index of the severity of diabetes. However, with a progressive disease like NIDDM, a single fixed glucose concentration is not sufficient for diagnosis (Turner and Nell, 1992).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Glucose concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Blood</td>
</tr>
<tr>
<td></td>
<td>Venous Capillary</td>
</tr>
<tr>
<td>Type I and II Diabetes</td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>≥6.7</td>
</tr>
<tr>
<td>2 hr after glucose load</td>
<td>≥10.0</td>
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<tr>
<td>Impaired glucose tolerance</td>
<td></td>
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<tr>
<td>Fasting</td>
<td>&lt;6.7</td>
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<tr>
<td>2 hr after glucose load</td>
<td>6.7-10.0</td>
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Table 2.1: WHO criteria for diabetes and impaired glucose tolerance: diagnostic values for a 75 g oral glucose tolerance test (OGTT) (WHO, 1985)

Etiology of non-insulin-dependent diabetes mellitus (NIDDM)

The major factors that may cause NIDDM or type II diabetes are genetics, environment, and nutrition. Studies of twins indicated a strong genetic influence on the incidence NIDDM. Barnett (1981) reported that among 200 pairs of twins, 48 out of 53 pairs (91%) of the twins were concordant for type II diabetes. In contrast, only 80 out of 147 pairs (54%) of twins developed type I diabetic. It has been suggested that genetic factors can have almost 100% penetrance for type II diabetes, especially at advanced ages. At least in homozygous twins, the concordance of NIDDM may reach 100 percent (Cerasi, 1992). Family studies also indicate a strong genetic influence. About 42% of
identical NIDDM twin pairs had first degree relatives with NIDDM. Individuals having both a sibling and a parent with NIDDM have higher fasting blood glucose, HbA1, and fasting and stimulated plasma insulin than controls (Leslie et al., 1986). Pima Indians have the highest prevalence of diabetes than any other population in the world (Knowler et al., 1978; Bogardus C, 1993). No associations between specific HLA type genes and NIDDM have been identified in Caucasian population. It is likely that the transmission of NIDDM is controlled by gene complexes (Taylor, 1989).

The early stage of NIDDM is usually asymptomatic. Two-hour oral glucose tolerance tests (OGTT) may be used for diagnosis (Table 1). It is not clear whether impaired glucose intolerance (IGT) is a necessary stage in the development of NIDDM. Not all people with IGT develop NIDDM (Harris, 1989). However, in high risk populations, there is a positive correlation between abnormal two hour OGTT and the future development of type II diabetes (Cerasi, 1992).

Diet and obesity are two environmental factors that may be related to the development of NIDDM. The incidence of NIDDM decreased during World War II perhaps due to deprivation of food. However, the lower calorie intake which decreased the occurrence of obesity rather than diet per se may have been the cause. Dietary therapy can improve measured insulin sensitivity, but this is a physical adaptation. Taylor (1989) suggested that dietary factors alone do not initiate NIDDM, but they may be likely to influence the rate of progression to clinical symptoms.

It has been commonly accepted that obesity plays a significant role in the development of type II diabetes (Cerasi, 1992; Turner and Neil, 1992). However, Taylor (1989) believed that there is little or no direct evidence that...
obesity plays an etiological role in the development of diabetes. In a study of 2000 NIDDM subjects, 1.54 times more males than females were diabetic even though males were less obese than females (121 vs 141% ideal body weight) and only marginally more obese than the general population. Taylor (1989) suggested that any theory relating obesity to the etiology of NIDDM should account for gender differences.

In contrast to Taylor (1989), Golay and Felber (1994) considered that obesity may be more than a risk factor but a causal factor for the development of NIDDM on a genetic base. According to Golay and Felber (1994), there are four stages of the evolution from obesity to diabetes (Fig. 2.1)

Obesity with normal glucose tolerance is the first stage. Obese people have higher lipid oxidation originating from muscle triacylglycerols that competitively lowers glucose oxidation. The decrease in glucose utilization affects the capacity for further glycogen synthesis and glucose storage. Less glucose is taken up by tissues, mainly skeletal muscle and adipose tissues. Higher amounts of insulin are released by β-cells in response to hyperglycemia. Thus hyperinsulinemia, one of the first anomalies of obesity, represents a consequence of tissue resistance to glucose uptake. Obesity with normal glucose tolerance shows a compensated state of insulin resistance via hyperinsulinemia. Following a glucose load, the elevation of plasma glucose induces an increase of glucose-6-phosphate (G6P), which stimulates glycogen synthase activity. When glycogen pools are full, the lowering of plasma glucose induces glycogenolysis and glycogen is released from storage pools (Golay and Felber, 1994).
Fig. 2.1: Evolution from obesity with normal glucose tolerance to Non-Insulin-Dependent Diabetes Mellitus. (Modified from Golay and Felber, 1994)
In obese people with impaired glucose tolerance, the insulin resistance is more profound. Increased levels of insulin cause down-regulation of insulin receptors on cell membranes of tissues. Therefore, hyperinsulinemia and hyperglycemia appear after a glucose load or a meal. Since lipid oxidation is the preferred energy source, the reduction of glucose utilization causes an elevation of glycogen storage, which inhibits glycogen synthase activity. In this stage, decreased glucose storage can still be compensated by hyperinsulinemia.

When obese people with impaired glucose tolerance develop diabetes, the metabolic changes exhibited are increased lipid oxidation, decreased glucose oxidation, and high basal and post-load insulin concentrations. The hyperglycemia of the basal state and post-glucose load reaches diabetic levels. In mild fasting hyperglycemia, elevated glucose levels result from decreased efficiency of glucose uptake by tissues such as muscle. Glucose storage is decreased in spite of hyperinsulinemia. The defect of glucose storage is related to the insulin-stimulated activity of skeletal muscle glycogen synthase, which is regulated by synthase phosphatase. Kida et al. (1990) found no increase in synthase phosphatase activity in obese Pima Indians with insulin resistance. Chronic hyperglycemia decreases glycogen storage and also inhibits the mobilization of glycogen due to decreased phosphorylase activity. In the final stage of progression from obesity to diabetes, the compensatory mechanism is no longer functional, and β-cells lose the ability to respond to hyperglycemia with insulin secretion. The lack of circulating insulin leads to increased hepatic glucose production which causes further hyperglycemia (Golay and Felber, 1994). The rate of glucose release from the liver is highly correlated with blood
glucose levels. Thus, it is suggested that the rate of glucose release from the liver is an important index of the severity of fasting hyperglycemia in diabetes (Simonson, 1993). Overall, the sustained increase of lipid oxidation and duration of obesity are two important factors in the progression from obesity to diabetes (Cerasi, 1992; Turner and Neil, 1992; Golay and Felber, 1994). People with abdominal obesity are more likely to develop diabetes than those who have peripheral obesity (Cerasi, 1992; Turner and Neil, 1992).

**Insulin secretion**

When ingested glucose is absorbed and reaches the blood stream, specific glucose transporter (Glut-2) located in the microvilli of the canaliculi between β-cells facilitate the diffusion of glucose into β-cells. Glucokinase is important in regulating the subsequent β-cell response. Phosphorylation of glucose by glucokinase is a rate-limiting step for insulin secretion and glucose oxidation. Through glycolysis and the citric acid cycle in the islet cells, ATP is produced from glucose oxidation. The elevation of ATP concentration causes an ATP-sensitive K⁺ channel to close. K⁺ efflux is suppressed, leading to the depolarization of β-cell membranes. This opens a voltage-regulated Ca²⁺ channel and results in a rapid increase of intracellular Ca²⁺, which activates myosin light-chain kinase. Secretory granules are then mobilized toward the membrane surface. Insulin is released from granules via exocytosis (Berne and Levy, 1993). One class of the oral hypoglycemic agents, sulfonylurea
drugs, stimulates insulin secretion by directly closing the K⁺ channel (Berne and Levy, 1993).

Following the exposure of β-cells to glucose, cAMP levels are elevated after adenylate cyclase activation. A cAMP-dependent protein kinase (protein kinase A) also stimulates the release of insulin from granules by phosphorylating proteins that are involved with insulin exocytosis. Besides glucose, many substances can stimulate or inhibit the secretion of insulin. For instance, glucagon stimulates insulin release via stimulatory G protein (Gs protein) located on the membrane of islet cells. Gs protein acts as a secondary messenger to activate adenylate cyclase. Acetylcholine stimulates a Gs protein which activates phospholipase C to generate inositol 1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol (DG) from phosphatidylinositol. IP₃ facilitates the increase of intracellular Ca²⁺ and DG stimulates insulin release via the activation of protein kinase C. Somatostatin inhibits the secretion of insulin through the inhibitory G protein (Gi protein) which inhibits the activity of adenylate cyclase.

Insulin release exhibits a biphasic response. Within seconds after the exposure of β-cells to glucose, there is an immediate pulse of first-phase insulin release, peaking at 1 minute and then returning to baseline within 3 to 5 minutes. After 10 minutes of continuous glucose stimulation, a second phase of insulin secretion gradually rises to a plateau and is maintained for several hours in normal individuals (Berne and Levy, 1993; Felig and Bergman, 1995). There are two pools of insulin storage that contain granules with different sensitivities to glucose. First-phase insulin secretion may be the release of
granules which contain preformed insulin. The second phase response may represent newly synthesized insulin after stimulation by glucose. It is thought that glucose mainly stimulates post-transcriptional insulin synthesis. It is independent of new insulin mRNA synthesis.

Impaired or missing first-phase insulin response is one of the characteristics of NIDDM (Taylor, 1989; Cerasi, 1992). Normal basal insulin release corresponds with the normal basal fasting glucose concentration. The higher the fasting blood glucose, the flatter the curve of insulin response to glucose. Since NIDDM patients are hyperglycemic, their basal state is equivalent to a long-term exposure of glucose stimulation in normal people. The basal insulin level in NIDDM is the result of β-cell adaptation to hyperglycemia (Cerasi, 1992).

Insulin response plays an important role in the progression from glucose intolerance to NIDDM. Kadowaki et al. (1984) noted that individuals who had glucose intolerance and low insulin responses to glucose eventually developed NIDDM, whereas those who had glucose intolerance and high insulin responses did not progress to diabetes. Decreased insulin response precedes the appearance of glucose intolerance and hyperglycemia (Cerasi, 1992). Low insulin response to glucose may be a risk factor for the later development of glucose intolerance in a normal individual. For a glucose intolerant person, the low insulin response is a risk factor for the transition to NIDDM (Cerasi, 1992).

**Insulin resistance**

Insulin resistance is present when insulin is not able to stimulate normal cellular glucose uptake. It is highly associated with NIDDM, obesity.
hypertension, hyperlipidemia, and coronary artery disease. This complex is called Syndrome X, Reaven's syndrome, or insulin-resistance syndrome (DeFronzo and Ferrannini, 1991; Borkman et al., 1993; Simonson, 1993). Even in nonobese individuals with normal oral glucose tolerance, about 25% have insulin resistance.

There are two types of insulin resistance. One type involves decreased affinity and/or number of insulin receptors, or a defective receptor function (e.g., the failure of insulin to stimulate the autophosphorylation of tyrosine kinase of the receptor). This type of receptor-mediated insulin resistance is characterized by a shift of the insulin dose response curve to the right (i.e., increase Km), but the maximal response of tissue (Vmax) is not affected. This type is called decreased insulin sensitivity.

Another type of insulin resistance is characterized by a downward shift of insulin dose response curve (i.e., decreased Vmax) without the change of Km. It may result from a post-receptor defect, such as defective mobilization of glucose transporters from the interior of cells to the cell membranes, impairment of the metabolic pathways for glucose storage and/or oxidation, or the absence of sufficient receptor-binding capacity. This postreceptor-mediated insulin resistance is termed decreased insulin responsiveness. Most cases of NIDDM and obesity are of mixed type with both reduced insulin sensitivity and responsiveness. Due to the impairment of glucose uptake by tissues, insulin resistance results in hyperglycemia. In response to insulin resistance, pancreatic β-cells secrete more insulin that leads to compensatory hyperinsulinemia. Therefore, insulin resistance can be characterized by both hyperinsulinemia and hyperglycemia (Simonson, 1993; Felig and Berman, 1995).
Obesity is an acquired promoter of insulin resistance. Decreased numbers of insulin receptors have been observed in monocytes, adipocytes, hepatocytes, and muscle cells (Felig and Berman, 1995). Excess lipid oxidation, which usually occurs in obesity, can result in an impaired rate of insulin-mediated glucose storage and glucose oxidation. Decreased intracellular and plasma membrane pools of glucose transporters may also account in part for insulin resistance in NIDDM. Therefore, insulin resistance may be a consequence of a variety of mechanisms at the receptor and/or postreceptor sites.

Persistently high plasma insulin levels resulting from insulin resistance may contribute to the development of hypertension, abnormal plasma lipids, and atherosclerosis (DeFronzo and Ferrannini, 1991; Simonson, 1993). Fig. 2.2 summarizes the current understanding of causes and effects of insulin resistance.

![Diagram](image)

Fig. 2.2: Cause and effect of insulin resistance. Also considered as Syndrome of Insulin Resistance. (Modified from DeFronzo and Ferrannini, 1991)
Hypertension

Individuals with essential hypertension have a 30% to 35% reduction in glucose utilization. The degree of insulin resistance is positively related to blood pressure, especially systolic pressure (Ferrannini et al., 1987; DeFronzo and Ferrannini, 1991; Simonson, 1993). There are many possible mechanisms by which insulin resistance may be related to hypertension (DeFronzo and Ferrannini, 1991; Simonson, 1993).

Insulin decreases sodium excretion rate, which increases sodium retention. Consequently, extracellular volume expands, and blood pressure is elevated. The antinatriuretic effect of insulin is exerted on the proximal and distal parts of the nephron. A second possible mechanism is that insulin induces a dose-related release of norepinephrine from the sympathetic nervous system (Rowe et al., 1981; DeFronzo and Ferrannini, 1991; Simonson, 1993). Norepinephrine enhances cardiac output via increased cardiac contractility and heart rate. It also increases cardiopulmonary blood volume through constriction of the great veins. In addition, norepinephrine stimulates contraction of resistance vessels including renal vessels, and increases renal tubular sodium reabsorption and renin secretion. Therefore, the expansion of extracellular fluid volume resulting from elevation of norepinephrine concentrations contributes to higher blood pressure. Insulin may also affect the transport and distribution of cations, e.g., $K^+$, $Na^+$, and $Ca^{++}$, in the cell membrane, which influence the polarization of cell membranes. Altered polarization in membranes of cardiac muscle cells may interfere with the inotropic force and contraction of the heart. If the alterations occur in the membranes of vascular smooth muscle cells, these cells may be more responsive to vasopressor hormones such as catecholamines.
and angiotensin II. Another possible mechanism is that insulin may directly or indirectly stimulate the production of growth factors, such as insulin-like growth factor I (IGF-I). This could lead to hypertrophy of the vascular wall and narrowing of the lumen of the resistance vessels.

Hyperlipidemia

Characteristic lipid profiles in people with NIDDM include increased free fatty acids (FFA), increased serum very low-density lipoprotein (VLDL)-cholesterol, elevated serum low density lipoprotein (LDL)-cholesterol levels, and decreased serum high density lipoprotein (HDL)-cholesterol (DeFronzo and Ferrannini, 1991; Simonson, 1993).

The impaired ability of insulin to inhibit lipolysis in insulin resistance contributes to the release of FFA into circulation. When serum FFA are elevated, higher lipid oxidation overcomes glucose oxidation, and consequently reduces the utilization of glucose by tissues. In addition, the rate of hepatic gluconeogenesis is also increased due to higher availability of acetyl-CoA from FFA oxidation. Both conditions exacerbate the development of hyperglycemia. The elevated FFA concentrations may also interfere with the interaction of insulin with its receptor and the signal transduction from the receptor complex to the effectors. For instance, increased acetyl-CoA may inhibit the activity of pyruvate dehydrogenase (PDH) and, as a consequence, glucose oxidation. An excess of acetyl-CoA also increases the formation of citrate, which inhibits glycolysis via the inhibition of phosphofructokinase (PFK). Glucose-6-phosphate (G6P) then accumulates and inhibits hexokinase and formation of the substrate of glycogen synthase, uridine diphosphoglucose (UDPG). Since
G6P is also the activator of glycogen synthase, glycogen synthesis depends on the balance of these opposite effects (Bonadonna and DeFronzo, 1992; Simonson, 1993).

Increased serum FFA and glucose leads to more hepatic synthesis of triacylglycerol, which is combined with apoproteins to form VLDL. The catabolism of VLDL to LDL in the circulation is dependent on lipoprotein lipase, which requires normal insulin action. In a state of insulin resistance, lipoprotein lipase activity is suppressed, and VLDL-triglycerides cannot be removed efficiently from the circulation. Increased production in combination with decreased clearance of VLDL-triglyceride results in hypertriglyceridemia in patients with insulin resistance (DeFronzo and Ferrannini, 1991; Simonson, 1993).

LDL-cholesterol is derived from VLDL-cholesterol. Thus, high levels of VLDL-cholesterol increase the concentrations of LDL-cholesterol. In NIDDM patients, LDL-cholesterol is usually modified via glycosylation and/or oxidation. The glycosylated and oxidized LDL-cholesterol are more likely to be taken up by scavenging macrophages. This could lead to the formation of foam cells on the vascular walls, and to the stimulation of the secretion of macrophage growth factors, which induce the proliferation of smooth muscle cells (Jialal and Chait, 1989; Simonson, 1993).

Under normal condition, nascent HDL produced from either the liver or intestines interacts with triglyceride-rich lipoproteins such as VLDL and chylomicrons via the action of lipoprotein lipase to form mature HDL. In NIDDM patients, lipoprotein lipase is impaired and consequently reduces the formation of mature HDL. In addition, the activity of hepatic lipase is enhanced, thus the removal of HDL from the circulation may be accelerated. It is also likely that
HDL may be modified by glycosylation which may decrease its cholesterol scavenging effect (Simonson, 1993).

**Atherosclerosis**

Atherosclerosis is the major factor responsible for the high morbidity and mortality associated with insulin resistance (Jialal and Chait, 1989; Simonson, 1993). Plaques start with injury of endothelial cells on the arterial wall possibly caused by a combination of factors such as hypertension, hyperlipidemia, hyperinsulinemia, cigarette smoking, trauma. The arterial damage leads to a series of responses including adhesion of platelets, which release platelet-derived growth factors. This induces proliferation of smooth muscle cells in the lining of arterial walls, deposition of lipids or abnormal lipids such as oxidized LDL-cholesterol in macrophages to form the foam cells, and deposition of connective tissue collagen.

Hyperinsulinemia induced by insulin resistance enhances the formation of atherosclerosis. At elevated concentrations, insulin may act like a growth factor to stimulate the proliferation of medial and intimal smooth muscle cells and the growth of connective tissues. Insulin can also stimulate platelet aggregation. Cholesterol uptake into atherosclerotic lesions by LDL receptors can be enhanced by insulin since insulin increases LDL receptor activity and endogenous cholesterol synthesis.
**DESATURASE SYSTEMS**

**Physiological functions**

Saturated fatty acids (C4-C16) can be synthesized de novo from acetyl CoA by the cytosolic fatty acid synthase complex with palmitate (16:0) as the end product. Longer chain fatty acids, especially monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are synthesized by way of endogenous desaturation and elongation systems (Mead et al., 1986; Mayes, 1990a; Mayes, 1990c). In animals, only Δ5 desaturase (Δ5D), Δ6 desaturase (Δ6D), and Δ9 desaturase (Δ9D) exist. Additional double bonds can only be introduced between the existing double bond and the carboxyl carbon at Δ5, Δ6 and Δ9 position counting from the carboxyl terminus. Desaturases in plants can introduce double bonds at the Δ6, Δ9, Δ12, and Δ15 positions (Mayes, 1990a; Mayes, 1990b).

Animals are able to synthesize the ω9 family of unsaturated fatty acids but unable to synthesize linoleic acid (18:2, ω6) and α-linolenic acid (18:3, ω3). These two PUFA are essential and must be provided in the diet to animals.

Desaturation and elongation occur in endoplasmic reticulum membranes of many tissues, mainly in the microsomes of the liver (Gonzalez et al., 1986; Dillon, 1987). The first double bond introduced into a saturated fatty acid is usually in the Δ9 position. Monounsaturated fatty acids (MUFA), specifically palmitoleic acid and oleic acid are converted from palmitic acid and stearic acid,
respectively (Mayes, 1990b). $\Delta 6D$, $\Delta 5D$ and elongase catalyze the biosynthesis of polyunsaturated fatty acids including $\omega 3$, $\omega 6$, and $\omega 9$ families. Substrates and products of these enzymes are shown in Fig. 2.3.

In higher plants and animals, the desaturase enzymes require oxygen. Elongase requires the absence of oxygen and presence of malonyl-CoA (Mead et al., 1986). Desaturase enzymes are located in the lipid bilayer of microsomes. $\Delta 6$ desaturase is a single polypeptide containing one non-heme iron atom (Dillon, 1987). Desaturase enzymes need a source of electrons, mainly supplied by NADH or NADPH through NADH-cytochrome c reductase and NADH-Cytochrome b5 electron transfer system (Wahle, 1983; Dillon, 1987).

![Diagram](image)

Fig. 2.3: Biosynthesis of the $\omega 6$, $\omega 3$, $\omega 9$ families of polyunsaturated fatty acids.
In the reduced state, desaturase enzymes react with molecular oxygen and acyl-CoA substrates to produce unsaturated cis-fatty acids. Since the electron flux supplied by this system is many times greater than the requirement of desaturases, the rate-limiting step of the desaturase system is thought to be the desaturase enzymes, per se (Brenner, 1981).

It has been proposed that there may be two separate desaturation systems in the endoplasmic reticulum (Mead et al., 1986). One may require a thiol ester as the substrate, and the other can act on the esterified fatty acids of phospholipids in membrane. The latter desaturase system allows a fatty acid of a membrane lipid to be transferred to the desaturase and the altered fatty acid transferred back to the membrane lipid without the intermediates leaving the membrane and equilibrating with a free fatty acid pool. This membrane desaturase system would use lower energy and have a more rapid response for modification of membrane lipids than the former desaturase system (Mead et al., 1986).

\( \Delta 6 \)D reaction is the rate-limiting step in the conversion of linoleic acid (LA; 18:2, \( \omega 6 \)) to arachidonic acid (AA; 20:4, \( \omega 6 \)) (Brenner, 1981; Dillon, 1987; Mayes, 1990b; Yamazaki et al., 1992). The desaturation of n3 fatty acids is also catalyzed by \( \Delta 6 \)D. Yamazaki et al. (1992) compared the rate of eicosapentaenoic acid (EPA; 20:5, \( \omega 3 \)) production by feeding rats on diets supplemented with either \( \alpha \)-linolenic acid (LNA; 18:3, \( \omega 3 \)) or stearidonic acid (18:4, \( \omega 3 \)), using lard as a control. The authors suggested that the reaction of \( \Delta 6 \)D is also the rate-limiting step in the conversion of LNA to EPA.
There is a competition between fatty acids of different families for the desaturases. In general, \( \omega 3 \) family fatty acids have the greatest affinity for desaturase enzymes, and \( \omega 6 \) has higher affinity than \( \omega 9 \) family. By measuring the ratios of the concentrations of daughter (derivative) fatty acids to parent (precursor) fatty acids within each family, Siguel et al. (1987) confirmed that the order of relative pathway activity of unsaturated fatty acid in humans is \( \omega 3 > \omega 6 > \omega 9 > \omega 7 \). The major competition occurs at the \( \Delta 6 \). Each product also has feedback inhibition in its own family at both the desaturase and acyltransferase levels (Mead et al., 1986). Saturated fatty acids have greater inhibition on the elongation from LA to AA than on the desaturation of LA to \( \gamma \)-linolenic acid (GLA; 18:3, \( \omega 6 \)).

The fatty acid composition of tissue phospholipids (PL) may be influenced by many factors: the composition of fatty acids in the diet, the rate of fatty acid oxidation before the incorporation into phospholipids; the retroconversion of certain fatty acids; the rates of desaturation and elongation; the activity of specific acyltransferases for incorporation of fatty acids; and the competition among families of fatty acids for desaturation, elongation, and incorporation (Wahle, 1983; Mead et al., 1986). Once PUFA are formed via desaturation and elongation, they are incorporated into PL on the glycerol molecule, especially in position 2. Arachidonic acid is the most abundant and important long-chain PUFA in tissue lipids (Dillon, 1987). Although the rate of elongation from AA to 22:4, \( \omega 6 \) is higher than the desaturation rate from dihomo-\( \gamma \)-linolenic acid
(DGLA; 20:3, ω6), the loss of AA is recovered mainly due to the retroconversion of 22:4, ω6 to AA.

Desaturase enzymes and membrane fluidity

The fluidity of membranes is dependent on the fatty acid and cholesterol content in the membrane (Marra and Alaniz, 1990). The activity of desaturase enzymes affects the fatty acid composition of membrane phospholipids and indirectly influence the formation of cellular membranes as well as the properties of membranes (Garda and Brenner, 1985; Laganiere and Yu, 1993; Borkman et al., 1993). The viscosity of the membrane lipid bilayer is important in controlling the osmotic properties of the membrane. In essential fatty acids (EFA) deficiency, LA and LNA cannot be sufficiently provided and the formation of AA is also limited. Lack of unsaturated fatty acids in the membrane results in increased membrane viscosity, consequently affecting the transport function of membranes, enzymatic functions of transmembrane proteins, and regulation of surface receptors such as the insulin receptor (Mead et al., 1986; Borkman et al., 1993). Ginsberg et al. (1987) demonstrated that the increasing content of PUFA within cell membranes in cultured cells increases membrane fluidity, the number of insulin receptors, and the action of insulin. In EFA deficiency, dermatitis and high skin permeability to water are related to loss of membrane fluidity because of low unsaturated fatty acid contents in membrane phospholipids (Mead et al., 1986). Higher desaturase activity increases the amount of long chain PUFA in membranes and consequently increases the fluidity of membrane. The uptake of extracellular substances
such as glucose, and hormones like estrogens and insulin are enhanced. Marra and Alaniz (1990) demonstrated that the reduction of microsomal Δ6D activity and alteration of fatty acid composition of plasma and different liver fractions are responsible for the decrease in liver microsomal membrane fluidity. However, Mandon et al., (1987) did not find apparent alterations of fluidity in the microsomal membrane of kidney and lung despite changes in Δ6D activity due to the effects of high carbohydrate and high protein diets. Other than certain acyltransferases which modulate the incorporation of fatty acids to membrane phospholipids, the activity of desaturases also affects the distribution of saturated and unsaturated fatty acids in phospholipids of membrane (Wahle, 1983). The fluidity of membranes may affect the conformation and function of transmembrane enzymes. Desaturases, per se, are an examples ( Rogers and Strittmatter, 1975; Wahle, 1983; Mead et al., 1986; Zevallow and Farkas, 1989). Rogers and Strittmatter (1975) demonstrated that the fluidity of endoplasmic reticular membrane affected the phase separation and mobility of NADH-cytochrome b5 and NADH-cytochrome c reductase leading to an influence on desaturase activity. When membrane fluidity is decreased, desaturase activity is enhanced to compensate for decreased unsaturated fatty acids in membrane phospholipids, and vice versa (Mead et al., 1986). This may serve as a regulator of membrane fluidity. Zevallos and Farkas (1989) confirmed this observation. They found that higher fluidity of endoplasmic reticulum membrane depressed the conversion of stearic acid to oleic acid and LA to GLA.
Regulation of desaturase activity

The activity of Δ6D may be regulated by many factors as shown in Fig. 2.4.

Insulin

Fasting-induced reduction of Δ6D activity in rats has been shown to be reversed after glucose refeeding due to the secretion of insulin (Brenner et al., 1968). The activities of Δ5D and Δ6D were reported to be partially inhibited by diabetes during normo- and hyperglycemic periods in spontaneously diabetic Wistar Bio-Breeding (BB) rats (Mimouni and Poisson, 1992). In this study, conditions of hyper-, normo-, and hypoglycemia were manipulated by injecting different doses of insulin. Rats that received insulin treatment at a dose of 10 I.U./kg body weight twice a day for two days had restored Δ6D and Δ5D activity confirming the regulatory role of insulin in desaturase function. This is not directly associated with glycemic status. Insulin apparently increases desaturase protein synthesis and leads to stimulation of fatty acid desaturation (Brenner, 1990).

Δ5D activity in liver and adrenal gland microsomes was inhibited in streptozotocin-induced diabetic rats, but restored by treatment with insulin (Brenner, 1990). El Boustani et al. (1989) also demonstrated that Δ5D activity was decreased in human type I diabetes in vivo and was increased after insulin injection.
Fig. 2.4: Factors that regulate Δ6 desaturase activity. (Modified from Brenner, 1981)
Inhibition of Δ6D activity can modify the composition of fatty acids in membranes. However, Mimouni and Poisson (1992) demonstrated that the fatty acid composition of microsomes was not consistent with the desaturase activities in different glycemic states. They suggested that there may be multiple factors involved in the alteration of fatty acid composition besides fatty acid desaturation. These factors could include the composition of fatty acids in the diet, the rate of fatty acid oxidation, the retroconversion of certain fatty acids, the activity of specific acyltransferases for incorporation of fatty acids; and the competition among different families (ω3, ω6 and ω9) of fatty acids for desaturation, elongation, and incorporation.

Even though the increased insulin levels may enhance Δ5D and Δ6D activity, insulin resistance may blunt the effect. Borkman et al. (1993) reported that insulin sensitivity inversely correlated with Δ5D activity expressed as the ratio of AA/DGLA from muscle phospholipids. This inverse correlation was observed in the patients with coronary heart disease who received coronary artery by-pass surgery. When normal men were studied, the insulin sensitivity was positively correlated with the indicator of Δ5D activity expressed as AA/DGLA ratio. Skeletal muscle is insulin dependent for glucose uptake but liver is not. Therefore, insulin resistance may not influence the desaturase activity of liver microsomes.

**Estrogen**

Different responses of Δ6D activity were noted between male and female rats when they were exposed to warm and cold temperature (Gonzalez et al., 33).
Only female rats had different responses of Δ6D activity to changes of temperature. An effect of estrogen on Δ6D activity was proposed by the researchers (Gonzalez et al., 1983). In their later study using normal and ovariectomized female rats, Gonzalez et al. (1986) found that the amount of AA in microsomal phosphatidylcholine from normal female rats exposed at 30°C was lower than that of rats at 15°C and ovariectomized rats at both temperatures. Ovariectomized rats did not show changes in Δ6D activity at different temperatures. After they gave 17β-estradiol to normal and ovariectomized rats and kept them at 30°C and 15°C temperature, normal female rats with higher plasma estrogen levels showed lower Δ6D activity compared to ovariectomized rats. Both types of rats had a lower estradiol levels at 15°C temperature and showed higher Δ6D activity than those at 30°C temperature. Estradiol may have played a role in the regulation of Δ6D activity by inhibiting its activity. Marra et al. (1988) demonstrated that the incubation of hepatoma tissue culture and liver microsomes from female Wistar rats with 17β-estradiol showed suppressed Δ5D desaturation of labeled eicosa-8,11,14-trienoic acid (same as DGLA) (p<0.01). The incorporation of DGLA in both types of cells was also inhibited by 17β-estradiol (p<0.01). Progesterone did not inhibit Δ5D activity. Estrogen apparently does not cause a direct inhibitory effect on Δ5D activity, but rather requires interaction of estrogen and its cytoplasmic receptors and changes in gene transcription and/or translation.
Other hormones

Insulin treatment restores Δ5D and Δ6D activities in diabetic rats (Brenner et al., 1968; Jones et al., 1986; Mimouni and Poisson, 1992). Glucagon (Gomez Dumm et al., 1976), epinephrine (Gomez Dumm et al., 1976), corticoids (Marra et al., 1989) and thyroxine (Kuhn et al., 1983), which have an antagonistic effect on insulin, decrease the activity of Δ6D (Brenner, 1990). The depressing effect of glucagon and epinephrine on Δ6D activity is mediated by the stimulation of adenyl cyclase, which increases the production of cAMP (Brenner, 1981; Brenner, 1990). Glucocorticoids and mineralcorticoids inhibit desaturase activity via a genomic mechanism. Steroid hormones interact with nuclear receptors and activate DNA to synthesize mRNA for the synthesis of certain protein factors. These protein factors are synthesized in the ribosomes to inhibit the activities of Δ6D and Δ5D (Brenner, 1990). After intraperitoneal injection of 11-deoxycorticosterone or aldosterone, Δ6D activity in rat liver microsomes decreased approximately 4-fold over 24 hours (Marra et al., 1990).

Testosterone injection also decreased the activities of Δ5D and Δ6D of liver microsomes in both male and female rats. The depressing effects were more evident in female rats than in males (Marra et al., 1990). It is generally believed that increased production of testosterone is related to the increased incidence of atherosclerosis in men (Marra et al., 1990). They suggested that the changes in desaturase activities induced by testosterone and the difference observed between female and male animals may be the reason for sexual difference in the occurrence of atherosclerosis (Marra et al., 1990). Therefore,
the sexual differences in the regulation of Δ6D activity and the prevalence of coronary heart disease (CHD) deserve further investigation.

**Dietary regulation of Δ6D activity**

Different dietary components can also influence the activity of Δ6D.

Reduction of Δ6D activity in kidneys and lungs of male rats fed a high-carbohydrate diet (69% starch vs 55% starch) was observed by Mandon et al. (1988). A high-protein diet was shown to increase the activity of Δ6D in the liver microsomes of female rats (Pelluffo and Brenner, 1974). The increase only occurred, however, when dietary protein reached 35% or more. The response to dietary protein on Δ6D activity was higher in old rats than in young ones. An increase in the concentration of protein in the diet increased Vmax, but did not modify Km of Δ6D. Mandon, et al. (1988) also found the same high-protein diet effect on Δ6D activity by feeding rats 32% casein. The source of protein also influences desaturase activity and consequently alters the fatty acid patterns of lipids in various tissues. In the study of Lindholm et al. (1991), rats fed a casein diet had higher Δ6D activity than those fed a soybean protein diet. No clear explanations of these observations were provided in these studies.

Dietary cholesterol inhibits the activity of Δ5D and Δ6D in rat liver microsomes when rats were fed with control diet, or in combination with SFA or ω6 fatty acids such as linoleic acids (Garg et al., 1986; Garg et al., 1988a). Garg et al. (1986) reported that Δ5D and Δ6D activities were enhanced when dietary cholesterol was removed by feeding rats a sterol-free diet. Garg et al.
(1988a) suggested that fish oil might prevent the cholesterol inhibition on \( \Delta 5D \) activity in rat liver microsomes. This conclusion was based on the observation that the depressing effect of dietary cholesterol was not evident with a fish-oil diet in contrast to a diet containing beef tallow or linseed oil supplemented with 2% cholesterol. Cholesterol may decrease membrane fluidity of liver microsomes and interfere with activity of enzymes located in microsomal membranes such as desaturase enzymes (Garg et al., 1986).

Garg et al. studied the effect of different \( \omega 3 \) fatty acids on the activities of \( \Delta 5D \) (1988a) and \( \Delta 6D \) (1990) as well as AA content in serum lipids and hepatic microsome phospholipids (PL). Reductions of \( \Delta 6D \) activity and AA content in serum lipids and PL of liver microsomes were seen in rats fed linseed oil (a source of C18:3\( \omega 3 \)) and fish oil (a source of C20:5\( \omega 3 \) and C22:6\( \omega 3 \)), but only when combined with SFA (beef tallow) in the diets. Fish oil had a greater effect on the inhibition of \( \Delta 6D \) activity. No inhibiting effect of \( \omega 3 \) fatty acids was found when diets were also high in \( \omega 6 \) fatty acids (safflower oil). The activity of \( \Delta 5D \) in the desaturation of DGLA to AA was higher in the linseed oil-fed group but lower in the fish oil-fed group compared with beef tallow-fed group (Garg et al., 1988a). This may be related to the competition between \( \omega 6 \) fatty acids and \( \omega 3 \) fatty acids as substrates for desaturase enzymes. In the study of Christiansen et al. (1991), \( \Delta 6D \) activities were stimulated 1.5 to 2.5-fold after linseed or sunflower seed oil feeding when compared to a pelleted reference diet. The activities of \( \Delta 5D \) were increased 3.5-fold and 2.5-fold in the linseed oil and sunflower oil diets, respectively. However, \( \Delta 5D \) and \( \Delta 6D \) activities were
considerably reduced when the diet was supplemented with fish oil as compared to linseed and sunflower seed oil diets. Apparently there is a difference in the activity of the desaturation system when linoleic versus linolenic acid is used as substrate for Δ6D. This may account for the conflicting results related to the effects of various dietary fatty acids on the activity of Δ6D in some studies (Garg et al., 1990).

**Aging and desaturase activity**

Since Ayala et al. (1973) first reported that rat testis Δ6D activity decreased with age, many studies have repeated this observation (Peluffo and Brenner, 1974; Horrobin, 1981; Bordoni et al., 1988; Hrelia et al., 1989; Bourre et al., 1990; Ulmann et al., 1991a; Maniongul et al., 1993). Bordoni et al. (1988) studied Δ6D activity in male Wistar rat liver microsomes and fatty acid composition of liver microsomal lipids at different ages (13 days, 1 month, 4 months, 14 months, and 22 months old). The enzyme activity was significantly lower in microsomes from 4 month-old rats than that from 1 month-old rats. A more dramatic reduction of Δ6D activity was observed in the microsomes of 14 and 22 month-old rats. There was a linear correlation between Δ6D activity and animal age (y=106.96 - 2.85x , r²=0.91).

Even though ω3 and ω6 fatty acids are catalyzed by the same desaturation and elongation systems, the response of Δ6D activity differs when different substrates are used. In the study of Hrelia et al. (1989), the activity of Δ6D in liver microsomes from male Wistar rats decreased at a rate proportional to the
animals’ age when linoleic acid was the substrate. Compared to 1 month-old rats, Δ6D activity decreased 25% in 6 month-old, 35% in 10 month-old, and 60% in 25 month-old rats. A high linear correlation between Δ6D activity and animal age was noticed (y=108.46 - 2.55 x, r²=0.93). Using α-linolenic acid (LNA) as the substrate, less reduction in Δ6D activity occurred and the reduction occurred later in life. Only a 15% decrease was observed in 10 month-old rats and 23% in 25 month-old ones. A linear correlation between Δ6D activity and animal age was not evident. Maniogui et al. (1993) used the same strain of male rats ranging from 1.5 to 24 months. Δ6D activity using 1-14C linoleic acid as the substrate increased up to 6 months old and stayed constant through the age of 24 months. Activity of Δ5D decreased dramatically from 1.5 months to 3 months, then increased gradually until reaching the same level as in 1.5 month-old rats at 24 months. Using 1-14C α-linolenic acid as the substrate, Δ6D activity increased from 1.5 months to 3 months, but decreased linearly until 24 months to reach the same level as was observed at 1.5 months. Ulmann et al. (1991a) also observed that Δ6D activity decreased with age when 3, 6, and 9 month-old male Wistar rats were compared. However, they found that the Δ6D using 1-14C α-linolenic acid as the substrate is more sensitive to the age factor than the Δ6D using 1-14C linoleic acid.

A kinetic analysis conducted by Hrelia et al. (1991) using 1-14C linoleic acid as a substrate showed that the Km values of Δ6D increased with age in male
Wistar rats. A linear correlation between Km and age was reported \( r=0.998, p<0.05 \). The Vmax values of Δ6D were similar in 11 and 25 month-old rats, but only 25 month-old rats had a 28% decrease in Vmax value compared to 2 months old rats. Using 1-\(^{14}\)C α-linolenic acid as a substrate, Km values of Δ6D were not significantly different among young and old rats. On the contrary, Vmax was significantly decreased in both 11 and 25 month-old rats. No linear correlation was noted between Vmax values and animal's age when 1-\(^{14}\)C α-linolenic acid was used. The researchers concluded that the effect of aging on affinity of Δ6D to ω3 fatty acids was not as strong as the affinity of Δ6D to ω6 fatty acids. The loss of substrate affinity by Δ6D may be an important factor in aging.

γ-Linolenic acid (GLA) increased the Vmax value of Δ6D in old rats but not in young rats (Hrelia et al., 1991). GLA might be able to prevent the loss of substrate affinity in aging animals. Biagi et al. (1991) showed that diet supplemented with GLA in the form of evening primrose oil had no specific effect on Δ6D activity in the young male Wistar rats, but the GLA supplementation significantly increased the desaturation of LA and LNA in old rats. When Δ6D activity is at or near its peak, it is likely that the supplementation of GLA does not influence Δ6D activity.

Bourre et al. (1990) measured Δ6D activity in the mouse brain and liver using LA as a substrate, and found that activity of Δ6D in brain decreased dramatically during early development up to postnatal day 21 and remained nearly stable during adulthood and aging. This is mainly because brain development in the early stage requires a large amount of PUFA for membrane
synthesis. Hepatic Δ6D activity increased approximately 9-fold between day 3 before birth and day 7 after birth. Activity then decreased by 44% up to weaning and was constant up to 4 months. After 4 months, Δ6D activity decreased by 40% with age.

Aging also affects Δ6D activity in heart microsomes, but to a lesser extent than that observed with liver microsomes. Lopez Jimenez et al. (1993) reported that heart Δ6D activity in old male Wistar rats (18 months) was lower compared to young animals (3 months) (p<0.02), but the decrease in heart is less than in liver (15% and 63% decrease, respectively). Despite the decrease of heart Δ6D activity in old rats, the fatty acid composition of heart phospholipids did not show significant alteration in old and young rats. It may be likely that the heart does not exclusively depend on hepatic supply of fatty acids or its own endogenous synthesis.

Several studies have demonstrated that the fatty acid composition of liver microsome membranes is consistent with the age-related alterations in fatty acid desaturase activity (Bordoni et al., 1988; Hrelia et al., 1989; Biagi 1991; Maniongul et al., 1993). A significant increase in the proportion of LA and LNA, and a significant decrease in DHA were observed in 6, 10, and 25 month-old male rats (Hrelia et al., 1989). Lower AA/LA ratio and ω3 PUFA/LNA ratio were noted in 6, 10, and 25-month-old rats compared to 1 month-old rats. Bordoni et al. (1988) also found that the AA/LA ratio was lower in 4, 14, and 22 month-old rats than in 1 month-old rats. Biagi et al. (1991) demonstrated that the unsaturation index and membrane micro-viscosity increased and decreased respectively especially in older rats when Δ6D activity was improved by
supplementation of GLA in the diet. Other studies did not find a consistent change of fatty acid composition of membrane with desaturase activity (Ullmann et al., 1991a). Many factors may contribute to the alterations of fatty acid composition in membranes. It is possible that aging changes the structural and physicochemical properties of the membrane such as fluidity, which then influences the activity of Δ6D. Whether the age-related changes in physicochemical properties of membranes are a consequence or a cause of alterations in desaturase activity still needs to be determined (Hrelia et al., 1989; Diagi et al., 1991).

Consequences of loss of desaturase activity

A decrease in Δ6D activity deserves special concern since Δ6 desaturation is the major regulating step in EFA metabolism. As a consequence of losing Δ6D activity, the production of GLA, dihomo-γ-linolenic acid (DGLA), and AA via desaturation and elongation decrease in aging. AA can be sufficiently provided from animal sources of a regular diet. Other fatty acids (GLA, DGLA) only exist in a few types of food. It is more likely that deficiencies of GLA and DGLA occur in older age and lead to the deficit of series 1 of prostaglandin synthesis such as PGE1 (Horrobin, 1981). It has been suggested that the loss of Δ6D activity, especially the ability to convert LA to GLA, is a key factor in aging through altered membrane lipid profiles (Horrobin, 1981; Bordoni et al., 1988; Hrelia et al., 1989). The activity of Δ5D is usually higher than the activity of Δ6D.
Under the condition of low Δ6D activity, a certain amount of AA can still be converted from DGLA. The higher level of 2-series PGs and relatively low level of 1 series PGs may accelerate aging.

PGE1 is the major product in series 1 PGs from DGLA. It enhances the activity of T-lymphocytes, lowers blood pressure, and inhibits the proliferation of vascular smooth muscle cells. It can also inhibit cholesterol synthesis and platelet aggregation through the stimulation of cAMP production. Therefore, PGE1 can enhance immunity of cells and prevent the development of atherosclerosis and thrombosis (Horrobin, 1981). The decrease of Δ6D activity may lead to the reduction of DGLA and consequently result in the deficit of PGE1 production and aging conditions.

The loss of Δ6D and Δ5D activity may result in the alteration of fatty acid composition in tissue and membrane phospholipids and consequently affect the membrane fluidity and synthesis of eicosanoid such as TXA2 and PGI2. The degree of membrane fluidity may affect the uptake of materials (such as glucose) and hormones (such as insulin) and cause sequential conditions.

**EICOSANOIDS & PLATELET AGGREGATION**

**Eicosanoids**

Eicosanoids are a class of lipids synthesized in most tissues of the body from 20-carbon fatty acids. They are biologically active compounds that include...
prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and lipoxins (Dillon, 1987; Mayes, 1990a; Ney, 1991). The prostanoid group includes PG and TX. Different fatty acids as substrates produce different groups of eicosanoids as shown in Fig. 5.

The alphabetic designation for PG generally refers to the substitutions occurring on the cyclopentane ring at positions 9 and 11 (Longenecker, 1985). However, for LT, the alphabetic designations involve the status of the parent molecule at positions 5 and 6 as well as the status of the substitution on position 6. The terminal subscript number indicates the number of double bonds remaining in the molecule. For the prostanoids, products usually have two double bonds less than the fatty acid substrates. In the ω6 family, dihomo-γ-linolenic acid (DGLA) with three double bonds is converted by cyclooxygenase into prostanoids with one double bond. PGE1 in this group is a potent inhibitor of platelet aggregation. The conversion of AA which contains four double bonds produces prostanoids with two double bonds. In this group of prostanoids, thromboxane A2 (TXA2) produced in blood platelets is a potent platelet aggregating agent and vasoconstrictor. It is as effective as Angiotensin II (Dillon, 1987) in increasing blood pressure. Prostacyclin (PGI2), the major metabolite of AA synthesized by the endothelium of vascular walls, is an antiaggregatory and vasodilator compound. In the family of ω3 fatty acids, eicosapentaenoic acid (EPA), found abundantly in marine oils, is the precursor of PGs with three double bonds. Prostacyclin 13 (PGI3) has equipotent antiaggregation as PGI2. Thromboxane A3, however, is inactive and tends to decrease platelet aggregation (Dillon, 1987; Kwon et al., 1991).
Fig. 2.5: Synthesis of prostaglandins, thromboxanes, and leukotrienes from different origins. PG, prostaglandin; TX, thromboxane; LT, leukotriene; (1), cyclooxygenase pathway; (2), lipooxygenase pathway. (modified from Mayes, 1990a)
The discovery of prostaglandins was made by Kurzrok and Lieb in the early 1930s (Kurzrok & Lieb, 1930). They noticed that certain substances in fresh human semen caused rhythmic contraction and relaxation of myometrium. Von Euler (1936) named the lipid-soluble acidic substances prostaglandin and vesiglandin because he considered the substances were originated in the prostate gland. However, Eliasson (1958) in the later work determined that prostaglandins in semen were not from the prostate gland but were made by the seminal vesicles and secreted into the seminal fluid. In 1966, the biological activity of prostaglandins from platelets was first reported at the Second Nobel Symposium (Myers et al., 1985). The inhibitory activity of PGE$_1$ on platelet aggregation and adhesion was described by Klowze (Myers et al. 1985). Earlier research was focused on the effects of PGEs on platelet function, and the discoveries of PGI$_2$ and TXA$_2$ led to a special interest on the clinical importance of eicosanoids in platelet function.

The steps in the synthesis of PGs were determined by researchers using labeled precursors. Silver et al. (1973) demonstrated that immediate addition of arachidonic acid (AA) to platelets resulted in PG formation and platelet aggregation. Thromboxane A$_2$ (TXA$_2$) was discovered by Hamberg et al. (1975) after its stable metabolite thromboxane B$_2$ (TXB$_2$) was discovered (Hamberg 1974). TXA$_2$ is the major AA product of platelets, also the main substance by which AA induces platelet aggregation. The aggregatory effect of TXA$_2$ on platelets may be due to the increase of intracellular Ca$^{2+}$ availability (Longenecker, 1985). Thromboxane synthetase (TXS) is a microsomal enzyme, which requires cofactors such as methemoglobin/hematin/hemin and tryptophan/3-indoleacetic acid/ catecholamines (Longenecker, 1985). It can be
inhibited by substrate analogs but not by non-steroidal anti-inflammatory drugs (NSAID) such as aspirin.

Prostacyclin (PGI₂), which originates primarily from vascular endothelium, is the most potent antiaggregator in addition to being a vasodepressant (Longenecker, 1985; Moncada et al., 1986; Mayes, 1990a). The inhibitory effect of PGI₂ is mainly due to the activation of the adenylate cyclase system leading to elevation of intracellular cAMP in platelets. It also enhances fibrinolysis and stimulates cholesterol metabolism. Even though smooth muscle cells can convert AA to PGI₂, the endothelial cells on vessel walls are the most active producers of PGI₂ (Moncada et al., 1986). PGI₂ is very labile at physiologic pH with a half life of 10 min. It is quickly metabolized to the stable inactive derivative, 6-keto-PGF₁α. Both TXA₂ and PGI₂ are derived from endoperoxides PGH₂, which originates from AA. Under conditions where platelet capacity to produce TXA₂ is limited, the utilization of platelet endoperoxides by vascular endothelium to produce PGI₂ increases. It has been suggested that PGI₂ acts only as a local regulator of platelet function because of its low level in circulation (Longenecker, 1985). One of its conversion products, 6-O-PGE₁ is also a potent antiaggregator.

The endoperoxides, PGG₂ and PGH₂, can also induce platelet aggregation and release of granule materials. However, whether the effect of endoperoxides on platelet aggregation is via their conversion to TXA₂ or their direct action per se remains controversial (Longenecker, 1985). Using specific inhibitors of TXS to determine the roles of endoperoxides and TXA₂ in platelet aggregation, Gorman (1983) demonstrated that the inhibition of aggregation
occurred only when the endoperoxides were converted to inhibitory PGD$_2$.

There may be a direct role of endoperoxides in platelet aggregation. It is certain that the effect of endoperoxides and TXA$_2$ on platelet aggregation is mainly due to the enhancement of intraplatelet Ca$^{2+}$ availability (Longenecker, 1985).

Other PG can also influence platelet aggregation. PGE$_1$, derived from dihomo-$\gamma$-linolenic acid, is an inhibitory factor, whereas, PGE$_2$ has biphasic effects, i.e., stimulatory at lower and inhibitory at higher concentrations. The inhibitory effect of PGE$_1$ indicates the potential antiaggregatory role of dihomo-$\gamma$-linolenic acid (Longenecker, 1985). The effect of PGF$_{2\alpha}$ on platelet aggregation is ambiguous (Longenecker, 1985). Hung et al (1982) suggested that PGF$_{2\alpha}$ might be a weak incomplete agonist of TXA$_2$ receptors. The stimulation of PGF$_{2\alpha}$ is partially sensitive to aspirin. As mentioned before, PGD$_2$ is a potent inhibitor of platelet aggregation, second only to PGI$_2$.

Current studies show that there is a single receptor (stimulatory platelet receptor, also called thromboxane receptor) for the stimulation of platelets by TXA$_2$ and endoperoxides, whereas, there are two types of inhibitory platelet receptors for inhibitory PGI$_2$, PGE$_1$ and PGD$_1$ (Myers, 1985). The PGI$_2$/PGE$_1$ receptors includes two classes: high-affinity, low-capacity site, and low-affinity, high-capacity site. Binding of PGI$_2$ and PGE$_1$ is rapid, reversible, and saturable. The inactive metabolite of PGI$_2$, 6-keto-PGF$_{1\alpha}$ has very little or no affinity for platelet PGI$_2$/PGE$_1$ receptor. It is thought that both classes of receptors are associated with the activation of adenylate cyclase, and they may represent two populations of platelets instead of two types of receptors on each
platelet. The PGD$_2$ receptor is reversible and saturable, with a single class of high-affinity, low-capacity sites.

The structural characteristics that may influence the aggregatory and antiaggregatory activity of eicosanoids include the ring structure, the length of the side chains, the position and number of double bonds, and substituents on the ring or side chains (Myers, 1985). The ring structure may be the most important characteristic in differentiating the stimulatory and inhibitory eicosanoids. Modification of the ring of eicosanoids reduces their individual effect.

**Platelet aggregation**

Platelet aggregation is one of a series of processes by which blood vessels stop bleeding in order to prevent blood loss (Sweeney et al., 1989; West, 1990). The platelet plug (or aggregation mechanism) is extremely important in preventing blood loss from minute ruptures in very small blood vessels that occur hundreds of times each day. The pathways of autoactivation of platelets mainly involve the release of ADP, the liberation and metabolism of AA, and the synthesis of platelet-aggregating factor (PAF, 1-O-alkyl-2-acetyl glycerophosphocholine). When a blood vessel is ruptured, the damaged vessel wall exposes collagen in the subendothelium. After contacting collagen, platelets begin to swell and form multiple projecting pseudopods. Platelets become sticky and adhere to collagen to form temporary platelet plugs. This process is referred to as phase I aggregation or primary aggregation. It is reversible if the
concentration or extent of stimulus is low. However, if the stimulus is sufficiently high, an irreversible secondary series of reactions will be triggered (Jones, 1985). The availability of AA is enhanced due to the activation of phospholipase A2. Phosphorylation of cAMP-dependent myosin light chain kinase is induced. Calcium, ADP, ATP and serotonin contained in dense granules in platelets are then released. The endogenous ADP also acts as a physiologically significant agonist for the secondary-phase aggregation to amplify platelet activation. Release of ATP accompanying ADP secreted from dense granules is usually used as one of the indicators for determining platelet aggregation. Instead of being used for normal phospholipid synthesis or cAMP production, endogenous ATP acts as a phosphate and energy donor in the process to trigger cellular responses. Therefore, production and utilization of energy by platelets are rapidly increased (Sweeney, 1989; West, 1990).

Besides collagen, initial thrombin formed at an injury site is also considered to be a primary agonist for triggering platelet aggregation. Combined with the secondary agonist, ADP released from activated platelets, thrombin interacts with specific membrane receptors to activate guanine nucleotide-binding protein (Gs-protein) in the platelet membrane as a secondary messenger which leads to the activation of phospholipase C (West, 1990; Winocour, 1989b). The pathways of platelet activation are shown in Fig. 6. (Winocour, 1989b).

Hydrolysis of membrane phosphatidylinositol catalyzed by phospholipase C forms two secondary messengers, 1,2-diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP3). DG activates platelet protein kinase C, which catalyzes a phosphorylation of a 47 K protein which is essential for the response of platelet activation. DG can also be degraded by diglyceride lipase and monoglyceride
Fig. 2.6: Pathways of platelet activation. (Modified from Winocour, 1989b)
lipase to release AA (Winocour, 1989b). IP3 causes Ca^{2+} to enter the cytosol from the dense tubule system in endoplasmic reticulum in the platelets. The elevation of cytosolic Ca^{2+} triggers the subsequent reactions and allows platelet aggregation to occur: (1) Ca^{2+}/calmodulin dependent protein kinase activate myosin light chain kinase to catalyze phosphorylation of myosin light chain which is needed for platelet shape change, secretion and contraction. (2) Activation of calpain (a Ca^{2+}-dependent protease) to activate other platelet enzymes. (3) Intraplatelet Ca^{2+} is required to activate phospholipase A_{2}, which catalyzes the release of AA from platelet membrane PL. In the platelets, AA is converted into TXA_{2} by cyclooxygenase, endoperoxidase and thromboxane synthase. TXA_{2} is an agonist for platelet aggregation. It binds to a specific platelet membrane receptor (stimulatory receptor) and activates phospholipase C to amplify platelet activation through further generation of DG and IP3 (refer to Fig.6) (Sweeney, 1989; Winocour, 1989b; West, 1990).

Because platelet aggregation is involved in many cardiovascular diseases, drugs that can influence the production or action of eicosanoids on platelets become important. Non-steroidal anti-inflammatory drugs, such as aspirin, inhibit platelet aggregation by inactivating platelet cyclooxygenase, thereby reducing the production of TXA_{2} (West, 1990; Ratnatunga, 1992). However, since cyclooxygenase is associated with the production of both TXA_{2} and PGI_{2}, whether aspirin can consistently inhibit platelet formation of TXA_{2} without affecting the endothelial production of PGI_{2} is questionable. It may be more effective to use the thromboxane synthase inhibitors which only inhibit the production of TXA_{2} without influencing the formation of PGI_{2} (Myers, 1985).
Role of Ca\textsuperscript{2+} and cyclic AMP in platelet functions

In platelets, Ca\textsuperscript{2+} and cyclic AMP (cAMP) serve as second messengers in regulating the activation and inhibition of platelet aggregation, respectively (Feinstein et al., 1985).

The action of Ca\textsuperscript{2+}

The effect of Ca\textsuperscript{2+} on platelet aggregation occurs mainly through activation of Ca\textsuperscript{2+}/calmodulin-dependent myosin light-chain kinase which phosphorylates myosin, and through Ca\textsuperscript{2+}/phosphatidylinerine (PS)-dependent protein kinase C which phosphorylates a cytosolic polypeptide essential for platelet activation (Feinstein, 1985; Jones, 1985). Ca\textsuperscript{2+} can also enhance the activity of phospholipase C and A\textsubscript{2}. It is required for the maximal activity of phospholipase A\textsubscript{2}, which is the rate-limiting enzyme in AA release from membrane phospholipids. Since AA is the precursor of prostaglandins such as TXA\textsubscript{2} and PGI\textsubscript{2}, the concentration of intracellular Ca\textsuperscript{2+} can indirectly regulate the production of TXA\textsubscript{2} and PGI\textsubscript{2}. Phospholipase C can be activated under the stimulation of collagen, thrombin, ADP, and other aggregators such as TXA\textsubscript{2} due to the increase of intracellular Ca\textsuperscript{2+}. This enzyme cleaves phosphatidylinositol from membranes of activated platelets to form 1,2-diaclylglycerol (DG) and inositol 1,4,5-triphosphate (IP\textsubscript{3}). Both Ca\textsuperscript{2+} and DG activate protein kinase C. DG can decrease the phospholipase C requirement for Ca\textsuperscript{2+} and increase the hydrolyzation of membrane phospholipids, i.e., phosphatidylinositol, by increasing the affinity of protein.
kinase C for Ca²⁺. Sufficient DG produced from membrane phospholipids by the stimulation of aggregators such as collagen and thrombin can activate protein kinase C without the elevation of intracellular Ca²⁺ concentration ([Ca²⁺]). The combination of DG and Ca²⁺ synergistically increases the rate of platelet secretion, primarily due to the increased phosphorylation of myosin (Feinstein et al., 1985). There is no enhanced phosphorylation of the cytosolic polypeptide by protein kinase C in response to the elevation of DG and [Ca²⁺]. The protein kinase C pathway may be necessary for platelet secretion, but is not sufficient.

Extracellular Ca²⁺ is also required for aggregation. However, its presence is not necessary for the responses of platelets such as shape change, granule release, and thromboxane production. It is likely that extracellular Ca²⁺ is required only for final aggregation and acts by mediating the surface interactions between cells (Jones, 1985).

Intracellular Ca²⁺ appears to be more important than extracellular Ca²⁺ in controlling platelet aggregation based on the following evidence: 1) Calcium ionophores are mobile carriers that selectively promote permeability of membranes to Ca²⁺. They are able to trigger many responses of platelet activation even when extracellular Ca²⁺ is not present; 2) Intracellular Ca²⁺ antagonists such as 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate-HCl inhibit platelet activation. Extracellular Ca²⁺ chelators such as EDTA can also prevent platelet aggregation, but the response is less effective; 3) mobilization of Ca²⁺ into platelets occurs prior to responses of platelet activation (Jones, 1985). Intracellular Ca²⁺ is involved in the stimulus-response pathway since
platelet activation can be triggered by the intracellular Ca\textsuperscript{2+} without the presence of a physiological stimulus. This also shows that the internal storage of Ca\textsuperscript{2+} is sufficient for initiating platelet responses. Different concentrations of calcium ionophores trigger different responses of platelets. At low concentrations of calcium ionophores, only primary aggregation occurs. When the concentration of calcium ionophores increases, granule release (platelet secretion) and secondary aggregation occur following the elevation of [Ca\textsuperscript{2+}]\textsubscript{i}.

The stimulation of contractile proteins in platelets, i.e., myosin, during platelet activation is a major process contributing to shape changes, granule release, and aggregation (Jones, 1985). It is controlled by regulation of cellular actinomyosin complexes. Under the stimulation of Ca\textsuperscript{2+} and calmodulin complex, platelet Ca\textsuperscript{2+}/calmodulin-dependent myosin light-chain kinase phosphorylates the light chain of myosin. Phosphorylation of myosin light chain is required for the activation and association of myosin and actin. Contractility of platelet actinomyosin complex is then enhanced. In the absence of intracellular Ca\textsuperscript{2+}, calmodulin has low affinity of binding to the kinase. However, the presence of sufficient intracellular Ca\textsuperscript{2+} results in high-affinity binding of calmodulin. Therefore, the [Ca\textsuperscript{2+}] can regulate the phosphorylation of platelet Ca\textsuperscript{2+}/calmodulin-dependent myosin light-chain kinase and association of myosin and actin. The increase of contractility of actinomyosin complex can then further enhance the activation of platelets leading to the shape changes, secretion and aggregation. The resting [Ca\textsuperscript{2+}]\textsubscript{i} in platelets is about 0.1 uM. In response to certain aggregators such as thrombin and ionomycin, [Ca\textsuperscript{2+}]\textsubscript{i} rises rapidly to higher than 2-3 uM. The threshold for shape change of platelets is
between 0.4 and 0.6 μM, but aggregation requires a further elevation of about 2 μM (Feinstein et al., 1985).

Due to the important role of cytosolic Ca²⁺ in platelet aggregation, the association of hyperaggregability seen in diabetic patients and the homeostasis of platelet Ca²⁺ concentrations has been investigated (Ishii et al., 1991; Pellegatta et al., 1993). In the study conducted by Pellegatta et al. (1993), platelet [Ca²⁺]ᵢ was measured using a fluorescent probe, fura-2, in 60 IDDM patients and 31 age-matched healthy controls under both resting and thrombin-stimulated conditions. Patients with a poor metabolic control with or without complications showed significantly higher [Ca²⁺]ᵢ in resting platelets compared with controls. After the stimulation of thrombin, the significant elevation of [Ca²⁺]ᵢ was observed only in patients with good metabolic control and without complications. However, a significant correlation was found between resting platelet [Ca²⁺]ᵢ and both blood cholesterol and glycosylated hemoglobin (HbA₁c) levels. High blood cholesterol concentration has been noted to be associated with platelet hyperaggregability (Viener et al., 1984). Different from the observations of Pellegatta et al. (1993), Ishii et al. (1991) demonstrated that the basal [Ca²⁺]ᵢ of platelets from NIDDM patients with high platelet aggregation rates (DM-A), NIDDM patients with normal platelet aggregation rates (DM-B), and age-matched healthy controls were similar. However, there was significantly higher platelet [Ca²⁺]ᵢ in the DM-A group of patients compared to the DM-B group and control subjects when platelets were stimulated by thrombin and platelet-activating factor (PAF) with or without the presence of extracellular Ca²⁺. There was no significant difference in [Ca²⁺]ᵢ between the
DM-B group and controls. The researchers suggested that the platelet hyperfunctions observed in DM patients may be associated with the enhanced intracellular Ca²⁺ mobilization via Ca²⁺ influx and Ca²⁺ release. In the presence of extracellular Ca²⁺, the [Ca²⁺]i depends on both Ca²⁺ influx and release from intracellular Ca²⁺ pool, i.e., endoplasmic reticulum. Whereas, when extracellular Ca²⁺ is absent, platelet [Ca²⁺]i depends only on intracellular Ca²⁺ release. The secondary messenger inositol 1,4,5-trisphosphate derived from phosphatidylinositol (PI) can cause the release of Ca²⁺ from intracellular pools. Turnover rate of phosphatidylinositol has been noted to increase in platelet membranes from NIDDM patients with high platelet aggregation (Ishii et al., 1991).

The action of cAMP on platelet aggregation

Platelet cyclic AMP (cAMP) levels affect the responses of platelet activation (Feinstein et al., 1985; Jones, 1985; West, 1990; Johansson et al., 1992; Tao et al., 1992). Phosphorylation of platelet Ca²⁺/calmodulin-dependent myosin light-chain kinase by a cAMP-dependent protein kinase decreases the activity of myosin light-chain kinase. Sufficient intracellular cAMP concentration impairs the contraction of actinomyosin and consequently inhibits the activation of platelets. In addition, elevation of platelet cAMP decreases hydrolysis of phosphatidylinositol to form inositol 1,4,5-triphosphate, reduces the release of Ca²⁺ from endoplasmic reticulum, and thus decreases the elevation of cytosolic Ca²⁺ concentration and inhibits the activity of phospholipase A2 and phospholipase C. In the resting state cytosolic Ca²⁺ levels are determined by
the balance between passive Ca\(^{2+}\) influx through a channel leaking system and active extrusion system through Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchanger activities (Tao et al., 1992). Johansson et al. (1992) and Tao et al. (1992) reported that the elevation of cAMP decreases cytosolic Ca\(^{2+}\) concentrations by increasing the maximal velocity of the Ca\(^{2+}\)-ATPase pump located in the platelet membrane. The Km (the cytosolic Ca\(^{2+}\) concentration giving half-maximal rate of extrusion) of Ca\(^{2+}\)-ATPase however is not affected. Therefore, the elevation of cAMP may interfere with platelet aggregation.

During the activation of platelets, intracellular cAMP is kept at a low level via the action of a calcium-sensitive cyclic nucleotide phosphodiesterase. This enzyme is activated in response to platelet stimulation when [Ca\(^{2+}\)] is sufficiently high. It degrades cAMP to maintain intracellular cAMP at a low level appropriate for the activation of Ca\(^{2+}\)/calmodulin-dependent myosin light-chain kinase.

Prostaglandins are regarded as modulators of adenyl cyclase activity (Mayes, 1990a). Thromboxane A\(_2\) decreases the production of cAMP by inhibiting the activity of adenyl cyclase and increasing the tendency for platelet aggregation. In contrast, PGI\(_2\) inhibits aggregating action of platelets by stimulating the activity of adenyl cyclase (Dillon, 1987).

**Influence of diet on platelet function**

**Influence of diet on fatty acid composition of serum and platelets**

Alteration of fatty acid composition of platelet membrane phospholipids (PL) may affect platelet responsiveness (Socini et al., 1983; McDonald et al., 1989;
Kwon et al., 1991). Many studies have shown that dietary fatty acids may be reflected in the fatty acid composition of lipids from various tissues, including serum and platelet membranes (Hornstra et al., 1983; Socini et al., 1983; Weaver et al., 1985; Vericel et al., 1987; McDonald et al., 1989; Agren et al., 1990; Phinney et al., 1990; Piche and Mahadevappa, 1990; Agren et al., 1991; Kwon et al., 1991; Hansen et al., 1993). Piche et al. (1990) demonstrated that rats fed a cod-liver-oil diet containing low LA had less AA in phospholipids of platelet membrane than controls. The fatty acid composition of platelet PL from healthy males on a diet containing canola oil showed lower ω6 fatty acids, especially LA and AA (Kwon et al., 1991). The amount of fatty acids in a diet may also modify the fatty acid composition in circulating lipid fractions. When compared with omnivorous people, semi-vegetarians had significantly lower AA concentrations in their serum PL, and vegetarians had a considerable reduction of AA in serum PL and cholesteryl esters (CE) (Phinney et al., 1990). In the study conducted by Socini et al. (1983), the AA levels in plasma and platelet PL of rats fed fish oil (10% of total energy) were lower than those from rats in the basal diet (5% from corn oil and 5% from fish oil) group. Linoleic acid levels were lower in plasma PL of the fish oil group, but no significant difference was observed in platelet PL between these two groups.

**Influence of dietary fatty acids on eicosanoid synthesis and platelet aggregation**

The amount of fatty acids in the diet strongly influences eicosanoid concentration since certain fatty acids such as AA and EPA in those tissues are the precursors of eicosanoid synthesis (Hornstra et al., 1983; Socini et al., 1983;
Dillon, 1987; McDonald et al., 1989; Piche and Mahadevappa, 1990; Agren et al., 1991; Ney, 1991) Diet-induced alterations on fatty acid composition in platelet membranes may affect the function of platelets including aggregation, secretion, and the biosynthesis of eicosanoids (During et al., 1983; Hornstra, 1983; Longenecker, 1985; Lee et al., 1988; Piche and Mahadevappa, 1990; Burri et al., 1991). McDonald et al. (1989) reported that healthy young men who received a diet prepared with high oleic acid canola oil had longer bleeding time and higher production of PGI2 than when they received a diet containing a mixture of fats. The production of TXB2, the stable metabolite of TXA2, was significantly decreased when subjects were fed a sunflower-oil-based diet. Compared with a diet high in SFA, platelet aggregation was reduced significantly when those young men received a diet containing canola oil during the first 3 weeks of a 8-week period. However, only an acute effect of antiaggregation was shown in the canola oil diet group when 2 mg/L of collagen was used as the aggregator. The secretion of ADP from platelets was diminished during the experimental period when a canola-oil-based diet was provided. There observations may be because that higher amount of oleic acid compete with desaturation and consequently reduce the production of AA for synthesis of prostaglandins and platelet aggregation. Lee et al. (1988) investigated the effects of various combinations of ω3 and ω6 fatty acids with saturated fat on eicosanoid production in rats using safflower oil (SFO), linseed oil (LSO), palm oil (PLO) and a 1:1 combination of these oils. The activity of phospholipase A2 was significantly lower in the LSO group than in the SFO and/or PLO groups. Phospholipids from serum, liver, and aorta showed significantly lower concentrations of AA in LSO group compared to other
groups. The vascular production of PG\textsubscript{I\(_2\)} and platelet production of TXA\textsubscript{2} were also significantly decreased in LSO group. The depressing effect of LSO diet was reversed when it was combined with SFO, but not with PLO. Meydani et al. (1992) demonstrated that rats fed fish oil had significantly lower concentrations of TXB\textsubscript{2} and PG\textsubscript{I\(_2\)} than those fed corn oil and/or coconut oil. Linseed and fish oils are good sources of \(\omega3\) fatty acids, which may compete with \(\omega6\) fatty acids for desaturation of fatty acids and prostaglandin synthesis. Consequently, less TXA\textsubscript{2} and PG\textsubscript{I\(_2\)} were produced.

**Arachidonic acid metabolism and platelet aggregation**

Arachidonic acid, the precursor of PG\textsubscript{I\(_2\)} and TXA\textsubscript{2}, is the major long chain PUFA found in tissue lipids, especially membrane PL. AA is available from PL by phospholipase A\textsubscript{2} for the synthesis of PGs, TXs, and LTs (Mayes, 1990a). It originates from either the diet or endogenous synthesis by desaturating and elongating LA (Dillon, 1987; Mayes, 1990a). The concentration of AA in tissue membranes can be affected by the activities of \(\Delta6D\), \(\Delta5D\), and phospholipase A\textsubscript{2} (Dillon, 1987; Lee, 1988; Mayes, 1990). Only animal products provide AA, mainly meats and eggs; foods from plant sources do not contain C\textsubscript{20} and C\textsubscript{22} PUFA except seaweeds (Horrobin, 1981; Phinney, 1990). Vegetarians have to rely on endogenous synthesis of C\textsubscript{20} fatty acids by way of elongation and desaturation from C\textsubscript{18} fatty acids for the biosynthesis of physiologically active eicosanoids (Phinney, 1990).

Arachidonic acid is found in abundance in membrane phospholipids, especially in phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE).
Phospholipids in human platelets are composed of about 40 mole % of PC, 23-28 mole % of PE, 9-11 mole % of phosphatidylserine (PS), 3-6 mole % of phosphatidylinositol (PI), and 18-20 mole % of sphingomyelin (Adams, 1985). Arachidonic acid is usually found at the sn-2 position of all the phospholipids to a certain degree.

Activation of phospholipase A$_2$ and C (PLA$_2$ and PLC) requires high cytosolic Ca$^{2+}$. PLA$_2$ acts primarily on the sn-2 position of PC and PE to liberate AA from the membranes. PLC acts on phosphatidylinositol (PI) to produce arachidonoyl diglyceride, which then releases free AA via the action of diglyceride lipase (Longenecker, 1985).

The effect of fats on prostaglandin production may lead to different responses of platelet aggregation due to the changes of AA availability from diets. By feeding rats with different proportions of fat (10%, 30%, and 50% of energy from beef tallow, olive oil, peanut oil or butter) in the diet, Steel et al. (1990) demonstrated that butter- and beef tallow-feeding decreased aortic production of PGI$_2$ and collagen-induced TXA$_2$ production in a dose-dependent manner as the level of fat increased. The reduction of PGI$_2$ and TXA$_2$ formation was accompanied by dose-dependent decreases in aortic and plasma phospholipid AA content and increases in EPA and DHA. Neither olive oil nor peanut oil showed a significant effect on production of PGI$_2$ and TXA$_2$. Butter-feeding, but not any of other fats, also decreased collagen-induced platelet aggregation. They suggested that the effects of butter-feeding on prostaglandin production and platelet aggregation were mediated through a decreased availability of AA for incorporation into tissue PL and, consequently, decreased
production of PGI₂ and TXA₂ and platelet aggregation. In the later work, Steel et al. (1993) observed significant reductions of AA content in aortic and plasma PL, PGI₂ and TXA₂ production, and collagen-induced platelet aggregation in rats fed a butter-enriched diet containing 50% fat as compared with control rats a 5% butter diet. ADP-induced platelet aggregation was not affected by the butter-enriched diet. Results from both studies show that the AA depletion in butter diets leads to a greater reduction of TXA₂ production compared with the decrease of PGI₂ production. They also found a dose-dependent reversal of those changes by the daily oral administration of ethyl arachidonate (9, 18, 36, or 72 mg). Results show that higher amounts (18 mg/rat/day) of ethyl arachidonate are required to reverse the reduction of PGI₂ compared to the amount of AA (9 mg/rat/day) required for reversal of TXA₂ production.

The effect of ω3 family fatty acids on platelet aggregation

ω3 fatty acids are reported to have an anti-thrombotic effect due to the competition with AA biosynthesis (Siguel and Maclure, 1987) and/or the substitution for AA in platelets and vascular tissues. Lee et al. (1988) compared the production of PGI₂ and TXA₂ in rats fed linseed oil (mainly containing linolenic acid; 18:3ω3), safflower oil (a good source of linoleic acid; 18:2ω6), or palm oil (mainly provides SFA such as palmitic acid; 16:0). The authors found that linseed oil feeding caused lower production of PGI₂ and TXB₂ with accompanying reductions of AA in tissue PL and the decrease of phospholipase A₂ activity. The activity of Δ6D was significantly higher in the linseed oil group. The inhibiting effect of linseed oil on phospholipase A₂
disappeared when safflower oil was combined with linseed oil. However, the combination of palm oil and linseed oil did not significantly change the inhibiting effect of linseed oil. The concentration of AA in tissue membranes can be affected by the activities of Δ6D, Δ5D, and phospholipase A2 (Dillon, 1987; Lee, 1988; Mayes, 1990). Phospholipase A2 is thought to play an important role in regulating the availability of AA for eicosanoid synthesis.

It has been proposed that EPA might have a higher affinity for the cyclooxygenase enzyme and might competitively inhibit the formation of PG\(I_2\) and TX\(A_2\) (Dusing et al., 1983). Socini et al. (1983) studied the influence of fish oil on PG synthesis and platelet aggregation by feeding rats fish oil, corn oil, or a combination of corn oil and fish oil. The administration of fish oil in combination with corn oil decreased PG\(I_2\) significantly (90% lower) and marginally decreased the synthesis of TX\(A_2\). The total antiaggregatory activity of the aortic wall was remarkably reduced by the administration of fish oil. When the fish oil was given alone, the production of PG\(I_2\) and TX\(A_2\) was equally decreased due to the reduction of AA in platelets. Those researchers also noted that when LA was adequate (5% of total energy), supplementation of fish oil inhibited the synthesis of PG\(I_2\) and antiaggregatory activity of aorta wall more effectively than it did the production of TX\(A_2\). In rabbits fed diets containing 40% fat (8% from sunflower-seed oil and 32% from either fish oil, linseed oil, olive oil, palm oil, or sunflower-seed oil) for one and one-half years, thromboxane A\(2\) production was lowest after feeding fish oil (Hornstra et al., 1983). The linseed-oil diet had an intermediate effect on TX\(A_2\) synthesis. TX\(A_2\) production was highest in the remaining dietary groups. There was a significant positive correlation between the AA concentration of vascular PL and the formation of eicosanoids.
PGI\textsubscript{2} (r=0.78, p<0.01). It was reported that the reduction of AA due to the substitution of \(\omega 3\) fatty acid in the vascular PL, especially 20:5\(\omega 3\), 22:5\(\omega 3\) and 22:6\(\omega 3\), influenced PGI\textsubscript{2} synthesis (Hornstra et al., 1983).

The PGI\textsubscript{3} production in fish oil feeding was insufficient, however, to compensate for the reduction of PGI\textsubscript{2} even though PGI\textsubscript{3} is considered to be as equipotent with PGI\textsubscript{2} (Hornstra, 1983). The synthesis of 3-series prostaglandins after the consumption of EPA from fish oil may be influenced by the presence of different dietary linoleic acid levels. Therefore, before advocating the use of fish oil as an antithrombotic agent, the effect of relative amounts of dietary EPA and linoleic acid on the differential synthesis of both 2 and 3 series prostaglandins should be further investigated (Socini et al., 1983). In addition, cod liver oil, which is a major source of fish oil, is high in unsaturated fatty acids but low in Vit. E (McDowell, 1989). This may increase the tendency of lipid peroxide production.

Based on the above findings, it is reasonable to consider that the incorporation of dietary lipids into tissues may influence the health of humans as well as animals due to induced physiological alterations.

**Effect of essential fatty acid deficiency**

Deficiency of dietary essential fatty acid (EFA), mainly LA, may result in decreased production of PGI\textsubscript{2} and TXA\textsubscript{2} (Blomstrand et al., 1985). Different families of fatty acids compete for the same elongation and desaturation enzyme systems (Siguel and Maclure, 1987; Mayes, 1990). When dietary LA is insufficient, AA can not be effectively synthesized due to a deficiency of substrate. Under these circumstances eicosatrienoic acid (20:3, \(\omega 9\)) production
from oleic acid (18:1ω9) is induced, resulting in an elevated triene: tetraene ratio. This is regarded as an indicator of EFA deficiency (Holman, 1971). Rats fed with partially hydrogenated low erucic acid rapeseed oil without LA supplementation had higher 20:3ω9 accumulation in liver microsomes PL compared to those fed with LA-supplemented oil or olive oil (Blomstrand et al., 1985). Eicosatrienoic acid has been reported to inhibit the function of cyclooxygenase, which is the key enzyme for the synthesis of prostanoids (Ziboh et al., 1974).

**Supplementation of intermediate fatty acids**

As reported in certain studies, the activity of Δ6D, a rate-limiting step in the conversion of linoleic acid to AA, is reduced with aging. The biosynthesis of γ-linolenic acid (GLA) from LA is decreased. Consequently, elderly people may be exposed to dihomo-γ-linolenic acid (DGLA) and AA deficiency. AA can be obtained from diets containing animal products easily, but there is little GLA and/or DGLA present in the human diet except human milk (which contains both GLA and DGLA), evening primrose oil and borage oil (which contain GLA) (Horrobin, 1981). Therefore, the impaired synthesis of DGLA is of greater consequence since this fatty acid is the precursor of PG with one double bond (PG1 series). Prostaglandin E1 is a strong antiaggregator and vasodilator. Therefore, DGLA is a potentially anti-thrombotic PUFA. Prostaglandin E1 may also lower blood pressure, activate T-lymphocytes, inhibit smooth muscle proliferation and thrombosis, and increase cAMP levels, which may inhibit
cholesterol formation in many tissues (Horrobin, 1981). It is possible that the loss of PGE1 is a crucial factor in aging (Horrobin, 1981; Hrelia et al., 1989).

Since AA may be obtained from animal sources in the diet, but the human diet contains very little GLA and/or DGLA, it is reasonable to assume that supplementation of GLA and/or DGLA may by-pass the rate-limiting step. Sixteen elderly people (mean age: 85 years) consumed a diet supplemented with either primrose oil capsules containing 1 gm of GLA and 1 gm of LA or sunflower seed oil capsules containing 2 gm of LA for 2 months in a cross-over design study (Murota et al., 1983). Both DGLA and AA were increased in plasma lipid and platelet PL when primrose oil was supplemented. However, no such alterations were observed with sunflower seed oil supplementation. As mentioned above, DGLA is a potent anti-thrombotic PUFA. Therefore, DGLA and AA have opposite potential effects on platelet aggregation. Because DGLA and AA both increased in plasma lipids and platelet PL, the supplementation of primrose oil in this study did not actually show alteration of platelet aggregation in elderly people.

After reviewing several studies, it was presumed by Dillon (1987) that the ratio of DGLA and AA in the PL might determine the series of PG synthesized. In the study conducted by Sim et al. (1977), the supplementation of GLA and DGLA showed an inhibiting effect on platelet aggregation due to the production of PGE1. The effects of dietary lipids on physiological reactions of male subjects who received a canola-oil-based or a safflower-oil-based diet also showed that AA is positively associated with platelet aggregation, whereas DGLA has a negative effect possibly through the syntheses of different PG (Kwon et al., 1991).
Effect of Vit. E on prostaglandin synthesis and platelet aggregation

Karpen et al. (1982) reported that chronic administration of Vit. E in streptozotocin-diabetic rats reversed the decreased PGI2 production. This suggests the possible role of Vit. E in platelet aggregation, and the role of lipid peroxide formation in decreasing the biosynthesis of PGI2. Lipid peroxides have been noted to decrease PGI2 formation due to the damage of endothelium on vessel wall caused by the lipid peroxides.

Under normal physiological conditions, the activity of cyclooxygenase can be limited by the availability of fatty acids as substrates and lipid peroxides as activators (Warso and Lands, 1983). The straight-chain hydroperoxides are more effective as an activator of cyclooxygenase. The efficiency of activation is proportional to the chain length of lipid peroxides. However, these lipid peroxides have dual effects on the regulation of enzyme activity. When the concentrations of lipid hydroperoxides are low (10-100 nmol/L), they activate cyclooxygenase; but when the concentrations are high (above 1 mmol/L), they inhibit the activity of cyclooxygenase.

High concentrations of lipid peroxides (1 mmol/L) also inhibit the activity of prostacyclin synthase (Warso and Lands, 1983; Packer, 1991; Kubow, 1993). The administration of Vit. E is reported to alleviate the loss of prostacyclin synthase activity caused by lipid peroxides (Warso and Lands, 1983; McDowell, 1989; Pyke and Chan, 1990). In the study of Pyke and Chan (1990), effect of Vit. E on PGI2 release in the rats with ischemia-reperfusion myocardial injury. After 4 months of feeding a semi-purified diet containing 0, 30, and 3000 ppm of R,R,R,-α-tocopherol acetate, rat hearts were treated with Langendorff perfusion.
The release of PGI$_2$, measured as 6-keto-PGF$_{1α}$, from abdominal aorta was increased by the addition of dietary Vit. E in a dose-dependent manner. These researchers suggested that Vit. E is important in maintaining the synthesis of PGI$_2$, especially in the conditions of oxygen stress. Vit. E can also protect arachidonic acid, a substrate for the formation of PGI$_2$ and TXA$_2$, from peroxidation. Therefore, it is considered to be required for the production of prostaglandins associated with platelet aggregation (McDowell, 1989; Kubow, 1993).

The inhibiting effect of Vit. E on TXA$_2$ formation is thought to be due to the inhibition of cyclooxygenase activity by decreasing the concentration of lipid peroxides, which act as activators, rather than any effect on the thromboxane synthase (TXS) (Warso and Lands, 1983). Kawaguchi et al. (1982) studied hypercholesterolemic rabbits and reported that the levels of glutathione and glutathione peroxidase were greatly reduced (70% and 92%, respectively) in the hyperaggregable platelets. They found that the addition of glutathione had no direct effect on TXA$_2$ synthesis from PGH$_2$, but it decreased TXB$_2$ and PGH$_2$ synthesis from AA. These results show that the reduction of TXB$_2$ synthesis is due to the suppression of cyclooxygenase influenced by the insufficient concentration of lipid peroxides as activators. Therefore, the protective effect of Vit. E on reducing TXA$_2$ formation is mainly on the suppression of cyclooxygenase activity. Evidently, vit. E inhibits platelet aggregation by both lowering platelet TXA$_2$ formation and raising arterial PGI$_2$ production (Karpen et al., 1981; Warso and Lands, 1983; McDowell, 1989).

The inhibitory effect of Vit. E on platelet aggregation was also observed in NIDDM patients (Kunisaki et al., 1990). Diabetic patients exhibit
hyperaggregability due to imbalanced TXA$_2$ and PGI$_2$ formation (Lagarde et al., 1980; Davi et al., 1982; Akai et al., 1983; Winocour et al., 1985). Kunisaki et al. (1990) investigated the influence of Vit. E supplementation on the abnormalities of platelet function and prostaglandin metabolism in 14 NIDDM patients with proliferative retinopathy. Vit. E was administered to both diabetic patients and healthy controls in a daily dose of 600 mg. The in vitro addition of Vit. E inhibited platelet aggregation in a dose-dependent manner. At lower concentrations of Vit. E, greater inhibition of platelet aggregation was seen in diabetic patient than in controls. However, at higher concentrations, no significant difference in the degree of inhibition on platelet aggregation was observed between diabetic patients and controls. It was noted that dietary supplementation of Vit. E primarily inhibited the secondary phase of platelet aggregation of diabetic patients induced by ADP. The reduction of platelet aggregation was proportional to the plasma concentration of Vit. E. Production of TXB$_2$ was significantly lower during the administration of Vit. E. Plasma levels of TXB$_2$ in diabetic patients were significantly higher than in controls. There was a significant increase in plasma levels of 6-keto-PGF$_{1\alpha}$ after the supplementation of Vit. E that led to a higher 6-keto-PGF$_{1\alpha}$/TXB$_2$ ratio in plasma from diabetic patients.

Aging and prostaglandin synthesis

It is believed that aging is accompanied by a decreased ability to synthesize prostaglandins and by an imbalance between the production of TXA$_2$ and PGI$_2$
(Dillon, 1987). Since PG\textsubscript{I\(2\)} and TXA\textsubscript{2} have opposite effects on platelet aggregation, it is suggested that the balance in the synthesis of TXA\textsubscript{2} by platelets and PG\textsubscript{I\(2\)} by the endothelial cells of blood vessel wall is a critical factor in determining a tendency for thrombus formation (Dillon, 1987; Notarbartolo et al., 1992). Many studies have shown the age-dependent decline in PG\textsubscript{I\(2\)} production and increased TXA\textsubscript{2} production (Chang et al., 1980; Kent et al., 1981; Vericel et al., 1985; Moncada, 1986; Dillon, 1987; Meydani et al., 1992). Kent et al. (1981) incubated aortic strips from young (6 months) and mature (2-4 years) pigs either in the presence of added AA or in the absence of AA. Labeled 6-keto-PGF\textsubscript{1\(\alpha\)} was detected in all groups. There was no significant difference in the accumulation of 6-keto-PGF\textsubscript{1\(\alpha\)} in aortic intimal strips from young pig and mature pigs when AA was not added to the incubation mixtures. Under the presence of exogenous AA, twice as much total 6-keto-PGF\textsubscript{1\(\alpha\)} was produced by the aortic strips of young pigs as was produced by tissues from older animals. Identical fatty acid composition of aortae from young and old animals were noted. Therefore, the possibility of dietary influence on prostaglandin synthesis could be ruled out. In the study of Chang et al. (1980), intact cells and cell-free homogenates from cultured rat aortic smooth muscle cells were assayed. Radioactive-labeled AA and prostaglandin H\textsubscript{2} (PGH\textsubscript{2}) were used as substrates to detect the activity of cyclooxygenase and the production of PG\textsubscript{I\(2\)}. The total cyclooxygenase activity for PG synthesis is the same in cells from young and old rats. However, the age-related reduction of PG\textsubscript{I\(2\)} synthesis was observed both in intact cell and cell-free homogenate assays. After reviewing studies related to PG\textsubscript{I\(2\)} production in response to age, Notarbartolo et al. (1992) suggested that human plasma showed a stimulatory activity on PG\textsubscript{I\(2\)}
production that may play an important role in the regulation of PGI₂ synthesis in the vascular wall. A reduction of stimulatory activity observed in aging endothelial cells may contribute to the development of atherosclerosis in elderly humans. In contrast, the synthesis of TXA₂ from AA was reported to be increased with age by Vericel et al. (1985). Platelets from middle-aged (30-50 years) and elderly humans (74-95 years) were incubated for 4 minutes using radioactive-labeled AA. The incorporation of AA into phospholipids (PL) and production of TXB₂ were noted to be significantly higher in the elderly people. No further explanation about the increased incorporation of AA into PL was given in this study.

When investigating the effect of age, dietary fat type and different doses of vitamin E supplementation on the synthesis of lung eicosanoids in young (3-mo-old) and old (24-mo-old) mice, Meydani and his co-workers (1992) observed that the ratio of TXB₂ and 6-keto-PGF₁₅ was significantly higher in old male C57BL/6NIA mice no matter what kind of dietary fat was added (corn oil, coconut oil, or fish oil). Murota et al. (1983) also proposed that there is an age-related shift from the biosynthesis of PGI₂ to TXA₂ when the PG synthesis in human diploid fibroblasts was studied. Production of PGI₂ on the first day was much higher in young cells than in old cells. Young cells produced more PGI₂ than TXA₂, but the old cells produced more TXA₂ than PGI₂ each time cells were stimulated with 0.25% trypsin for 6 sec. In the same study, the production of cyclic AMP was also found to decrease with age. In young cells, the intracellular cyclic AMP level was increased 170-fold over the initial level, whereas the intracellular cAMP in old cultures increased only 45-fold over the initial level due to the decreased production of PGI₂ with age.
It has been suggested that hyperaggregability of platelets may be a significant risk factor for vascular diseases in elderly subjects (Murota et al., 1983). The altered balance of TXA₂ and PGI₂ may be one of the reasons (Dillon, 1987). This pathological alteration may account for the higher tendency of elderly people to develop vascular diseases, such as increased blood pressure and frequency of thrombotic events.

**Effect of estrogen on platelet aggregation**

Epidemiologic studies have shown that women are prone to have osteoporosis after menopause and have higher morbidity and mortality due to cardiovascular disease than pre-menopausal women. To decrease the risk resulting from estrogen deficiency, hormone replacement therapy has been prescribed for some menopausal women. However, pathological conditions may develop after estrogen replacement therapy. As reviewed by L'Hermite (1991), various types of estrogens may have different effects on prostaglandin syntheses (mainly PGI₂ and TXA₂) and platelet aggregation. All estrogens increase the local production of PGI₂, resulting in vasodilation and prevention of platelet aggregation. But certain estrogens, such as conjugated equine estrogens, can also induce the hepatic synthesis of renin and angiotensinogen. This may lead to the activation of platelet TXA₂ and hypertension. Therefore, the effect of estrogens on platelet aggregation depends on the balance of PGI₂ and TXA₂ production influenced by various estrogens. Synthetic estrogens activate both PGI₂ and TXA₂ and may cause
vasospasm and platelet aggregation. Natural estrogens increase the formation of PGI$_2$ and result in vasodilation and anti-aggregation. On the other hand, progesterone does not have a stimulating effect on PGI$_2$ production and may counteract the effect of estrogens so as to increase the aggregability of platelets (L'Hermite, 1991).

Due to the different effects of various estrogens on prostaglandin syntheses, several studies show contradictory results and conclusions on the possibility of thromboembolism induced by estrogen administration (Rosenblum et al., 1985; Inauen et al. 1991; Young et al., 1991). Inauen et al. (1991) exposed vascular subendothelium of rabbit aorta to flowing human blood obtained from females subjects before drug ingestion, during low intake (20 ug ethinyl estradiol and 150 ug desogestrel per day), or high intake (50 ug ethinyl estradiol and 125 ug desogestrel per day) of oral contraceptives (OC). Both low and high doses of OC decreased anti thrombin III activity, but increased fibrinogen. Fibrin deposition on vascular subendothelium was enhanced only by the high dose of OC. No alterations of subendothelial deposition of platelets and platelet thrombi were observed. This study demonstrated that the high dose of OC, but not the low dose, led to a significant increase of fibrin-subendothelial interactions. Huch et al. (1987) studied the coagulant activity (CA) of platelets from OC users. The CA of platelets was measured using epinephrine, ADP, and collagen as aggregators. They observed that the asymptomatic OC users had hypercoagulability in both collagen-stimulated and unstimulated platelets. They also incubated platelets obtained from normal males with estradiol and/or progesterone for one hour to investigate the effect of estradiol and progesterone on platelets. No significant difference in CA of platelets was found in the hormone treated platelets compared with untreated platelets from the same
donor, even though there was a dose dependent effect. The authors considered that the increased CA of platelet observed in OC users may not be due to the direct effect of hormones on platelets. This observation is consistent with the results of the study conducted by Rosenblum et al. (1985). They demonstrated different effects of estrogen on platelet aggregation in both in vivo and ex vivo experiments. In the in vivo study, after mice were implanted subcutaneously with a pellet containing 0.5 mg of estradiol or with a placebo for 8 to 12 days, platelet aggregation in mesenteric arterioles was induced by injuring the endothelium. The time of the onset of platelet aggregation was significantly shortened in the estradiol treated mice compared to the placebo group. However, when platelet rich plasma was used in the ex vivo experiment, the platelet aggregation induced by arachidonic acid was not effected by the treatment of estradiol. The researchers suggested that the enhanced aggregation effected by estradiol may be due to the direct effect on endothelium, but not a direct effect on platelets.

Various studies demonstrated controversial results of the effects of estrogens on the formation and release of PGI₂ and TXA₂. A study conducted by Berge et al. (1990) studied the interaction of estradiol and progesterone and endothelial cell by measuring the collagen-induced platelet aggregation, as well as the production and release of PGI₂ and TXA₂ after thrombin stimulation. Under basal conditions, endothelial cells grown with added progesterone released significantly less PGI₂ than did controls. After the stimulation of thrombin, endothelial cells grown with estradiol or a combination of estradiol and progesterone produced significantly less PGI₂ than controls. There was no significant effects of estradiol and progesterone on the platelet aggregation or
platelet formation of TXA$_2$ observed. The results may explain the increased risk of vascular disease in pregnant women and oral contraceptive users. However, they cannot explain the consequences occurring in postmenopausal women due to the hormonal loss. Balteskard et al. (1993) observed the production of TXA$_2$ by platelets from healthy men and postmenopausal women aged 50-73 years. No significant difference in TXA$_2$ production was observed between the men and women. This finding is different from their previous observation (Balteskard et al., 1989) that young men had higher TXA$_2$ production compared with young women (under age 40). A strong positive correlation ($r=0.7$, $p<0.0001$) with the increased generation of TXA$_2$ and years after menopause was noted. However, no such correlation was found in men. There was no significant correlation between the TXB$_2$ production and the number of platelets. This suggests that the increased production of TXB$_2$ may be due to the enhanced ability of platelets in producing TXB$_2$ in older women. Combining both of their studies, young women had lower TXA$_2$ production, but gradually increased production with age after menopause. This may explain the protective effect of natural estrogens against atherosclerosis in women.

Platelet functions in Diabetes Mellitus (DM)

Both IDDM and NIDDM patients have been noted to have platelet hypersensitivity, and enhanced platelet aggregation as well as adhesion, which may be associated with the increased incidence of vascular complications such as diabetic microangiopathy, micro-thromboses and atherosclerosis. Many
studies have demonstrated an increased sensitivity of platelets obtained from diabetic patients and rats (Winocour et al., 1985; Winocour, 1989b; Watala, 1991; Mandal et al., 1993).

In earlier studies, platelet hyperaggregation observed in diabetic patients were correlated with the degree of vascular diseases (Davis et al., 1985). However, other studies show that increased platelet aggregation could occur before clinical manifestations of vascular diseases. Mandal et al. (1993) investigated platelet aggregation in newly diagnosed NIDDM patients using whole blood platelet aggregometry. When ADP (10 and 20 mmol/L) and arachidonic acid (25 mmol/L and 50 mmol/L) were used as aggregating agents, patients showed significant hyper-aggregation at the time of diagnosis compared with age matched healthy controls. After blood glucose was corrected using oral hypoglycemic agents and dietary control, there was a significant decrease in platelet aggregation. They suggested that platelet hyperaggregation in diabetic patients could occur without the presence of any vascular complications. The platelet abnormality may not occur as a result of vascular diseases. On the contrary, it may be partially responsible for the alteration of vessel wall seen in diabetic patients (Winocour, 1985).

Many studies have been conducted to investigate the mechanisms of the platelet hyperaggregation observed in diabetic patients (Lagarde, 1980; Betteridge et al., 1981; Halushka, 1981; Takeda et al., 1981; Axelrod and Levine, 1982; Davi' et al., 1982; Akai et al., 1983; Morita et al., 1983; Di Minno et al., 1985; Davi' et al., 1990; Modesti et al., 1991; Ratzmann et al., 1991; Watala, 1991). The following possible mechanisms have been suggested after observations: (1) increased TXA$_2$ production by platelets due to the alteration of
AA metabolism; (2) decreased vascular biosynthesis of PG\textsubscript{I}\textsubscript{2} as well as reduced sensitivity of platelets to PG\textsubscript{I}\textsubscript{2}; (3) metabolic anomalies associated with diabetes mellitus, such as hyperglycemia and hyperlipidemia, contribute to the altered platelet AA metabolism and production of TXA\textsubscript{2}; (4) increased platelet interaction with vessels such as high levels of von Willebrand Factor leading to enhanced platelet adhesion; (5) decreased platelet survival observed in human diabetics that may be due to hypercholesterolemia; (6) decreased membrane fluidity of platelets affecting membrane bound receptors.

It was observed that diabetic patients can synthesize more TXA\textsubscript{2} (TXB\textsubscript{2}) than healthy controls (Lagarde, 1980; Halushka 1981; Davi et al., 1982). Lagarde et al. (1980) labeled platelets obtained from IDDM patients with $[^{14}\text{C}]$ arachidonic acid, and stimulated the release of TXB\textsubscript{2} with thrombin. They found an increased metabolism of $[^{14}\text{C}]$ arachidonic acid to $[^{14}\text{C}]$ TXB\textsubscript{2} by platelets from diabetic patients compared to those from controls. Studies show that the enhanced platelet aggregation observed in diabetic patients can be reversed by providing aspirin as an inhibitor of cyclooxygenase due to the decreased formation of TXA\textsubscript{2} by platelets (Davi, 1990). Therefore, it was suggested that the metabolism of AA plays an important role in contributing the enhanced platelet aggregation seen in diabetic patients (Winocour et al., 1985; Winocour, 1989b).

Di Minno et al. (1985) suggested that the increased fibrinogen binding and platelet aggregation in diabetic subjects in response to ADP or collagen is due to the increased formation of PGH\textsubscript{2} and TXA\textsubscript{2}. This may be due to enhanced activation of the arachidonic acid pathway since the hypersensitivity of platelets were lost when they are pretreated with aspirin or imidazole (Winocour, 1989).
Davi' et al. (1982) also demonstrated that platelets obtained from NIDDM patients synthesized significantly higher TXB\(_2\) than those from IDDM and age-matched healthy subjects. Platelets from NIDDM patients required significantly less collagen and AA to aggregate, and synthesized more TXB\(_2\) than platelets from healthy subjects (Davi' et al., 1990). The excretion of 11-dehydro-TXB\(_2\) in urine from NIDDM patients with normal renal function and clinical evidence of macrovascular disease was significantly higher than healthy controls (Davi' et al., 1990). The increased excretion of 11-dehydro-TXB\(_2\) was due to the enhanced biosynthesis of TXA\(_2\) by platelets, regardless of the types of macrovascular complications including coronary heart disease, peripheral vascular disease, and cerebrovascular disease (Davi' et al., 1990).

Aggregating agents such as thrombin and collagen mobilize more AA and induce more TXA\(_2\) production in platelets from diabetics than from controls (Lagarde et al., 1980). The possible mechanisms for enhanced pathway of AA mobilization from platelet phospholipids may involve increased Ca\(^{2+}\) mobilization via the phosphatidylinositol conversion and activation of phospholipase A\(_2\) (Takeda et al., 1981; Winocour, 1989b). Increased AA availability for TXA\(_2\) production is another possibility (Morita et al., 1983; Winocour, 1989b). It has been reported that platelet phospholipid-AA pools in diabetic patients is increased compared to controls (Morita et al. 1983), even though other studies did not find the difference of pool size between diabetic patients and normal controls (Takeda et al. 1981). Morita et al. (1983) had diabetic subjects and healthy controls consume an almost identical diet for 1 week prior to the analysis. Fatty acid composition of platelet phospholipids had significantly more AA in diabetic patients compared to controls. The
researchers suggested that the incorporation of AA into platelet phospholipids is accelerated in the diabetic metabolic state. However, Takeda et al. (1981) did not find a difference in platelet phospholipid-AA pool size between diabetic patients and normal controls. Platelet phospholipase A2 activity in diabetic patients was significantly increased compared to controls and its activity was decreased by the administration of insulin in vitro or chronic insulin therapy in the diabetic subjects (Takeda et al., 1981).

The vascular biosynthesis of PGI2 was noted to decrease in both IDDM and NIDDM patients (Winocour et al., 1985). The decreased synthesis of PGI2 can be seen in the DM patients without vascular disease. However, the vascular complications commonly seen in diabetic patients can also contribute to the reduction of vascular biosynthesis of PGI2 due to the damage of vessel wall. It is recommended by Winocour et al. (1985) that PGI2 does not present an influential effect on platelets under normal conditions. Only when the injury occurs on the vessel wall do the levels of PGI2 rise to a level sufficient to inhibit the platelet aggregation. A greater reduction of sensitivity of platelets to PGI2 was also found in NIDDM and IDDM compared to normal controls (Davi et al., 1982; Akai et al., 1983). However, other studies found no significant difference in sensitivity of platelets between diabetic patients (both IDDM and NIDDM) and control subjects (Modesti et al., 1991). Davi et al. (1982) reported that the sensitivity of platelets to PGI2 was correlated with fasting plasma glucose (r=0.64) and HbA1% (r=0.48) in both NIDDM and IDDM patients. Increased glucose concentrations may cause damage of vessel wall and decrease the ability of PGI2 biosynthesis of endothelium. Acute insulin treatment did not normalize PGI2 formation in spite of a fall in blood glucose. Winocour et al.
(1985) suggested that there should be a longer duration of insulin treatment to allow the repair of vascular wall and a more effective lowering of blood glucose. Modesti et al. (1991) studied whether the changed sensitivity of platelets to PGI₂ in DM patients is due to the alteration of lipid composition of platelet membrane or the impairment of PGI₂ receptors on platelets membranes. They found no significant differences in platelet sensitivity to PGI₂ between both NIDDM and IDDM and controls. The numbers of high affinity/low capacity and low affinity/high capacity PGI₂ receptors in DM patients were slightly lower but did not differ significantly compared to controls. However, significant correlations between platelet sensitivity to PGI₂ and platelet total cholesterol (r=0.89, p<0.001) as well as PGI₂ receptor (high affinity low capacity receptors) and platelet total cholesterol (r=-0.80, p<0.001) were observed in this study. The platelet cholesterol/phospholipid ratio was significantly correlated to both platelet sensitivity to PGI₂ and PGI₂ receptors (r=0.94 and r=-0.85, respectively). They also noted that some patients who had a decreased platelet sensitivity to PGI₂ and a low PGI₂ receptor number showed a much higher level of total cholesterol in platelet membranes. Therefore, Modesti et al. (1991) concluded that a reduction of platelet sensitivity to PGI₂, possibly caused by decreased number of high affinity PGI₂ receptors, may be seen in certain diabetic patients. It may be related to the alteration of lipid composition of platelet membranes with an increased cholesterol and cholesterol/phospholipid ratio in platelets.

It was also suggested that metabolic anomalies associated with diabetes mellitus such as hyperglycemia and hyperlipidemia contribute to the altered platelet AA metabolism and formation of TXA₂ (Halushka et al., 1981; Axelrod
and Bevine, 1982; Winocour et al., 1985; Winocour, 1989b; Mandal et al., 1993). Different anomalies may be related to the hypersensitivity of platelets in response to different aggregating agents (Winocour, 1989b). Studies conducted by Winocour and his colleagues (1986a, 1986b) show that the hypersensitivity of platelets to ADP is related to hyperglycemia rather than to hyperlipidemia. Washed platelets from streptozotocin-induced diabetic rats returned to a normal response to ADP when plasma glucose was controlled to normal with insulin, even though plasma cholesterol level remained elevated (Winocour et al., 1986a). Platelets from non diabetic litters of spontaneously diabetic BB Wistar rats did not show hypersensitivity to ADP regardless of hyperlipidemia (Winocour et al., 1986b). Mandal et al. (1993) noted that there was a positive relationship between blood glucose concentrations and platelet aggregation using ADP (both 10 and 20 umol/L) as an aggregator. No relationship between aggregation and glycosylated hemoglobin levels was observed. Winocour (1989b) suggested that glucose may affect platelet response either directly or through non enzymatic glycation. Glucose or glycogen is required as an energy source for normal platelet aggregation. However, it is not clear whether excessive glucose may affect platelet response. Since non enzymatic glycation is typically a chronic occurrence, the long term effect of glucose on certain blood proteins, such as fibrinogen and lipoproteins, and on platelet membrane proteins may affect platelet aggregation. This has not been fully confirmed, however.

When arachidonic acid and/or thrombin are used, hypersensitivity of platelets are related to altered plasma lipids and/or lipoproteins rather than to hyperglycemia (Winocour et al., 1987b). Winocour and his colleagues (1987b) reported that non-diabetic litters of spontaneously diabetic BB Wistar rats
with elevated plasma cholesterol concentrations showed hypersensitivity of platelets to AA and thrombin, even though they were normoglycemic. Platelets from non-diabetic rats with diet-induced hypercholesterolemia are hypersensitive to thrombin (Winocour, 1989b).

Halushka et al. (1981) noted that platelet TXB\(_2\) biosynthesis in the IDDM diabetic subjects was positively correlated with the plasma glucose. However, the correction of platelet aggregation and TXB\(_2\) formation were not seen in the study conducted by Ratzmann et al. (1991) after their NIDDM subjects received metabolic control. They reported that platelet aggregation and TXB\(_2\) production from platelets were not correlated to the extent of metabolic control after following 25 NIDDM patients without angiopathy for 6 months. They did not find a correlation among ADP-induced platelet aggregation and blood glucose, glycosylated hemoglobin (HbA\(_1\)), serum lipids or fatty acid composition of platelets in diabetic patients. Therefore, Ratzmann et al. (1991) suggested that glucose metabolism in diabetes mellitus did not affect platelet aggregation and TXB\(_2\) production in NIDDM patients. Winocour et al. (1985) suggested that serum glucose may only be an indicator of the adequacy of metabolic control and there might be other metabolic factors that influence AA metabolism and platelet aggregation. In the study conducted by Axelrod and Levine (1982), plasma TXB\(_2\) was increased in diabetic ketoacidosis in the rats and decreased by treatment with insulin. However, in the rats with hyperglycemia but not ketoacidosis, plasma TXB\(_2\) levels were noted to be lower than control rats. It may be likely that certain, but not all, metabolic anomalies seen in DM lead to the altered platelet AA metabolism and platelet aggregation.
Platelet and vessel wall interactions as well as platelet adhesion increase in both IDDM and NIDDM patients (Winocour et al. 1985). The enhancing platelet adhesion might be associated with the increase of plasma levels of von Willebrand Factor (vWF) observed in diabetic patients (Winocour et al., 1985; Winocour, 1989b). The vWF is a glycoprotein present in plasma as part of the factor VIII complex. It is mainly produced by endothelium. It can also be found on the surface of, and within, platelets and megakaryocytes. This factor is involved in platelet adherence to the subendothelium of vascular wall in the areas with high shear. Increased vWF may lead to enhanced platelet adhesion to the vascular wall. Platelets usually do not adhere to normal endothelium. Increased vWF and decreased PGI$_2$ and plasminogen activator released by vessels from diabetic humans and animals indicate an altered endothelium. Subendothelium is also altered by diabetes based on the observations of increased non-enzymatic glycation of collagen, enhanced lipid and lipoprotein transport into subendothelium and increased glycosaminoglycans. The alteration of vessel walls by diabetes may be a contributing factor that causes enhanced platelet accumulation on injured vascular walls (Winocour, 1989b).

Platelet survival was noted to decrease in diabetic humans with or without vascular disease compared with non-diabetic controls (Winocour et al., 1985). However, some studies investigating platelet survival using diabetic rats showed that platelet survival may be related to duration of diabetes. Platelet survival in rats with streptozotocin-induced diabetes was reduced during the acute period of diabetes (< 2 weeks), but it was prolonged after a longer period of diabetes (> 4 weeks) (Winocour, 1989b). The platelet survival in rats with spontaneous diabetes for 3 to 4 months was also prolonged (Winocour et al., 1987b).
In vivo, the platelet survival is dependent on the balance between platelet production from megakaryocytes and platelet clearance from the circulation. Platelets can be cleared through the reticuloendothelial system or through irreversible platelet modification during thrombosis or platelet vessel wall interactions (Winocour, 1989b). Modification of platelet membrane glycoproteins by plasmin during fibrinolysis can shorten platelet survival, but ADP, thrombin, or TXA2 do not affect platelet survival. In chronically diabetic rats, the reticuloendothelial system and/or fibrinolysis may be impaired and decrease platelet clearance (Winocour, 1989b). In diabetic humans, atherosclerosis and microcirculatory changes which usually accompany diabetes may shorten platelet survival. Hypercholesterolemia commonly seen in diabetic humans may also contribute to shortened platelet survival. Diabetic rats may have enhanced plasma cholesterol, but the elevation may not be sufficient to decrease platelet survival.

The platelet aggregation enhancing factor (PAEF) present in the plasma of diabetic patients also potentiated ADP-induced platelet aggregation and release of ADP (Winocour et al., 1985). The administration of PGI2, eicosapentaenoic acid (EPA), or aspirin on the platelets from normal subjects can inhibit PAEF-induced platelet aggregation and granule release in vitro. PAEF may act on platelet aggregation via a prostaglandin-dependent pathway (Winocour et al., 1985). Certain platelet-specific proteins, β-thromboglobulin and platelet factor 4 (PF4), also increased in diabetic patients in the absence of vascular complications (Betteridge et al., 1981), with greater levels found in the presence of vascular disease. Both proteins are released from platelet granules during the second phase of platelet aggregation. However, due to the
inconsistent sampling techniques, there are substantial differences in the
degree of elevation observed in various studies (Winocour et al. 1985).

Caution should be taken to use the plasma β-thromboglobulin as an indicator of
platelet activation, because its concentration can increase due to impaired renal
function often observed in diabetic complications.

Lipid fluidity of platelet membranes may be involved in the hypersensitivity
of DM (Watala, 1991). When the lipid fluidity of membrane is decreased due to
the increased composition of saturated fatty acids, protein-lipid interaction is
weaker and protein-water association is stronger. Consequently, proteins such
as receptors are displaced toward the aqueous phase located on both sides of
membranes. In addition, the micro viscosity of lipids may influence the
rotational diffusion of protein receptors in the lipid matrix. Changes of
membrane lipid fluidity may affect the accessibility and transport rate of
membrane-bound receptors. An example is that receptors on platelet
membranes may respond to aggregating agents and certain hormonal
receptors. Alteration of membrane lipid composition and non-enzymatic
glycation of membrane proteins may be two main factors affecting membrane
fluidity (Watala, 1991). The membrane lipid fluidity was altered by the non-
enzymatic glycation of membrane proteins in diabetic patients (Lugli et al.,
1983; Sampietro et al., 1986). The decreased membrane fluidity of platelets
was related to hypersensitivity in response to thrombin (Winocour et al., 1989a).
Watala (1991) suggested that platelet hypersensitivity in diabetics is likely to
result from the alteration of membrane fluidity due to increased glycation of
membrane proteins, rather than the higher membrane cholesterol/phospholipid
ratio which can also change the membrane fluidity. Membrane glycoproteins
Ilb and Illa were observed to be involved in the glycation in diabetes mellitus (Cohen et al, 1989). The glycoprotein Ilb/IIla complex forms fibrinogen receptors in activated platelets (Winocour, 1989b; Watala, 1991). Therefore, glycation occurring in the diabetic state may affect the binding of fibrinogen and result in increased aggregation. Hypercholesterolemia has been noted to alter the endothelial cells and consequently shorten platelet survival (Winocour et al., 1987b). DM patients were found to have higher rate of platelet production (Winocour et al., 1985). The greater number of younger platelets may contribute to the enhanced sensitivity of platelets to aggregating agents seen in DM.

Winocour et al. (1985 and 1989b) suggested that certain caution should be taken when interpreting the data of platelet aggregation in human studies using ADP. In citrated human PRP, second-phase platelet aggregation and the release of platelet granules occurs only in low Ca$^{2+}$ concentration. Under physiological concentration of Ca$^{2+}$, only primary aggregation induced by ADP occurs without the release of granules. The changes in primary aggregation of diabetic patients are not consistent. In addition, the response of platelet aggregation from diabetic patients can be affected by different aggregating agents.

**Platelet function and atherosclerosis**

Platelet functions are highly associated with the development of atherosclerosis and thrombosis (Sussman, 1985; Mustard et al., 1986; Monocada et al., 1986; Kristensen et al., 1989; Notarbartolo et al., 1992). The arterial intimal injury and platelet-vessel wall interaction are the initiating events.
of atherosclerosis. When the arterial endothelial wall is injured, platelets are attracted by the exposure of subendothelial collagen and adhere to the area of damaged endothelium. Activated platelets release platelet derived growth factor which stimulates smooth muscle cell proliferation. Besides, the potent aggregatory agent, TXA$_2$, as well as ADP are secreted from platelet granules to enhance the aggregation and form a platelet plug. Smooth muscle cells and macrophages incorporate cholesterol to form a foam cell and deposit on the arterial wall as atheromatous plaque (atheroma). Advanced atherosclerosis narrows the vessels and roughens the surface. This leads to thrombus formation around the atheroma.

Prostaglandins are involved in the interaction of platelets and vessel wall. Both thromboxane A$_2$ (a potent vasoconstrictor and platelet aggregating agent produced by platelets) and prostacyclin (a strong vasodilator and inhibitor of platelet aggregation produced by vascular endothelium and smooth muscle cells) are the major prostaglandins modulating platelet-vessel wall interaction. Alterations in the balance of TXA$_2$ and PGI$_2$ may cause pathological events such as atherosclerosis. PGI$_2$ also inhibits platelet adhesion and thrombus formation (Sussman, 1985). Impairment of PGI$_2$ production may lead to the progression of atherosclerosis. It has been noted that smooth muscle cells obtained from atherosclerotic lesions produced less PGI$_2$ than normal vascular smooth muscle cells (Moncada, 1982). Moncada (1986) also stated that HDL stimulates the production of PGI$_2$, whereas, LDL inhibits its production by endothelial cells. Therefore, the aberration in lipoprotein concentrations not only promotes atherogenesis but also influences the production of PGI$_2$ and consequently leads to the occurrence of atherosclerosis.
CHAPTER 3

OBESITY WAS ASSOCIATED WITH ENHANCED Δ6 DESATURASE ACTIVITY BUT Δ5 DESATURASE ACTIVITY WAS DECREASED WITH AGE IN SHHF/MCC-FA<sup>CP</sup> FEMALE RATS

INTRODUCTION

Fatty acids are mostly provided by the diet but some can be synthesized endogenously. Longer chain fatty acids, especially monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) can be synthesized by desaturation and elongation systems in liver microsomes (Mead et al., 1986; Mayes, 1990a; Mayes, 1990c; Gonzalez et al., 1986; Dillon, 1987). In animals, only Δ5 desaturase (Δ5D), Δ6 desaturase (Δ6D), and Δ9 desaturase (Δ9D) exist (Fig. 3.1). Animals are able to synthesize the ω9 family of unsaturated fatty acids completely, but unable to synthesize linoleic acid (LA; 18:2ω6) and α-linolenic acid (LNA; 18:3ω3). These two PUFA are essential and must be provided in the diet. The main product of ω6 desaturation and elongation is arachidonic acid (AA; 20:4ω6).

Once PUFA are endogenously formed via desaturation and elongation, they are incorporated into different lipid fractions on glycerol molecules especially in
Fig. 3.1: Biosynthesis of polyunsaturated fatty acids by desaturation and elongation.

position 2 (Mayes, 1990a). Higher desaturase activity potentially increases the amount of long chain PUFA in membranes and consequently could increase the fluidity of cell membranes. The uptake of extracellular substances such as glucose, and hormones like estrogens and insulin would be enhanced with a more fluid membrane matrix. Lack of unsaturated fatty acids in the membrane results in increased membrane viscosity, consequently affecting the transport function of membranes, enzymatic functions of transmembrane proteins, and regulation of surface receptors such as the insulin receptors (Mead et al., 1986; Borkman et al., 1993). Ginsberg et al. (1987) demonstrated that increasing content of PUFA within cell membranes in cultured cells increases membrane fluidity, the number of insulin receptors, and the action of insulin.

Arachidonic acid (AA), either from dietary sources or endogenous synthesis, is the most abundant and important long-chain PUFA in tissue lipids (Dillon,
1987) and is a major fatty acid in membrane phospholipids. It serves as the precursor for both TXA2 and PGI2 (Mayes, 1990a). AA is released from platelet membrane phospholipids (PL) by phospholipase A2 and converted into TXA2 by cyclooxygenase, endoperoxidase and thromboxane synthase (Longenecker, 1985; Mayes, 1990b). The balance between TXA2, a strong aggregator produced by platelets, and prostacyclin (PGI2), an equally potent antiaggregator synthesized by vascular endothelial cells, exerts local control over platelet aggregation (Dillon, 1987; Notarbartolo et al., 1992). Out of balance, excessive production of thromboxane could promote thrombosis. The content of AA in platelet membrane may play an important role in platelet function.

The Δ6D reaction is the rate-limiting step in the conversion of LA to AA (Brenner, 1981; Dillon, 1987; Mayes, 1990b; Yamazaki et al., 1992). Fasting-induced reduction of Δ6D activity in rats has been shown to be reversed after glucose refeeding due to the secretion of insulin (Brenner et al., 1968). The activities of Δ5D and Δ6D were reported to be partially inhibited by diabetes during normo- and hyperglycemic periods in spontaneously diabetic Wistar Bio-Breeding (BB) rats (Mimouni and Poisson, 1992). Rats that received insulin treatment at a dose of 10 I.U./kg body weight twice a day for two days had restored Δ6D and Δ5D activity confirming the regulatory role of insulin over desaturase function. The changes of Δ6D and Δ5D were not directly associated with glycemic status. Insulin apparently increases desaturase protein synthesis and leads to stimulation of fatty acid desaturation (Brenner, 1990).
Since Ayala et al. (1973) first reported that rat testis Δ6D activity decreased with age, many studies have supported this observation (Peluffo and Brenner, 1974; Horrobin, 1981; Bordoni et al., 1988; Hrelia et al., 1989; Bourre et al., 1990; Ulmann et al., 1991a; Manlongui et al., 1993). However, almost all of these studies were conducted using males only or mixed gender populations. Different responses of Δ6D activity were noted between male and female rats when they were exposed to warm and cold temperatures (Gonzalez et al., 1983). An effect of estrogen on Δ6D activity was proposed by the researchers (Gonzalez et al., 1983).

The SHHF/Mcc-faCP (HF) rats have the corpulent (faCP) gene which is recessive. The HF rats that are homozygous for the corpulent gene (faCP/faCP) are obese and exhibit certain characteristics: hypertension, hyperinsulinemia, glucose intolerance, hyperlipidemia, and eventually die of congestive heart failure (CHF) (McCune et al., 1990; Hoversland, 1992). The obese rats demonstrate a type II diabetes with marked sex differences. The obese males exhibit hyperglycemia with overt diabetic symptoms of glycosuria, polyuria, and polydipsia, whereas the obese females demonstrate an impaired glucose tolerance with mild or no overt symptoms of diabetes as seen in obese males. Obese HF rats exhibit anomalies similar to Syndrome X: obesity, NIDDM, hyperlipidemia, and hypertension (DeFronzo and Ferrannini, 1991). It is a model to study impact of obesity and NIDDM on physiological metabolism.

We used lean and obese SHHF/Mcc-faCP female rats from different ages to study the effects of obesity and age on fatty acid desaturation and the metabolic function of platelets. The following hypotheses were tested in this study:
(1) Old and obese rats would have greater Δ6D and Δ5D activities. (2) Product fatty acids of lipid fractions from liver, serum, and platelets would be positively correlated with changes of desaturase activities. (3) Platelet aggregation would be greater and clotting would commence in less time in obese female rats. Age would exacerbate the genotype effect on platelet aggregation. (4) There would be positive correlations between measures of platelet aggregation and Δ6D and Δ5D activities and AA content in different tissue PL.

MATERIAL AND METHODS

Animals. Three to six obese or lean SHHF/Mcc-faCP female rats were selected based on three age groups, including 6 mo, 9 mo, and older than 12 mo of age (lean rats: 19-21 mo; obese rats: two 12 and one 17 mo). Rats at 6 mo were selected because they were still cycling, whereas, 9 mo rats were assumed to be a mixture of cyclic and acyclic females. Due to the strain characteristics which show that these obese female rats die of heart failure at an earlier age (14 - 18 mo) than lean female rats (~ 24 mo), it was not possible to choose acyclic, pre-failed lean and obese female rats in the same age range. (McCune et al., 1995) Thus, ranges of ages were included in the acyclic group, which is referred to as older than 12 mo group. Animals were housed three to a group in plastic cages with stainless steel covers in a well-ventilated room, equipped with a 12-hr light-dark cycle. A commercially prepared diet (protein, 22.5%; fat, 5.5%; linoleic acid, 1.5%; fiber, 4.6%; ash, 5.5%; NFE, 52.0%) (Agway PROLAB Rat/Mouse/Hamster 3000, Purina Mills, Inc., New Albany, IN) was fed to all rats. Body weight and tail blood pressure were measured before
sacrifice. Phenobarbital was used for anesthesia and blood was collected directly from the heart using syringes. Six mL blood was transferred into two tubes containing 3.8% sodium citrate solution. Approximately 2-3 mL of blood was collected in a tube to prepare serum. One gm of liver was immediately excised for the preparation of microsomes. Half of the heart, at least 3 gm of liver, and 1 gm of retroperitoneal fat were collected and stored in a -70 °C freezer for subsequent lipid extraction.

**Whole blood aggregation.** Platelet aggregation was measured using the Chronolog Model 500 Whole Blood Lumi Aggregometer (Chronolog Corp. Havertown, PA). Platelets were counted from one half mL of blood using a platelet counter (Coulter S-Plus with Diff, Coulter Electronics, Hialeah, FL). Counts were done by Dr. Judith Radin in the Department of Veterinary Bioscience, The Ohio State University. The remaining whole blood was used for platelet aggregation analysis and harvest of platelets for lipid extraction. Platelet aggregation was performed by Dr. Sonhee Park in the Department of Food Science and Technology, The Ohio State University, as described by Kwon et al. (1991) (Appendix A). For aggregation test, 0.55 mL of 0.9% freshly prepared saline solution was added to 0.45 mL of citrated blood in a plastic disposable cuvette (Chronolog Corp., Havertown, PA) that contained a siliconized stir bar. Prepared blood samples were warmed to 37 °C in incubator wells to achieve temperature equilibrium. Before the addition of collagen (Chronolog Corp., Havertown, PA), cuvettes containing blood samples were placed into a heater block and stirred at 1000 rpm. After a stable baseline was achieved, 2 to 8 μL of collagen (stock concentration 1 mg/mL) was
added depending on the degree of aggregation. Impedance was recorded for 6 min. The unit of measure for aggregation was expressed as the increase in impedance measured between two electrodes on which platelet aggregation occurred in the whole blood. The change in impedance was compared to that of a 20 Ohms internal standard. Centimeters of deflection from baseline were converted to Ohms for final expression of data. The procedure was completed within 2 to 3 hours after blood was drawn. The blood samples were kept at room temperature until the aggregation measurement was finished. The remaining blood was used for preparation of platelet fatty acids.

**Lipid extraction from serum:** Blood was allowed to stand for 30 to 40 min at room temperature, and centrifuged at 400 x g in a Beckman J2-21 centrifuge (Beckman, Palo Alto, CA) for 20 min at room temperature. One mL of the serum was transferred into a 15 mm x 200 mm culture tube, in duplicate, with a Teflon™-coated screw cap for lipid extraction according to Folch et al. (1957) (Appendix B). A mixture of 10 mL of chloroform/methanol (2:1, v/v) was added to 1 mL of fresh serum in a culture tube, which was then tightly capped with a Teflon™-coated cap and vortexed for 1 min. The protein fraction was removed by paper filtration. The same procedure was repeated using 5 mL of the C: M mixture. After 3 to 4 mL saline solution (0.9 %) was added and the tube shaken, the extract was centrifuged at 2000 rpm for 10 min at room temperature. The upper layer containing water soluble material in water-methanol-salt solution was discarded. One mL of chloroform/methanol/ saline (3:47:48, v/v/v) was added to wash the surface of the chloroform layer, and then discarded. The
lower layer containing PL in chloroform was then transferred to a vial with a Teflon™-coated screw cap, flushed with N₂ gas and stored at -20 °C.

**Preparation of platelets:** Platelets were harvested as described by Kwon et al. (1991). Remaining citrated blood was centrifuged at 200 x g for 10 min at 4°C (Appendix C). The supernatant (platelet-rich-plasma) was recentrifuged at 5000 x g for 10 min at 4 °C. Five mL of ice-cold Tris-HCl (154 mM, pH 7.5)/NaCl (0.9%) /EDTA (77 mM) buffer was used to wash platelets after the supernatant containing platelet-poor-plasma was discarded. Following centrifugation at 1000 x g for 10 min at 4 °C, platelet pellets were resuspended in 1 mL of saline solution. Lipids were extracted using the same procedure for serum (Folch et al., 1957).

**Lipid extraction from heart, liver and adipose tissue:** Lipids from heart, liver, and adipose tissue were extracted according to the procedures suggested by Christie (1982) (Appendix D). Approximately 0.5 gm of heart, 1 gm of liver, or 0.25 gm of retroperitoneal fat was homogenized using a Polytron homogenizer (Kinematica, Switzerland). Tissues were homogenized for 1 min with 10 mL of methanol. After adding 20 mL of chloroform, the homogenizing process was continued for 2 min. The mixture was then filtered and the solid residue resuspended in 30 mL of chloroform-methanol (2:1, v/v) solution and homogenized for 3 min. After filtering, the residue was washed with 20 mL of chloroform and then with 10 mL of methanol. The total volume of the filtrates was measured. One quarter of the filtrate volume of 0.88% potassium chloride in water was added. The mixture was thoroughly shaken and allowed to settle.
The upper layer was removed by aspiration, followed by the addition of water-methanol (1:1, v/v) solution (one quarter of the volume of the lower layer). The upper layer was removed and the lower layer containing lipids was then collected and stored at -20 °C for further procedures.

**Thin-layer chromatography separation:** Silica gel G Thin-Layer Chromatography (TLC) plates (1000 microns) (Analtech, Newark, Delaware) were used to separate different lipid fractions including phospholipids (PL), free fatty acids (FFA), triglycerides (TG), and cholesteryl esters (CE) according to the procedure suggested by Christie (1982) (Appendix E). Plates were scored to make 1/2 inch wide tracts and reactivated in a 100 °C oven for 2 hours. Fresh developing solvent (n-hexane : diethyl ether : glacial acetic acid = 80 : 20 : 1, v/v/v) was prepared in a TLC developing chamber and allowed to equilibrate for at least 1 hour until saturation was achieved. Extracts of lipid were obtained by evaporating the chloroform using a flash-evaporator (Buchler, Fort Lee, NJ). Spots of lipids were applied to the cooled TLC plates. The TLC plates were placed into the saturated chamber for developing, and then dried in the hood after development was completed. Visualization of lipid spots were performed according to Nakamura et al. (1994). Spots containing phospholipids, free fatty acids, triglycerides, or cholesteryl esters were visualized by spraying each plate with 2',7'-dichlorofluorescein (2% in ethanol). Each spot was scraped off and collected in culture tubes, which were then flushed with N₂ and stored at -20 °C for further procedures.
**Methylation of fatty acids:** This was carried out based on the method of Morrison and Smith (1964) except that borontrichloride methanol was used instead of borontrifluoride methanol as the methylation reagent. Two mL of borontrichloride methanol reagent (Sigma, St Louis, MO) was added to each tube containing silica gel and the lipid fraction (Appendix F). Fifty mL of heptadecanoic acid (C17:0) (Sigma, St Louis, MO) was added as an internal standard. The culture tubes were then placed in a hot water bath (over 50 °C, usually 90 °C) for a 30 min period with periodic vortexing. After cooling the culture tubes to room temperature, 5 mL of deionized-distilled water and 1.25 mL of hexane containing 0.05 % of BHT as an antioxidant were added. Following shaking in an electric shaker for 3 min, the culture tubes were centrifuged at 2000 rpm for 10 min. The top layer with fatty acid methyl esters (FAME) was then transferred to a clean culture tube and a small amount of sodium sulfate was added to absorb the residual water. The FAME were transferred to a 4 mL glass vial with a Teflon-coated screw cap, filled with N₂ gas, wrapped with parafilm and stored at -20 °C until gas chromatography analysis.

**Gas liquid chromatography analysis:** The FAME were analyzed using gas chromatograph (GC) (Hewlett Packard, 5890A, Series II) using a capillary column (J&W Scientific, DB-23, 30 m x 0.53 mm I.D. x 0.5 microns, Varian, San Fernando, CA) and a flame ionization detector. Injector temperature was 220 °C and detector temperature was controlled at 230 °C. The temperature program began at 125 °C, increased 10 °C per min, held at 175 °C for 5 min, increased at the rate of 5 °C per min, and held at 210 °C for 4 min. A multiple
fatty acid standard and single fatty acid standards (Matreya, Inc. Pleasant Gap, PA) were used to determine peaks for each fatty acid. Fatty acid distribution (wt %) was calculated by formula in Appendix G.

**Preparation of microsomes.** All procedures were carried out under ice-cold conditions as described by Nakamura et al. (1994) (Appendix H). About 1 gm of freshly cut liver was rinsed in 0.9% saline solution and minced on a piece of glass on the top of ice. It was then transferred and homogenized in a Tetlon™-glass homogenizer containing 12 mL ice-cold homogenizing buffer (Appendix H). After thorough homogenization, the homogenate was transferred to two 10 mL plastic ultracentrifuge tubes (Beckman Instruments, Inc., Irvine, CA). Tubes were balanced with homogenizing buffer and centrifuged in the ultracentrifuge (Beckman Instruments, Inc., Irvine, CA) at 9,000 rpm (12,000 x g) at 4 °C for 40 min. The supernatant was then transferred to a 10 mL plastic ultracentrifuge tube and the pellet containing debris and mitochondrial fraction was discarded. After balancing tubes with homogenizing buffer, the supernatant was centrifuged in the Beckman L7 Ultracentrifuge (Beckman Instruments, Inc., Irvine, CA) at 35,000 rpm (150,000 x g) at 4 °C for 120 min. The supernatant was discarded and the pellet containing microsomes was then resuspended in 3 mL of the incubation buffer (Appendix H). The microsomes were divided into 3 vials, flushed with N₂ gas and stored at -70 °C for microsomal protein quantification and Δ6D and Δ5D activity determination.

**Quantification of microsomal proteins:** The amount of microsomal proteins was determined using the Folin's method (Appendix I). Briefly, bovine
serum albumin (BSA) was used as the standard. After labelling all test tubes including blank, standards, and microsomal samples in duplicate, five different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL) of BSA were prepared with DDW. One part of microsomal suspension was diluted with 49 parts of deionized distilled water (DDW). Five mL of alkaline copper reagent (0.01% CuSO4.5 H2O, 0.02% potassium tartrate, 2% Na2CO3, 0.1N NaOH) was added to each tube, which was then vortexed and settled at room temperature for 15 min. Following the addition of 0.5 mL of Folin-phenol reagent, each tube was vortexed immediately and settled at room temperature for 30 min. The optical density of each tube was read in a Spectronic 301 spectrophotometer (Milton Roy, Inc., Rochester, NY) at 500 nm. Concentrations of microsomal proteins were calculated based on the standard curve plotted from the BSA standards (Appendix I).

Δ6 and Δ5 desaturase activity: Based on the procedure of Nakamura et al. (1994), the activity of Δ6 and Δ5 desaturase was estimated by measuring the microsomal desaturation of 1-14C-labeled linoleic acid (18:2ω6) and 1-14C-labeled dihomo-γ-linolenic acid (20:3ω6) (New England Nuclear, Poston, MA; 53 mCi/mmol in ethanol) (Appendix J).

The incubation buffer (Appendix H) was prepared in advance and the cofactor buffer (Appendix J) was prepared fresh daily. The substrate suspension was also prepared on the day of assay, including 1-14C-labeled linoleic acid (1.9 nmol, or 0.1 μCi per assay) for Δ6 and 1-14C-labeled dihomo-γ-linolenic acid for Δ5 desaturase, 50 nmol of unlabeled substrate (10 nmol/mL
dissolved in propylene glycol) and 100 mL incubation buffer for one assay. The substrate suspension provided 33 mM of substrate in the final volume of a 1.5 mL reaction mixture. An incubation mixture contained 100 mL of substrate suspension, 400 mL of microsome suspension for Δ6 desaturase and 200 mL for Δ5 desaturase, and 1 mL cofactor buffer. Each sample was incubated for both 15 min and 30 min (Δ6 desaturase) or 10 min (Δ5 desaturase) in the water bath at 37 °C. A blank without microsomal protein was incubated simultaneously. The incubation reaction was stopped by adding 2 mL of saponifying reagent (2N KOH in methanol).

After the incubation, 50 mL BHT (2% in methanol) was added to the mixture, which was then flushed with N2 gas, and saponified at 75 °C in a water bath for 1 hour. The mixture was acidified with 1 mL of 6N HCl after cooling. Two mL of petroleum ether was used to extract fatty acids. After the petroleum ether was evaporated under N2 gas, fatty acids were methylated with 3 mL of borontrichloride methanol in a water bath at 80 - 90 °C for 30 min. Methylated fatty acids were then extracted by adding 5mL water and 1.25 mL hexane.

The fatty acid methyl esters were separated on 5 % silver nitrate impregnated TLC plates. For the preparation of TLC plates, five thoroughly cleaned glass plates were placed on a plate guide (Appendix K). In a dark room, 2.25 gm of silver nitrate and 45 gm of silica gel G were gently mixed with 150 mL of deionized water. The mixture was then poured into a spreader and spread smoothly and quickly (within 5 sec) on glass plates. After leaving plates in the dark room for one and half hours, the plates were dried in an oven at 110 °C for 2 hours and stored in a dessicator.
The developing solvent, containing 60 mL of petroleum ether and 40 mL diethyl ether for Δ6D or 30 mL of petroleum ether and 70 mL of diethyl ether for Δ5D, was prepared in a chamber one hour before applying fatty acid methyl esters on the TLC plates. Before evaporating the hexane, 50 mL (1 mg/mL) of γ-linolenyl methyl ester was added to all samples and 50 mL (1 mg/mL) was added to blank tubes for visualization. After hexane was evaporated under N₂ gas, the fatty acid methyl esters were dissolved in 400 mL petroleum ether and transferred to TLC plates. The whole procedure was done under dim light conditions. The plates were then sprayed with 2',7'-dichlorofluorescein (0.2% in ethanol) and fatty acid methyl esters were visualized under UV-light. Each spot including a certain amount of Silica gel G was scraped from the plate and transferred to a scintillation vial. Scintillation cocktail was added and the radioactivity counted using a scintillation counter (Beckman Instruments, Inc., Irvine, CA). The activity was expressed in two ways based on the calculation of percent conversion of substrate to products (Appendix I). The average desaturase enzyme activity was defined as pmol of products per min per gm of microsomal proteins. The total desaturase enzyme activity was express as nmol of products per min per total liver microsomal proteins.

Statistical analysis. This study was a 2 x 3 factorial design with genotype (lean and obese) and age (6 mo, 9 mo and older than 12 mo) as independent variables. Fatty acid compositions of serum, platelet, heart, liver, and adipose tissue, platelet aggregation, and the activity of Δ6 desaturase and Δ5
desaturase were dependent variables. Results were analyzed by Analysis of Variance (ANOVA), using the GLM procedure of the SAS software package (SAS Institute, Inc., 1988). Differences between means were assessed by the least significant differences (LSD) test. ANOVA-derived probability levels were provided for main effects of genotype (lean vs obese) and age (6 mo, 9 mo, and older than 12 mo) as well as Genotype x Age interactions. Correlation coefficients were calculated using the PROC CORR procedure in SAS (SAS Institute, Inc., 1988). Regression equations for platelet aggregation and platelet counts were done with Microsoft Excel version 5.0 software package (Microsoft Corporation, 1994). All significant differences and effects were declared when \( p < 0.05 \).

RESULTS

Three to six female HF rats were used for each age/genotype group. All rats were fed with the same commercial food throughout the study period. Fatty acid composition of the diet (Agway PROLAB Rat/Mouse/Hamster 3000) is shown in Table 3.1. Small amounts of AA and \( \omega 3 \) fatty acids were detected because the food was formulated with animal fat and fish meal.

In Tables 3.2 to 3.12, ANOVA-derived probability levels are provided for main effects of genotype (lean vs obese) and age (6 mo, 9 mo, and older than 12 mo) as well as Genotype x Age interactions. Footnotes in these tables indicate the significant interaction of genotype and age. Body weight increased with age in both genotypes (\( p < 0.05 \)) (Table 3.2). Overall, obese rats were significantly heavier than lean rats (\( p < 0.05 \)). Since liver microsomes are the
major site of lipid desaturation, total liver weight may be related to the amount of lipids desaturated. Obese rats had greater liver weight than lean rats (p<.05). No significant difference was found in the average amount of microsomal proteins in one gram of liver between genotypes. However, obese rats had greater total liver microsomal proteins than lean rats in all age groups (p<.05). Age effect on total liver microsomal proteins was not significant (p>.05). All rats were hypertensive since it is the characteristic of this strain. No differences of blood pressure were found between age or genotype groups. Obese rats had greater (p<.05) serum glucose levels than lean rats in all age groups suggesting poorer glucose tolerance in the obese rats. Overall, obese rats had a greater amount of platelets than lean rats (p<.05). The age effect was also significant, 6 mo-old rats had greater amount of platelets than 9 mo and rats older than 12 mo in both genotypes (p<.05).

$\Delta 6D$ activity was measured using $^{14}C$-labeled linoleic acid and expressed in two ways. The average $\Delta 6D$ activity was expressed as pmol of product fatty acids synthesized per minute by desaturase enzymes per one mg of liver microsomal proteins (Fig. 3.2). The total $\Delta 6D$ activity was nmol of product fatty acids synthesized per minute by the desaturase enzyme per total liver microsomal proteins (Fig. 3.3). There was a significant genotype effect for average (Fig. 3.2) and total $\Delta 6D$ activity (Fig. 3.3) (p<.05). Overall, obese rats had greater $\Delta 6D$ activity than lean rats (p<.05), whether expressed on a per mg microsomal protein basis, or on total microsomal proteins basis. Age did not have a significant effect on average and total $\Delta 6D$ activity regardless of the methods of expression (p>.05). However, there was a significant age and
Obese 9 mo-old rats had greater Δ6D activity than lean 9 mo-old rats (Fig. 3.3).

Average Δ5 desaturase activity (Fig. 3.4) did not differ significantly between lean and obese rats (p>.05). However, the activity was consistently reduced with age in both genotypes. Six mo-old rats had significantly greater average Δ5D activity than rats older than 12 mo in both lean and obese rats (p<.05). When total liver microsomal proteins were considered, both genotype and age effects were significant for total Δ5D activity (p<.05) (Fig. 3.5). Overall, obese rats had greater Δ5D activity than lean rats (p<.05). The activity also decreased with age with 6 mo and older than 12 mo different significantly (p<.05).

Tables 3.3 through 3.5 indicate the substrate (18:2ω6 and 18:3ω3) and product (18:3ω6, 20:3ω6, 20:4ω6, 20:5ω3, and 22:6ω3) fatty acids of desaturase enzymes in liver phospholipid (PL), triglyceride (TG), and cholesterol ester (CE), respectively. The liver is the major site of lipid desaturation. In PL and TG, there was a significant genotype effect on the substrate fatty acid, 18:2ω6. Obese rats had lower 18:2ω6 than lean rats, but the ω3 substrate fatty acid 18:3ω3 was not significantly different between lean and obese (p<.05). Of the substrate fatty acids, 18:2ω6 in liver TG and CE differ significantly between 6 mo and 9 mo in both lean and obese rats. However, only 18:3ω3 from liver CE had significant age effect, 6 mo-old rats had greater 18:3ω3 than 9 mo and older than 12 mo rats. There was a genotype and age interaction for 18:2ω6 in liver TG, lean 9 mo and older than 12 mo rats had greater 18:2ω6 than obese rats in
the same age. In all three lipid fractions, the major desaturase products
20:4ω6 (AA) and 20:5ω3 did not differ significantly with age or genotype (p>.05).
Only 20:5ω3 in liver TG had significant genotype and age interaction. Lean 9
mo-old rats had greater 20:5ω3 than obese 9 mo-old rats. There was a
significant genotype effect with obese rats having more 18:3ω6 (direct product
of Δ6D) from liver PL and CE. Overall, lean rats had greater 22:6ω3 from liver
TG than obese rats.

Fatty acid compositions of serum PL, TG, and CE are shown in Tables 3.6 to
3.8, respectively. In CE, there was a significant genotype effect on the
substrate fatty acid, 18:2ω6. Obese rats had less 18:2ω6 than leans rats
overall. Nine mo old rats had significantly greater 18:2ω6 in serum PL and TG
than 6 mo and older than 12 mo old rats in both genotypes (p<.05). Of the
product fatty acids, 18:3ω6 from serum CE had genotype effect with obese rats
greater than lean rats in all age groups. Also 18:3ω6 from TG had significant
age effect and genotype x age interaction. Overall, rats older than 12 mo had
greater 18:3ω6 (p<.05). Obese rats older than 12 mo also had significantly
greater 18:3ω6 than other rats in different age groups. There were significant
genotype and age effects for AA from serum PL, TG, and CE (p<.05).
Throughout all age groups, obese rats had greater AA from serum PL and CE,
but lower in TG, compared to lean rats. Regardless of genotype, 9 mo-old rats
had greater AA than 6 mo-old rats in all serum lipid fractions. Significant
genotype effect and genotype x age interaction for 20:5ω3 and 22:6ω3 was
noted in serum PL. Obese rats had greater 20:5ω3 and 22:6ω3 than lean rats overall. Obese 9 mo-old rats had significantly greater 20:5ω3 than lean 9 mo-old and rats older than 12 mo (p<.05), but obese older than 12 mo rats had the highest 22:6ω3 and lean 6 mo the lowest. For both genotypes, rats older than 12 mo had significantly higher 22:6ω3 in serum PL than 6 mo- and 9 mo-old rats. In serum TG, obese rats had less 22:6ω3 than lean rats.

Tables 3.9 to 3.11 show the fatty acid compositions of platelet PL, TG, and CE, respectively. There were a significant genotype effect and a genotype x age interaction for 18:2ω6 in platelet TG with obese greater than lean rats overall (p<.05). Obese 9 mo-old rats had greater 18:2ω6 than lean 9 mo-old rats. Only AA in platelet PL had significant genotype and age effects and genotype x age interaction (p<.05). Throughout all age groups, obese rats had greater AA than lean rats. Six mo-old rats had significantly lower AA than the other two age groups for both genotypes. Obese 9 mo-old rats had higher AA than all lean rats and obese 6 mo-old rats (p<.05). Of the ω3 substrate and products, no significant differences were noted in all lipid fractions.

Polyunsaturated fatty acid/saturated fatty acid (P/S) ratios calculated from tissue PL were indicated on Table 3.12. There were no significant differences in platelet and liver PL.

Table 3.13 shows the correlation coefficients (r) between desaturase activity and product fatty acids of desaturase enzymes (18:3ω6, 20:4ω6, and 20:5ω3) from liver, serum, and platelet PL. AA and 18:3ω6 in liver PL were positively correlated with Δ6D activity; specifically, 18:3ω6 correlated with average Δ6D
activity (p<.05), and 20:4ω6 with total Δ6D activity (p<.05). However, these
products did not significantly correlate with Δ5D activity (p>.05). Both 18:3ω6
and 20:4ω6 in serum PL also had positive correlations with Δ6D activity.
Similar to liver PL, these three fatty acids did not correlate with Δ5D activity
significantly (p>.05). In platelet PL, only AA had a significant positive correlation
with Δ6D (p<.05). However, it had a negative correlation with average Δ5D
activity (p<.05). In liver, serum, and platelet PL, 20:5ω3 did not correlate with
Δ6D or Δ5D activity (p>.05).

Platelet aggregation did not differ significantly among groups. However,
lean rats tended to show an increase with age (Fig. 3.6). When platelet counts
were considered, a significant positive correlation between aggregation and
platelet count was found for obese rats (r=.71, p<.032), but a correlation was not
found for lean rats (r=-.22, p=.513) (Fig. 3.5 and 3.6, respectively). Lag time
represents the interval between the time collagen was added and the time
aggregation began. No significant differences were observed in any group (Fig.
3.7).

Table 3.14 indicates correlation coefficients between measures of platelet
aggregation and desaturase activities, AA (wt %) from liver, serum, and platelet
PL. Neither platelet aggregation nor lag time correlated with desaturase
activities (p>.05). Platelet aggregation did not correlate with liver AA. A non-
significant correlation was found with AA from serum and platelet PL (p>.05).
Lag time did not correlate with AA contents in all tissue PL, but a non-significant
negative correlation was found with both Δ6D and Δ5D.
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>wt % of fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>1.44</td>
</tr>
<tr>
<td>16:0</td>
<td>15.28</td>
</tr>
<tr>
<td>18:0</td>
<td>4.05</td>
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<tr>
<td><strong>Monounsaturated</strong></td>
<td></td>
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<tr>
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<td>3.36</td>
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<tr>
<td>18:1ω9</td>
<td>22.25</td>
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<tr>
<td><strong>Polyunsaturated (ω6)</strong></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
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<tr>
<td>18:3ω6</td>
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</tr>
<tr>
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<tr>
<td>20:4ω6</td>
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<tr>
<td><strong>Polyunsaturated (ω3)</strong></td>
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<tr>
<td>20:5ω3</td>
<td>0.11</td>
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<tr>
<td>22:6ω3</td>
<td>2.16</td>
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Table 3.1: Fatty acid composition of commercial diet (Agway PROLAB Rat/Mouse/Hamster 3000, Purina Mills, Inc., New Albany, IN) for SHHF/Mcc-faCP rats.
<table>
<thead>
<tr>
<th></th>
<th>Genotype</th>
<th>6mo</th>
<th>9mo</th>
<th>≥12mo</th>
<th>ANOVA</th>
<th>Genotype</th>
<th>Age</th>
<th>GenotypexAge</th>
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</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td>LEAN</td>
<td>220.17±5.82</td>
<td>241.20±4.32</td>
<td>266.00±14.10</td>
<td>0.0001</td>
<td>0.0405</td>
<td>0.6409</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>466.00±8.79</td>
<td>526.40±25.64</td>
<td>545.33±80.34</td>
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<tr>
<td><strong>Liver weight</strong></td>
<td>LEAN</td>
<td>8.59±0.22</td>
<td>9.11±0.20</td>
<td>10.22±0.82</td>
<td>0.0001</td>
<td>0.7071</td>
<td>0.2371</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>20.78±0.79</td>
<td>21.83±1.16</td>
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</tr>
<tr>
<td><strong>Microsomal protein</strong></td>
<td>LEAN</td>
<td>15.18±1.59</td>
<td>16.49±3.56</td>
<td>17.30±1.85</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>9.31±0.64</td>
<td>13.86±2.13</td>
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<tr>
<td><strong>Total Microsomal protein (mg)</strong></td>
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<td>149.67±32.59</td>
<td>175.73±21.48</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>194.81±19.07</td>
<td>301.08±44.71</td>
<td>335.10±117.69</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Blood Pressure</strong></td>
<td>LEAN</td>
<td>180.20±2.42</td>
<td>163.33±7.42</td>
<td>172.50±4.57</td>
<td>0.5072</td>
<td>0.2801</td>
<td>0.1454</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>175.67±2.96</td>
<td>175.00±8.50</td>
<td>169.25±3.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>LEAN</td>
<td>111.00±2.53</td>
<td>87.67±0.33</td>
<td>87.50±14.76</td>
<td>0.0009</td>
<td>0.0656</td>
<td>0.9195</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>156.33±7.53</td>
<td>130.60±12.97</td>
<td>123.00±23.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Platelet count</strong></td>
<td>LEAN</td>
<td>542.50±29.60</td>
<td>374.25±51.04</td>
<td>458.67±44.57</td>
<td>0.0002</td>
<td>0.0386</td>
<td>0.0914</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>649.00±20.72</td>
<td>632.67±28.81</td>
<td>560.00±23.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥12 mo (12 mo & 17 mo), n=3.
2 Lean rats: 6 mo, n=5; 9 mo, n=3; ≥12 mo (19 mo - 21 mo), n=4. Obese rats: 6 mo, n=3; 9 mo, n=3; ≥12 mo (12 mo & 17 mo), n=0.
3 Lean rats: 6 mo, n=4; 9 mo, n=4; ≥12 mo (19 mo - 21 mo), n=3. Obese rats: 6 mo, n=4; 9 mo, n=3; ≥12 mo (12 mo & 17 mo), n=2.
4 SEM = Standard Error of Mean.

Table 3.2: Description of SHHF/Mcc-facP female rats. (Means ± SEM)
Genotype p*=0.0265
Age p=0.9849
Genotype x Age
p=0.2357

1. Lean rats: 6 mo, n=5; 9 mo, n=5; ≥12 mo (19 - 21 mo), n=5.
Obese rats: 6 mo, n=5; 9 mo, n=4; ≥12 mo (12 & 17 mo), n=3.
2. SEM = Standard Error of Mean.

Fig. 3.2: Average Δ6 desaturase activity in
SHHF/Mcc-fa Opo female rats
(Mean ± SEM²).
1. Lean rats: 6 mo, n=5; 9 mo, n=5; ≥12 mo (19 - 21 mo), n=5.
Obese rats: 6 mo, n=5; 9 mo, n=4; ≥12 mo (12 & 17 mo), n=3.
2. SEM = Standard Error of Mean.

Fig. 3.3: Total Δ6 desaturase activity (adjusted by total liver microsomal proteins) in SHHF/Mcc-fa_CP female rats¹ (Mean ± SEM²).
Fig. 3.4: Average Δ5 desaturase activity in SHHF/Mcc-fa<sup>cp</sup> female rats<sup>1</sup> (Mean ± SEM<sup>2</sup>).

1. Lean rats: 6 mo, n=3; 9 mo, n=4; ≥12 mo (19-21 mo), n=4.
   Obese rats: 6 mo, n=3; 9 mo, n=4; ≥12 mo (12 & 17 mo), n=3.
2. SEM = Standard Error of Mean.
Fig. 3.5: Total Δ5 desaturase activity (adjusted by total liver microsomal proteins) in SHHF/Mcc- fa cp female rats (Mean ± SEM²).
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥ 12 mo</th>
<th>ANOVA probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyunsaturated (ω6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>11.80 ± 0.76</td>
<td>11.56 ± 1.07</td>
<td>12.81 ± 0.91</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>9.80 ± 0.87</td>
<td>8.64 ± 1.02</td>
<td>8.97 ± 0.49</td>
<td>0.6501</td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>0.26 ± 0.12</td>
<td>0.13 ± 0.08</td>
<td>0.06 ± 0.08</td>
<td>0.6342</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.16 ± 0.05</td>
<td>0.40 ± 0.08</td>
<td>0.48 ± 0.30</td>
<td>0.0772</td>
</tr>
<tr>
<td>20:3ω6</td>
<td>LEAN</td>
<td>0.36 ± 0.08</td>
<td>0.27 ± 0.11</td>
<td>0.17 ± 0.06</td>
<td>0.4660</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.63 ± 0.23</td>
<td>0.17 ± 0.17</td>
<td>0.33 ± 0.33</td>
<td>0.4245</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>LEAN</td>
<td>19.79 ± 0.82</td>
<td>21.94 ± 1.71</td>
<td>20.76 ± 3.23</td>
<td>0.4866</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>22.15 ± 1.18</td>
<td>26.82 ± 1.25</td>
<td>21.04 ± 1.59</td>
<td>0.1099</td>
</tr>
<tr>
<td>Polyunsaturated (ω3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>0.11 ± 0.04</td>
<td>0.15 ± 0.09</td>
<td>0.12 ± 0.08</td>
<td>0.6333</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.13 ± 0.07</td>
<td>0.12 ± 0.04</td>
<td>0.20 ± 0.06</td>
<td>0.8377</td>
</tr>
<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>0.64 ± 0.18</td>
<td>0.85 ± 0.32</td>
<td>0.27 ± 0.07</td>
<td>0.3367</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>1.18 ± 0.25</td>
<td>0.84 ± 0.31</td>
<td>1.05 ± 0.48</td>
<td>0.6529</td>
</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>14.15 ± 1.62</td>
<td>12.99 ± 0.62</td>
<td>18.28 ± 2.79</td>
<td>0.3468</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>12.51 ± 1.66</td>
<td>15.63 ± 0.76</td>
<td>16.41 ± 1.42</td>
<td>0.0911</td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.

Table 3.3: Fatty acid composition (wt %) of liver phospholipids from SHHF/Mcc-faCP female rats. (mean ± SEM)
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥12 mo</th>
<th>ANOVA probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Genotype</td>
</tr>
<tr>
<td>Polyunsaturated (ω6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>15.41 ± 2.18 b</td>
<td>25.79 ± 0.65 a</td>
<td>20.88 ± 2.11 a</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>13.58 ± 1.76 b</td>
<td>13.36 ± 1.82 b</td>
<td>14.53 ± 3.20 b</td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>0.20 ± 0.10</td>
<td>0.28 ± 0.11</td>
<td>0.42 ± 0.42</td>
<td>0.2894</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.66 ± 0.66</td>
<td>0.26 ± 0.17</td>
<td>0.10 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>20:3ω6</td>
<td>LEAN</td>
<td>0.21 ± 0.05</td>
<td>0.22 ± 0.04</td>
<td>0.21 ± 0.07</td>
<td>0.6759</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.18 ± 0.06</td>
<td>0.26 ± 0.04</td>
<td>0.25 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>20:4ω6</td>
<td>LEAN</td>
<td>2.08 ± 0.32</td>
<td>3.19 ± 0.28</td>
<td>4.73 ± 2.32</td>
<td>0.0817</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>1.44 ± 0.18</td>
<td>1.63 ± 0.23</td>
<td>2.27 ± 0.58</td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated (ω3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>0.50 ± 0.06</td>
<td>1.06 ± 0.21</td>
<td>0.63 ± 0.20</td>
<td>0.4320</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.44 ± 0.08</td>
<td>0.65 ± 0.15</td>
<td>0.77 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>0.33 ± 0.14 c</td>
<td>0.90 ± 0.13 a</td>
<td>0.80 ± 0.22 a</td>
<td>0.1176</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.63 ± 0.13 abc</td>
<td>0.44 ± 0.08 bc</td>
<td>0.37 ± 0.03 bc</td>
<td></td>
</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>4.20 ± 0.51</td>
<td>5.41 ± 0.70</td>
<td>6.47 ± 0.83</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>2.72 ± 0.37</td>
<td>2.05 ± 0.30</td>
<td>2.08 ± 0.82</td>
<td></td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.
*a,b* For each fatty acid, means followed by different letters had significant age and genotype interaction (p<0.05).

Table 3.4: Fatty acid composition (wt %) of liver triglycerides from SHHF/Mco-facP female rats 1. (mean ± SEM 2)
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥ 12 mo</th>
<th>Genotype</th>
<th>Age</th>
<th>Genotype x Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyunsaturated (ω6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>5.17 ± 1.05</td>
<td>2.70 ± 0.33</td>
<td>3.97 ± 0.84</td>
<td>0.1159</td>
<td>0.0485</td>
<td>0.7563</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>3.46 ± 0.64</td>
<td>2.03 ± 0.12</td>
<td>3.18 ± 1.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>---</td>
<td>0.01 ± 0.01</td>
<td>0.08 ± 0.05</td>
<td>0.0332</td>
<td>0.1334</td>
<td>0.8270</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.10 ± 0.07</td>
<td>0.06 ± 0.03</td>
<td>0.15 ± 0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3ω6</td>
<td>LEAN</td>
<td>0.13 ± 0.06</td>
<td>0.05 ± 0.02</td>
<td>0.38 ± 0.28</td>
<td>0.4781</td>
<td>0.1974</td>
<td>0.8017</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.10 ± 0.05</td>
<td>0.03 ± 0.02</td>
<td>0.20 ± 0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4ω6</td>
<td>LEAN</td>
<td>3.57 ± 1.15</td>
<td>1.67 ± 0.14</td>
<td>3.29 ± 1.24</td>
<td>0.1881</td>
<td>0.5253</td>
<td>0.3818</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>3.13 ± 0.68</td>
<td>4.02 ± 0.51</td>
<td>5.02 ± 2.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated (ω3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>0.17 ± 0.04</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.03</td>
<td>0.3561</td>
<td>0.0132</td>
<td>0.6737</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.12 ± 0.05</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>0.71 ± 0.18</td>
<td>0.23 ± 0.03</td>
<td>0.42 ± 0.15</td>
<td>0.7370</td>
<td>0.2718</td>
<td>0.1722</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.38 ± 0.10</td>
<td>0.41 ± 0.13</td>
<td>0.45 ± 0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>1.90 ± 0.40</td>
<td>1.58 ± 0.59</td>
<td>0.94 ± 0.29</td>
<td>0.4780</td>
<td>0.4302</td>
<td>0.2127</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>1.05 ± 0.08</td>
<td>2.64 ± 0.96</td>
<td>1.81 ± 1.11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.

Table 3.5: Fatty acid composition (wt %) of liver cholesterol ester from SHHF/Mcc-fatP female rats*. (mean ± SEM²)
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥12 mo</th>
<th>Genotype</th>
<th>Age</th>
<th>Genotype x Age</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyunsaturated (ω6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>8.08 ± 1.98</td>
<td>13.83 ± 0.79</td>
<td>7.73 ± 0.44</td>
<td>0.5563</td>
<td>0.0004</td>
<td>0.2965</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>9.29 ± 0.69</td>
<td>11.49 ± 1.41</td>
<td>7.17 ± 1.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>---</td>
<td>0.15 ± 0.15</td>
<td>0.23 ± 0.23</td>
<td>0.3843</td>
<td>0.5843</td>
<td>0.7995</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.23 ± 0.15</td>
<td>0.17 ± 0.12</td>
<td>0.26 ± 0.17</td>
<td>0.0742</td>
<td>0.0844</td>
<td>0.2433</td>
</tr>
<tr>
<td>20:3ω6</td>
<td>LEAN</td>
<td>0.14 ± 0.07</td>
<td>0.19 ± 0.07</td>
<td>0.04 ± 0.04</td>
<td>0.3843</td>
<td>0.5843</td>
<td>0.7995</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.10 ± 0.08</td>
<td>0.47 ± 0.15</td>
<td>0.26 ± 0.17</td>
<td>0.0742</td>
<td>0.0844</td>
<td>0.2433</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>LEAN</td>
<td>10.65 ± 2.57</td>
<td>16.70 ± 1.42</td>
<td>11.63 ± 2.59</td>
<td>0.0001</td>
<td>0.0187</td>
<td>0.0757</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>16.81 ± 2.11</td>
<td>21.80 ± 0.87</td>
<td>25.83 ± 0.78</td>
<td>0.0001</td>
<td>0.0187</td>
<td>0.0757</td>
</tr>
<tr>
<td><strong>Polyunsaturated (ω3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>0.15 ± 0.09</td>
<td>0.04 ± 0.04</td>
<td>0.33 ± 0.25</td>
<td>0.4760</td>
<td>0.4610</td>
<td>0.3084</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.67 ± 0.43</td>
<td>0.21 ± 0.12</td>
<td>0.07 ± 0.04</td>
<td>0.4760</td>
<td>0.4610</td>
<td>0.3084</td>
</tr>
<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>0.80 ± 0.11</td>
<td>0.39 ± 0.08</td>
<td>0.47 ± 0.10</td>
<td>0.0455</td>
<td>0.0501</td>
<td>0.0334</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.87 ± 0.14</td>
<td>1.09 ± 0.19</td>
<td>0.45 ± 0.18</td>
<td>0.0455</td>
<td>0.0501</td>
<td>0.0334</td>
</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>5.84 ± 1.14</td>
<td>8.15 ± 0.60</td>
<td>7.58 ± 1.34</td>
<td>0.0004</td>
<td>0.0156</td>
<td>0.0345</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>9.05 ± 0.30</td>
<td>8.69 ± 0.70</td>
<td>13.38 ± 1.25</td>
<td>0.0004</td>
<td>0.0156</td>
<td>0.0345</td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=4; 9 mo, n=5; ≥12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.
ab For each fatty acid, means followed by different letters had significant age and genotype interaction (p<.05).

Table 3.6: Fatty acid composition (wt %) of serum phospholipids from SHHF/Mcc-facp female rats1. (Means ± SEM 2)
Table 3.7: Fatty acid composition (wt %) of serum triglycerides from SHHF/Mcc-fACP female rats. (Means ± SEM)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥ 12 mo</th>
<th>ANOVA probability level</th>
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<tr>
<td></td>
<td></td>
<td>Genotype</td>
<td>Age</td>
<td>Genotype x Age</td>
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<tr>
<td>Polyunsaturated (ω6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>15.36 ± 1.97</td>
<td>20.48 ± 1.50</td>
<td>15.79 ± 0.70</td>
<td>0.5580 0.0079 0.3590</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>14.89 ± 0.83</td>
<td>18.00 ± 0.99</td>
<td>16.95 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>0.50 ± 0.10 bc</td>
<td>0.62 ± 0.23 bc</td>
<td>0.80 ± 0.22 b</td>
<td>0.2420 0.0027 0.0198</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.62 ± 0.22 bc</td>
<td>0.16 ± 0.10 c</td>
<td>1.87 ± 0.55 a</td>
<td></td>
</tr>
<tr>
<td>20:3ω6</td>
<td>LEAN</td>
<td>0.27 ± 0.09</td>
<td>0.31 ± 0.05</td>
<td>0.23 ± 0.08</td>
<td>0.8817 0.8938 0.6628</td>
</tr>
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<td></td>
<td>OBESE</td>
<td>0.24 ± 0.08</td>
<td>0.27 ± 0.07</td>
<td>0.32 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>20:4ω6</td>
<td>LEAN</td>
<td>2.38 ± 0.71</td>
<td>4.20 ± 0.54</td>
<td>3.51 ± 0.49</td>
<td>0.0028 0.0311 0.1875</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>1.38 ± 0.09</td>
<td>1.77 ± 0.23</td>
<td>2.82 ± 0.67</td>
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<td>Polyunsaturated (ω3)</td>
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</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>0.65 ± 0.10</td>
<td>0.92 ± 0.12</td>
<td>1.30 ± 0.23</td>
<td>0.1229 0.7182 0.0658</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>1.74 ± 0.21</td>
<td>1.29 ± 0.29</td>
<td>1.01 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>1.24 ± 0.38</td>
<td>1.35 ± 0.15</td>
<td>1.04 ± 0.41</td>
<td>0.0557 0.7434 0.7431</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.60 ± 0.17</td>
<td>0.86 ± 0.08</td>
<td>0.82 ± 0.17</td>
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</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>5.32 ± 0.79</td>
<td>5.48 ± 0.25</td>
<td>4.97 ± 0.54</td>
<td>0.0001 0.3593 0.8357</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>3.26 ± 0.30</td>
<td>3.17 ± 0.18</td>
<td>2.29 ± 0.99</td>
<td></td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=4; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=4; ≥ 12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.

For each fatty acid, means followed by different letters had significant age and genotype interaction (p<0.05).
Table 3.8: Fatty acid composition (wt %) of serum cholesterol esters from SHHF/Mcc-lacP female rats (Means ± SEM)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥ 12 mo</th>
<th>Genotype</th>
<th>Age</th>
<th>Genotype x Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyunsaturated (ω6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>6.34 ± 0.84</td>
<td>12.13 ± 1.42</td>
<td>8.97 ± 1.15</td>
<td>0.0001</td>
<td>0.6992</td>
<td>0.0556</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>8.10 ± 0.47</td>
<td>8.45 ± 0.63</td>
<td>7.43 ± 0.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>0.18 ± 0.06</td>
<td>0.52 ± 0.25</td>
<td>0.49 ± 0.15</td>
<td>0.0197</td>
<td>0.1589</td>
<td>0.9438</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.65 ± 0.17</td>
<td>1.15 ± 0.27</td>
<td>1.09 ± 0.26</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>20:3ω6</td>
<td>LEAN</td>
<td>---</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.02</td>
<td>0.1941</td>
<td>0.2863</td>
<td>0.4156</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.04 ± 0.03</td>
<td>0.01 ± 0.01</td>
<td>0.12 ± 0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4ω6</td>
<td>LEAN</td>
<td>27.59 ± 1.57</td>
<td>35.23 ± 2.69</td>
<td>25.89 ± 1.80</td>
<td>0.0001</td>
<td>0.0141</td>
<td>0.5955</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>40.29 ± 2.98</td>
<td>48.85 ± 3.79</td>
<td>40.05 ± 1.65</td>
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<td></td>
</tr>
<tr>
<td>Polyunsaturated (ω3)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>1.02 ± 0.55</td>
<td>0.18 ± 0.09</td>
<td>0.78 ± 0.39</td>
<td>0.4156</td>
<td>0.1944</td>
<td>0.5525</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.75 ± 0.62</td>
<td>0.60 ± 0.21</td>
<td>0.02 ± 0.02</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>1.75 ± 0.68</td>
<td>2.18 ± 0.30</td>
<td>2.95 ± 0.43</td>
<td>0.4239</td>
<td>0.9038</td>
<td>0.3234</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>2.63 ± 0.91</td>
<td>2.99 ± 0.54</td>
<td>2.06 ± 0.66</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>2.39 ± 0.36</td>
<td>2.25 ± 0.18</td>
<td>4.05 ± 1.65</td>
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<td>0.1460</td>
<td>0.6945</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>2.30 ± 0.15</td>
<td>2.97 ± 0.37</td>
<td>4.40 ± 1.10</td>
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<td></td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=4; 9 mo, n=5; ≥12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.
### Table 3.9: Fatty acid composition (wt %) of platelet phospholipids from SHHF/Mcc-αCP female rats

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥ 12 mo</th>
<th>ANOVA probability level</th>
<th>Genotype</th>
<th>Age</th>
<th>Genotype x Age</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyunsaturated (ω6)</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>6.44 ± 1.44</td>
<td>5.51 ± 0.98</td>
<td>5.51 ± 1.20</td>
<td>0.9891</td>
<td>0.6265</td>
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<td>0.7278</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>5.18 ± 1.30</td>
<td>3.98 ± 0.27</td>
<td>3.47 ± 0.69</td>
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<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>0.20 ± 0.12</td>
<td>0.31 ± 0.22</td>
<td>0.61 ± 0.35</td>
<td>0.3510</td>
<td>0.6277</td>
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<td>0.2332</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>0.66 ± 0.24</td>
<td>0.07 ± 0.07</td>
<td>0.09 ± 0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3ω6</td>
<td>LEAN</td>
<td>0.57 ± 0.16</td>
<td>0.09 ± 0.07</td>
<td>0.28 ± 0.22</td>
<td>0.8444</td>
<td>0.4729</td>
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<td>0.4506</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>0.35 ± 0.13</td>
<td>0.17 ± 0.09</td>
<td>0.23 ± 0.12</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>20:4ω6</td>
<td>LEAN</td>
<td>10.43 ± 2.03c</td>
<td>15.35 ± 3.12bc</td>
<td>15.25 ± 1.42bc</td>
<td>0.0004</td>
<td>0.0001</td>
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<td>0.0070</td>
</tr>
<tr>
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<td>OBESE</td>
<td>13.90 ± 2.18a</td>
<td>23.75 ± 1.86a</td>
<td>20.13 ± 0.45ab</td>
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<tr>
<td><strong>Polyunsaturated (ω3)</strong></td>
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<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>4.32 ± 2.41</td>
<td>0.57 ± 0.40</td>
<td>2.08 ± 1.00</td>
<td>0.0908</td>
<td>0.3413</td>
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<td>0.4867</td>
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<tr>
<td></td>
<td>OBESE</td>
<td>1.80 ± 1.07</td>
<td>0.33 ± 0.25</td>
<td>0.02 ± 0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>0.92 ± 0.38</td>
<td>1.94 ± 1.04</td>
<td>0.75 ± 0.16</td>
<td>0.5255</td>
<td>0.4856</td>
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<td>0.8152</td>
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<tr>
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<td>OBESE</td>
<td>0.94 ± 0.32</td>
<td>0.65 ± 0.12</td>
<td>0.49 ± 0.07</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>4.65 ± 1.50</td>
<td>2.48 ± 0.75</td>
<td>4.24 ± 1.89</td>
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<td>0.0910</td>
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<td>0.2670</td>
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<tr>
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<td>OBESE</td>
<td>9.90 ± 3.82</td>
<td>2.52 ± 0.90</td>
<td>3.84 ± 1.31</td>
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</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=4. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.

For each fatty acid, means followed by different letters had significant age and genotype interaction (p<0.05).
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥ 12 mo</th>
<th>ANOVA probability level</th>
<th>Genotype</th>
<th>Age</th>
<th>Genotype x Age</th>
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<tbody>
<tr>
<td>Polyunsaturated (ω6)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>LEAN</td>
<td>2.95 ± 0.95 c</td>
<td>2.11 ± 0.47 c</td>
<td>2.26 ± 0.62 c</td>
<td>0.0001</td>
<td>0.1145</td>
<td>0.0352</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>4.79 ± 1.03 bc</td>
<td>8.16 ± 1.79***</td>
<td>6.58 ± 1.47 bc</td>
<td>0.5055</td>
<td>0.3339</td>
<td>0.7486</td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>LEAN</td>
<td>1.20 ± 0.37</td>
<td>1.30 ± 0.42</td>
<td>1.19 ± 0.69</td>
<td>0.0001</td>
<td>0.1145</td>
<td>0.0352</td>
<td></td>
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<tr>
<td></td>
<td>OBESE</td>
<td>0.67 ± 0.38</td>
<td>0.16 ± 0.14</td>
<td>0.53 ± 0.06</td>
<td>0.5055</td>
<td>0.3339</td>
<td>0.7486</td>
<td></td>
</tr>
<tr>
<td>20:2ω6</td>
<td>LEAN</td>
<td>1.81 ± 0.43</td>
<td>1.91 ± 1.25</td>
<td>1.87 ± 1.45</td>
<td>0.0001</td>
<td>0.1145</td>
<td>0.0352</td>
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</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.09 ± 0.06</td>
<td>0.48 ± 0.29</td>
<td>0.06 ± 0.04</td>
<td>0.5055</td>
<td>0.3339</td>
<td>0.7486</td>
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<tr>
<td>20:3ω6</td>
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<td>1.86 ± 0.66</td>
<td>3.74 ± 1.33</td>
<td>4.00 ± 2.86</td>
<td>0.0001</td>
<td>0.1145</td>
<td>0.0352</td>
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<td>OBESE</td>
<td>1.03 ± 0.11</td>
<td>1.26 ± 0.30</td>
<td>0.78 ± 0.22</td>
<td>0.0001</td>
<td>0.1145</td>
<td>0.0352</td>
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<tr>
<td>Polyunsaturated (ω3)</td>
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<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>LEAN</td>
<td>3.01 ± 1.42</td>
<td>3.84 ± 1.10</td>
<td>1.41 ± 0.62</td>
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<td>0.9864</td>
<td>0.3463</td>
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</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>1.52 ± 0.43</td>
<td>1.68 ± 0.94</td>
<td>2.37 ± 1.62</td>
<td>0.4272</td>
<td>0.9864</td>
<td>0.3463</td>
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</tr>
<tr>
<td>20:5ω3</td>
<td>LEAN</td>
<td>1.43 ± 0.65</td>
<td>1.01 ± 0.98</td>
<td>4.60 ± 1.70</td>
<td>0.3660</td>
<td>0.6272</td>
<td>0.2047</td>
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</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.25 ± 0.19</td>
<td>0.69 ± 0.17</td>
<td>0.69 ± 0.17</td>
<td>0.3660</td>
<td>0.6272</td>
<td>0.2047</td>
<td></td>
</tr>
<tr>
<td>22:6ω3</td>
<td>LEAN</td>
<td>5.44 ± 0.86</td>
<td>3.72 ± 2.45</td>
<td>11.10 ± 2.84</td>
<td>0.1611</td>
<td>0.5345</td>
<td>0.1347</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>3.96 ± 0.57</td>
<td>4.08 ± 1.12</td>
<td>4.38 ± 0.95</td>
<td>0.1611</td>
<td>0.5345</td>
<td>0.1347</td>
<td></td>
</tr>
</tbody>
</table>

1 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=4. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 mo & 17 mo), n=3.
2 SEM = Standard Error of Mean.
3 For each fatty acid, means followed by different letters had significant age and genotype interaction (p<.05).

Table 3.10: Fatty acid composition (wt %) of platelet triglycerides from SHHF/Mcc-lacZP female rats. (mean ± SEM)
Table 3.11: Fatty acid composition (wt %) of platelet cholesterol ester from SHHF/Mcc-faCP female rats (mean ± SEM)
<table>
<thead>
<tr>
<th>P/S ratios</th>
<th>Genotype</th>
<th>6 mo</th>
<th>9 mo</th>
<th>≥ 12 mo</th>
<th>ANOVA probability level</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Genotype</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>LEAN</td>
<td>1.14±0.07</td>
<td>1.24±0.03</td>
<td>1.28±0.10</td>
<td>0.2705</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>1.21±0.08</td>
<td>1.38±0.03</td>
<td>1.29±0.08</td>
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</tr>
<tr>
<td><strong>Platelets</strong></td>
<td>LEAN</td>
<td>0.55±0.03</td>
<td>0.52±0.08</td>
<td>0.61±0.04</td>
<td>0.2093</td>
</tr>
<tr>
<td></td>
<td>OBESE</td>
<td>0.74±0.10</td>
<td>0.66±0.09</td>
<td>0.52±0.01</td>
<td></td>
</tr>
</tbody>
</table>

1 SEM = Standard Error of Mean.
2 P/S ratio = (18:2ω6+18:3ω6+20:3ω6+20:4ω6+18:3ω3+20:5ω3+22:6ω3)/(14:0+16:0+18:0).
3 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=5. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 mo & 17 mo), n=3.
4 Lean rats: 6 mo, n=6; 9 mo, n=5; ≥ 12 mo (19 mo - 21 mo), n=4. Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 mo & 17 mo), n=3.

Table 3.12: Polyunsaturated/Saturated (P/S) ratios of liver and platelet phospholipids from SHHF/Mcc-faCP female rats. (Means ± SEM 1)
<table>
<thead>
<tr>
<th></th>
<th>Δ6D 1</th>
<th>Δ6D 2 total</th>
<th>Δ5D 3</th>
<th>Δ5D 4 total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>.43 *</td>
<td>.26</td>
<td>-.03</td>
<td>.03</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>.37</td>
<td>.49 *</td>
<td>-.24</td>
<td>.09</td>
</tr>
<tr>
<td>20:5ω3</td>
<td>.32</td>
<td>-.02</td>
<td>.24</td>
<td>.16</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>.50 *</td>
<td>.18</td>
<td>-.09</td>
<td>-.19</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>.29</td>
<td>.51 *</td>
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<td>.22</td>
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<tr>
<td>20:5ω3</td>
<td>.08</td>
<td>.03</td>
<td>.18</td>
<td>.31</td>
</tr>
<tr>
<td>Platelet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>-.06</td>
<td>-.12</td>
<td>.25</td>
<td>-.02</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>.40 *</td>
<td>.59 *</td>
<td>-.47 *</td>
<td>-.12</td>
</tr>
<tr>
<td>20:5ω3</td>
<td>-.11</td>
<td>-.16</td>
<td>-.17</td>
<td>-.26</td>
</tr>
</tbody>
</table>

1 Δ6D = average Δ6D activity (pmol/min/mg liver microsomal proteins)
2 Δ6D total = total Δ6D activity (nmol/min/total liver microsomal proteins)
3 Δ5D = average Δ5D activity (pmol/min/mg liver microsomal proteins)
4 Δ5D total = total Δ5D activity (nmol/min/total liver microsomal proteins)
* p < .05.

Table 3.13: Correlation coefficients (r) between Δ6D and Δ5D activity and fatty acids from liver, serum, and platelet phospholipids.
Genotype p = 0.4948
Age p = 0.7061

Genotype x Age p = 0.2707

1. Lean rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (19 - 21 mo), n=5.
Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 & 17 mo), n=3.
2. SEM = Standard Error of Mean.

Fig. 3.6. Platelet aggregation in SHHF/Mcc-fa<sup>cp</sup> female rats<sup>1</sup>
(Mean ± SEM<sup>2</sup>).
Fig. 3.7: Platelet count and platelet aggregation of obese SHHF/Mcc-fa cp female rats (n=9).
Fig. 3.8: Platelet count and platelet aggregation of lean SHHF/Mcc-fa £p female rats (n=10).
Genotype $p=0.3144$
Age $p=0.2953$
Genotype x Age $p=0.4102$

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 12 mo</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Lean rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (19 - 21 mo), n=5.
Obese rats: 6 mo, n=5; 9 mo, n=5; ≥ 12 mo (12 & 17 mo), n=3.

2. SEM = Standard Error of Mean.

Fig. 3.9: Lag time of platelet aggregation in SHHF/Mcc-fa $^{cp}$ female rats$^1$ (Mean ± SEM$^2$).
<table>
<thead>
<tr>
<th></th>
<th>Impedance</th>
<th>Lag time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Desaturase activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta 6D$ ¹</td>
<td>-.02</td>
<td>-.24</td>
</tr>
<tr>
<td>$\Delta 6D$ total ²</td>
<td>.12</td>
<td>-.04</td>
</tr>
<tr>
<td>$\Delta 5D$ ³</td>
<td>.01</td>
<td>-.14</td>
</tr>
<tr>
<td>$\Delta 5D$ total ⁴</td>
<td>-.06</td>
<td>.09</td>
</tr>
<tr>
<td><strong>Arachidonic acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver phospholipid</td>
<td>-.08</td>
<td>.17</td>
</tr>
<tr>
<td>Serum phospholipid</td>
<td>.34</td>
<td>-.07</td>
</tr>
<tr>
<td>Platelet phospholipid</td>
<td>.29</td>
<td>.14</td>
</tr>
</tbody>
</table>

¹ $\Delta 6D$ = average $\Delta 6D$ activity (pmol/min/mg liver microsomal proteins)
² $\Delta 6D$ total = total $\Delta 6D$ activity (nmol/min/total liver microsomal proteins)
³ $\Delta 5D$ = average $\Delta 5D$ activity (pmol/min/mg liver microsomal proteins)
⁴ $\Delta 5D$ total = total $\Delta 5D$ activity (nmol/min/total liver microsomal proteins)
* $p < .05$.

Table 3.14: Correlation coefficients ($r$) between measures of platelet aggregation and $\Delta 6D$ and $\Delta 5D$ activity, fatty acids from liver, serum, and platelet phospholipids.
DISCUSSION

Different dietary components can influence the activity of Δ6D and Δ5D including carbohydrates (Mandon et al., 1988), proteins (Pelluffo and Brenner, 1974; Mandon et al., 1988; Lindholm et al., 1991), cholesterol (Garg et al., 1986; Garg et al., 1988a), and different fatty acid compositions in the diet (Garg et al., 1988a; Garg et al., 1990; Christiansen et al., 1991). All rats were fed with the same commercial rat diet. Therefore, dietary influence was minimized in this study. Animal products including fish meal were used in the commercial rat diet as the sources of ω3 fatty acids and AA. However, most of the differences in tissue fatty acid contents were found to be AA, but not ω3 fatty acids. This would suggest that the differences observed in tissue fatty acids, especially AA, were mainly due to the influence of endogenous desaturase activity.

Several studies found decreased Δ6D activity with age (Peluffo and Brenner, 1974; Horrobin, 1981; Bordoni et al., 1988; Hrella et al., 1989; Bourre et al., 1990; Ulmann et al., 1991a; Maniongui et al., 1993). In this study, there were no consistent changes with age for average and total Δ6D in both genotypes. Expression of average Δ6D activity as pmol of product fatty acids synthesized per minute by desaturase enzymes per mg of liver microsomal proteins was also used by other researchers as a way to express desaturase activity (Nakamura et al., 1994). Total Δ6D activity expressed as nmol of product fatty acids synthesized per minute by desaturase enzymes per total liver microsomal proteins was used in the study of Blond et al. (1989) to indicate the
total capacity of Δ6 desaturation. Obese rats had greater liver weight than lean rats over all ages but average microsomal protein per gram of liver weight did not differ significantly. This may imply greater accumulation of triglycerides and/or glycogen in the obese rat liver. However, total liver microsomal proteins in obese rats was significantly greater than lean rats indicating that microsomal mass was also increased in obese rats. Regardless of methods of expression, age did not have a significant effect on Δ6D activity of female rats, which is different from the hypothesis that old rats would have greater desaturase activity. Other studies mainly used males only or mixed gender humans or animals. Since estrogen has been noted to have an inhibitory effect on Δ6D and Δ5D activity (Brenner, 1981; Gonzalez et al., 1986), Δ6D and Δ5D activities may differ by gender (Gonzalez et al., 1986). However, estrogen was not measured in these rats. So confirmation of an estrogen effect on desaturase activity is limited in this study. Different from Δ6D activity, both average and total Δ5D activities decreased with age in lean and obese rats, with the significance seen between 6 mo and older than 12 mo rats. It appears that when rats reached 12 mo or older, desaturase activity may decline due to an aging effect on cell and enzyme functions that is unrelated to estrogen levels. However, there was a considerable age difference between two 12 mo and one 17 mo obese rats included in the older than 12 mo group. Further study using rats in a closer age range should be conducted to confirm whether Δ5 desaturase activity declines at the later age.
Insulin was reported to enhance Δ6D (Mimouni and Poisson, 1992) and Δ5D (El Boustani et al., 1989; Brenner, 1990) activities. Insulin was not measured in these female SHHF/Mcc-faCP (HF) rats, but hyperinsulinemia due to insulin resistance has been noted in HF obese rats (McCune et al., 1990; Hoversland, 1992; Radin et al., 1993). There was a significant genotype effect for Δ6D activity (both average and total activities) and total Δ5D activity in these female rats (p<.05) with obese rats having the greater desaturase activity.

In liver lipid fractions, the content of 18:3ω6 as the product of Δ6 desaturase was greater and the substrate fatty acid 18:2ω6 was lower in obese rats over all age groups. This is consistent with the observation that obese rats had greater total Δ6D activity than lean rats. More 18:2ω6 was converted to 18:3ω6 in obese rats. However, the major product fatty acids AA and 20:5ω3 did not appear to increase in obese rats, despite the observation of greater total Δ6D and Δ5D activity in obese rat liver microsomes. A significant genotype effect with increased AA contents in serum and platelets PL of obese rats was noted (p<.05), however. Greater amount of AA produced in the liver of obese rats may have been delivered to other tissues. This may be one of the reasons that differences in AA from liver lipid fractions between lean and obese rats were not significant. It was thought that when substrate or product is low or absent, Δ6D activity may increase to enhance PUFA synthesis, whereas, Δ5D activity may only increase when sufficient substrate is provided to avoid the accumulation of 20:3ω6 (Brenner, 1981). This may in part explain why obese rats had greater
Δ6D and Δ5D activity when they had lower 18:2ω6 and the non-significant differences of 20:3ω6 from different tissue lipid fractions in all groups. There was a significant genotype and age interaction for 18:3ω6 from serum TG, 20:5ω3 and 22:6ω3 from serum PL, and AA from platelet PL. Obese 9 mo and/or older than 12 mo rats tended to have greater amount of these product fatty acids than lean 6 mo or same age rats. It appears that genotype and age interact to regulate Δ6D and/or Δ5D activity. However, the age difference between two 12 mo and one 17 mo obese rats may need to be considered before a definitive conclusion can be drawn.

The activity of desaturase enzymes affects the fatty acid composition of membrane phospholipids and influences the properties of membranes (Garda and Brenner, 1985; Laganiere and Yu, 1993; Borkman et al., 1993). The viscosity of membrane lipid bilayer is important in controlling the osmotic properties of the membrane. Lack of unsaturated fatty acids in the membrane results in increased membrane viscosity, consequently affecting the transport function of membranes, enzymatic functions of transmembrane proteins, and regulation of surface receptors such as the insulin receptors (Mead et al., 1986; Borkman et al., 1993). Higher desaturase activity increases the amount of long chain PUFA in membranes and consequently increases the fluidity of membrane. The uptake of extracellular substances such as glucose, and hormones like estrogens and insulin are enhanced. In this study, P/S ratios calculated from fatty acids of tissue phospholipids showed no significant differences in platelet and liver phospholipid. Changes in liver microsomes
and platelet membrane viscosity may not be significant enough to influence the action of insulin receptors and enzyme functions.

Among three major product fatty acids for desaturase enzymes (18:3\(\omega6\), 20:4\(\omega6\), and 20:5\(\omega3\)) from liver, serum, and platelet PL, 18:3\(\omega6\) and AA had positive correlations with \(\Delta6D\) activity (\(p<.05\)). This is consistent with the hypothesis that product fatty acids would be positively correlated with desaturase activity. However, 20:5\(\omega3\) from all three tissue PL did not correlate with \(\Delta6D\) activity. \(\omega3\) family fatty acids were thought to have greater affinity for desaturase activity and compete with \(\omega6\) fatty acids for desaturation (Siguel et al., 1987; Garg et al., 1988a; Garg et al., 1990). Even though the rat diet contained fish meal, dietary 18:3\(\omega3\) was much lower than dietary 18:2\(\omega6\) as the substrates for desaturation. The competition for desaturase enzymes between 18:3\(\omega3\) and 18:2\(\omega6\) as a substrate may not be significant. \(\Delta5D\) activity did not correlate with any of the product fatty acids in all three tissue PL. The reason may be that it is not the rate limiting enzyme for desaturation. \(\Delta5D\) activity was extensively higher than \(\Delta6D\) activity (Fig. 3.2 to 3.5). Despite the fact that \(\Delta5D\) activity decreased with age, it was apparently high enough to convert 20:3\(\omega6\) to 20:4\(\omega6\) efficiently in older age rats. The strong correlations of \(\Delta6D\) activity with 18:3\(\omega6\) and AA further confirm that \(\Delta6D\) is the determination enzyme of lipid desaturation (Brenner, 1981; Dillon, 1987; Mayes, 1990a; Yamazaki, 1992).
Platelet aggregation did not differ between lean and obese rats over age. There was no significant genotype or age effect on platelet aggregation (p>.05). No correlations were observed between platelet aggregation and desaturase activities and AA in liver PL. AA in platelet phospholipids is the precursor of TXA2 production (Mayes, 1990a), which is a potent aggregator produced by platelets (Mayes, 1990a; Dillon, 1987; Notarbartolo et al., 1992). It is expected that an increase of AA in platelet phospholipids may lead to greater formation of TXA2 and result in stronger platelet aggregation. AA is also the substrate for prostacyclin (PGI2) production. PGI2 is a potent antiaggregator produced by endothelial cells on the vascular wall, so it acts more like a local modulator. Whole blood was collected directly from the rat heart and aggregation was performed in vitro using the aggregometer. The influence on platelet aggregation from PGI2 would not be as strong as from TXA2, since TXA2 could be produced by platelets continuously during the process of aggregation. However, TXA2 was not determined in this study, so the confirmation is not possible. Platelet aggregation was positively correlated with AA contents in serum and platelet phospholipids, but it was not significant. EPA is thought to have higher affinity for the cyclooxygenase enzyme and compete with AA for synthesis of prostaglandins (PG) and thromboxanes (TX) (Dusing et al., 1983; Siguel and Maclure, 1987; Lee et al., 1988). TXA3 synthesized from EPA is inactive and thought to decrease platelet aggregation (Dillon, 1987; Kwon et al., 1991). In platelet PL, AA content was far higher than EPA (Table 3.9), TXA2 production may not be interfered significant enough to influence platelet aggregation. Despite the significant genotype effect on Δ6D activity, total Δ5D activity, and AA content in phospholipids from different tissues, platelet
aggregation did not show a consistent pattern in all groups. Also, no
genotype and age effects were found in lag time.

When platelet counts were considered, obese rats showed a significantly
positive correlation between aggregation and platelet count. It appears that
platelet aggregation increased when the amount of platelets was increased in
obese rats. The possible explanation may be that more TXA₂ can be produced
by greater amount of platelet, and the interaction between platelets may be
enhanced when platelet amount is high. However, the correlation was not
observed in lean rats. Many factors may effect platelet aggregation besides
production of PG and TX and platelet counts. Platelet size may affect the
adhesion of platelets, larger platelets increase the possibility of platelet
interactions and increase the aggregation. The glycoprotein von Willebrand
Factor (vWF) produced by endothelium and platelets acts as a part of the factor
VIII complex (Winocour et al., 1985; Winocour, 1989b). High plasma level of
vWF may increase platelet aggregation. Platelet survival (Winocour et al.,
1987b) and plasma levels of β-thromboglobulin, platelet aggregation
enhancing factor (PAEF) and platelet factor 4 (PF4) (Winocour, 1985) are
thought to influence platelet aggregation. This may explain why platelet
aggregation was not significantly different in all groups and the correlations
with AA content in liver, serum, and especially platelet PL were not significant.
Also, platelet counts in lean rats did not correlate with platelet aggregation. It
appears that platelet aggregation was not a very sensitive functional indicator of
desaturase activity for the genotype and age effects in this rat strain.
In conclusion, obese rats had greater average and total Δ6 desaturase activity, but the difference over age groups was not significant. Δ5 desaturase activity decreased with age in both lean and obese rats. Overall, obese rats had greater total Δ5D activity. Product fatty acids of desaturase enzymes in liver, serum, and platelet phospholipids correlated with Δ6D, but not Δ5D, activity. However, the product fatty acids, especially AA, did not correlate with platelet aggregation. Platelet aggregation may not be a very sensitive functional indicator of pathology in desaturase activity as influenced by obesity and age in female SHHF/Mcc-fa<sup>OP</sup> rats.
CHAPTER 4

DIABETIC CONDITION, BUT NOT MENOPAUSAL STATUS, WAS ASSOCIATED WITH INCREASED SYNTHESIS OF ARACHIDONIC ACID AND PLATELET AGGREGATION IN PRE- AND POST-MENOPAUSAL TYPE II DIABETIC WOMEN

INTRODUCTION

The prevalence of non-insulin dependent diabetes mellitus (NIDDM), hypertension (HTN), dyslipidemia, and atherosclerosis increases with progressive obesity and advancing age (Debry, 1987; Kannel, 1988; Reaven, 1988; DeFronzo and Ferrannini, 1991; Simonson, 1993). In the United States, 6 to 7 million people have diabetes and 90% of them are NIDDM (American Diabetes Association, 1993). By age 70, 45 to 50% of the elderly are obese and hypertensive, 10 to 12% are NIDDM, and over 50% have evidence of atherosclerotic cardiovascular disease (ASCAD) (DeFronzo and Ferrannini, 1991). Obesity, NIDDM, HTN, dyslipidemia, and atherosclerosis are closely related and usually referred to as insulin-resistance syndrome or syndrome X (DeFronzo and Ferrannini, 1991). Hyperinsulinemia resulting from insulin resistance plays an important role in the association of these disorders.
The major causes of death in both type I and type II diabetes are ASCAD related such as myocardial infarction due to coronary artery atheroma (Jialal and Chait, 1989; Kristensen et al., 1989; Turner and Neil, 1992). Diabetic patients also have an increased risk of developing peripheral vascular disease which may lead to stroke (Jialal and Chait, 1989). Diabetes occurring at an earlier age presents a greater health risk, especially in females. Diabetic premenopausal women have a 16-fold increased risk of heart disease compared to the healthy population. Overall mortality in early-onset patients is increased 5-fold in men, but 11-fold in women (Dorman, 1984). The mechanism of increased risk for vascular disease and stroke in diabetic patients, especially in women is not completely understood.

Thrombosis plays an important role in the pathogenesis of atherosclerosis (Crowley, 1983; Sussman, 1985; Mustard et al., 1986; Monocada et al., 1986; Ross, 1986; Jialal and Chait, 1989; Notarbartolo et al., 1992). The balance between thromboxane A$_2$ (TXA$_2$), a strong aggregator produced by platelets, and prostacyclin (PGI$_2$), an equally potent antiaggregator synthesized by vascular endothelial cells, effects thrombosis formation from platelets (Dillon, 1987; Notarbartolo et al., 1992). Abnormalities in platelet function, platelet aggregation, and fibrinolysis are seen in experimental diabetic animals (Ruf et al., 1991; Iida et al., 1993) and human diabetes (Winocour, 1985; Davis et al., 1985; Mandal et al., 1993). Diabetic patients synthesize more TXA$_2$ than healthy individuals (Lagarde, 1980; Halushka, 1981; Davi et al., 1982); but, vascular biosynthesis of PGI$_2$ decreases in both IDDM and NIDDM patients (Winocour et al., 1985).
Age is also associated with abnormal TXA$_2$ and PGI$_2$ production. With advancing age, the ability of vascular tissue to produce PGI$_2$ decreases but platelet formation of TXA$_2$ increases (Chang et al., 1980; Kent et al., 1981; Murota, 1983; Vericel et al., 1985; Moncada, 1986; Dillon, 1987; Meydani et al., 1992). This may account in part for a higher incidence of coronary artery disease in old and NIDDM patients compared to young and healthy individuals.

Arachidonic acid (20:4ω6; AA) is the metabolic precursor for both TXA$_2$ and PGI$_2$ (Mayes, 1990a). It is released from platelet membrane phospholipids (PL) by phospholipase A$_2$ and converted into TXA$_2$ by cyclooxygenase, endoperoxidase and thromboxane synthase (Longenecker, 1985; Mayes, 1990b). The content of AA in serum and platelets especially membrane may play an important role in platelet function. AA originates from either the diet or endogenous synthesis via microsomal desaturation and elongation (Dillon, 1987; Mayes, 1990a). Other than dietary influence, the composition of fatty acids in tissues, e.g., serum, platelet, and hepatocytes, may be effected by desaturase activities (Wahle, 1983; Mead et al., 1986; Marra and Alaniz, 1990).

Δ6 desaturase (Δ6D) is the rate-limiting enzyme in the biosynthesis of AA from linoleic acid (18:2ω6; LA) (Brenner, 1981; Dillon, 1987; Mayes, 1990a; Yamazaki, 1992). Activity of Δ6D was reported to decrease with age in human and rats (Peluffo, 1974; Horrobin, 1981; Brenner, 1981; Bordoni, 1988; Blond, 1989; Hrella, 1989; Kalen, 1989; Hrella, 1990; Bourre, 1990; Hrella, 1991; Ulmann, 1991a; Ulmann, 1991b). However, these studies were done with
either male subjects only or mixed gender. In a previous study (Liu and Medeiros, 1995) using female subjects, no evidence of decreased Δ6D activity was found in older women.

Estrogen has been found to suppress Δ6D and Δ5D activity (Brenner, 1981; Gonzalez et al., 1986). Since loss of estrogen is one manifestation of aging in women, estrogen loss may affect Δ6D activity differently and consequently affect the availability of AA for TXA2 synthesis. In a previous study, none or a weak correlation between estrogen level and indices of Δ6D and Δ5D activity was found in aging women (Liu and Medeiros, 1995). However, there was no control for estrogen replacement therapy and peri-menopausal status of subjects in this study.

Insulin increases Δ6D and Δ5D activity (El Boustani et al., 1989; Brenner, 1990; Mimouni and Poisson, 1992). Hyperinsulinemia is commonly seen in obese humans (Reaven, 1988; DeFronzo and Ferrannini, 1991) and rats (McCune et al., 1990; Radin et al., 1993) and is often associated with insulin resistance. Enhanced activity of Δ6D observed in obese humans and animals may be in part due to hyperinsulinemia. In the previous studies, obese women (BMI > 30) had significantly higher insulin levels (p<.05) than lean women (Medeiros et al., 1995). Insulin level was positively correlated with serum fatty acid 20:4ω6/18:3ω6 ratio, an index of Δ5D activity. However, none or a weak correlation was observed between insulin and serum or platelet 18:3ω6/18:2ω6, an index of Δ6D activity. Obese women also had significantly greater platelet aggregation and shorter lag time compared to lean women (P<.05). A possible
explanation for the altered platelet function in obese women could be increased AA formation (Δ6D and Δ5D activity) and metabolism (production of PGI₂ and TXA₂). Both desaturase activity and eicosanoid formation may be affected by hormonal activity, specifically insulin and estrogen.

This study was designed to clarify the role of estrogen and insulin in regulation of fatty acid desaturation. A model of estrogen loss (pre- and post-menopausal) and hyperinsulinemia (lean healthy and obese NIDDM) in human females was studied for possible changes in indices of Δ6D and Δ5D activities. Platelet function was studied to determine potential functional impact of alterations in desaturase activity. The following hypotheses were tested: (1) Older and obese diabetic women would have greater indices of Δ6 and Δ5 activity. (2) Indices of Δ6 and Δ5 desaturase activity would be positively correlated with measures of diabetic condition and negatively correlated with estrogen levels. (3) Platelet aggregation and TXB₂ production would be greater and clotting would commence in less time in obese diabetic women. Menopausal status would exacerbate the effect of diabetes on platelet aggregation. (4) Measures of platelet aggregation would be positively correlated with measures of diabetic condition and negatively correlated with estrogen levels. (5) TXA₂ production would be positively correlated with measures of diabetic conditions, indices of Δ6D and Δ5D activity, AA content in serum and platelet phospholipids and measures of platelet aggregation, but negatively correlated with serum estrogen levels.
MATERIALS AND METHODS

Subjects. Control subjects were recruited through ads placed in newspapers and fliers (Appendix L) posted The Ohio State University campus. Obese NIDDM subjects were selected from diagnosed outpatients in the Diabetes and Endocrine Clinic, Division of Endocrinology and Metabolism in The Ohio State University Medical Center. Ten obese female subjects with NIDDM aged 33 to 48 years (premenopausal group), and 11 NIDDM subjects aged 50 to 71 years (postmenopausal group) were recruited for this study. Fourteen lean healthy premenopausal (aged 21 to 45) and 12 postmenopausal females (aged 57 to 91) were recruited as control subjects. Obese subjects had a body mass index (Refer to Appendix G for calculation) equal to or greater than 30 and had normal blood pressure (systolic pressure less than 160 mm-Hg and/or diastolic pressure above 95 mm-Hg) with or without medication. Lean control subjects were non-diabetic, with body mass index less than 30, and had normal blood pressure. All subjects were non-smokers and non-vegetarians. Lean and obese subjects in the younger group were not pregnant, did not use oral contraceptives, or did not have any anomalies in their monthly menstrual cycle. Older lean and obese subjects were post-menopausal and not using estrogen replacement therapy. A pre-screening form (Appendix M) was used for the recruitment of subjects. The objectives and protocols of this study and expected results were explained in detail to subjects. A written informed consent (Appendix N) was obtained before any data collection.
Data collection. There were two visits for each subject. Subjects were requested to fill out a medical information form (Appendix O) and a one-year semi-quantitative food frequency questionnaire (Appendix P), and were instructed how to fill out a three-day dietary record. Height and weight were recorded and body mass index calculated (Appendix G). Histories of medications and disease conditions were obtained from the medical information form in order to judge factors that may influence the results of this study. Information on vitamin and mineral supplement utilization were obtained. Within one week after the collection of three-day dietary records, subjects returned for blood collection. A total of 32 mL of twelve-hour fasting blood were obtained via venipuncture into six tubes with three tubes containing 3.8 % sodium citrate as an anticoagulant (4.5 mL blood plus 0.5 mL anticoagulant each) and three tubes without anticoagulant. The citrated blood was used for analysis of whole blood aggregation and the preparation of platelets for lipid extraction. Serum was used for lipid extraction, and quantification of glucose, insulin, estrogen, and thromboxane B₂ (TXB₂). For subjects in the younger groups, the second visit was arranged on the second or third day of their menstrual period so that estrogen concentrations would be synchronized over the entire subject group. Blood pressure was measured using a sphygmomanometer on the right arm after subjects remained seated for at least 10 min. If subjects were taking aspirin, aspirin-based or anti-inflammatory drugs, there was at least a two-week interval between abstinence from the drug and blood collection.
**Dietary Information.** A semi-quantitative one-year food frequency questionnaire, modified from Eck (1989), was completed by subjects. The questionnaire was designed to provide a profile of long-term nutrient intake and to focus on the foods that contain arachidonic acid. A three-day dietary record (Appendix Q) was filled out by subjects to understand recent dietary intake. Subjects were requested to record all foods and beverages on two weekdays and one weekend before blood collection.

Reminder phone calls were made one or two days prior to beginning their dietary record to ensure the completion of the instrument and to answer all questions. Information on dietary nutrient content was assessed using the Nutritionist IV Dietary Analysis software (N² Computing, Salem OR). There was no information on arachidonic acid (AA) in the original software database. Foods containing AA and their amounts were obtained from Agriculture Handbook No. 8 series (Human Nutrition Information Service, 1976; Human Nutrition Information Service, 1980; Human Nutrition Information Service, 1983; Human Nutrition Information Service, 1984; Human Nutrition Information Service, 1987; Human Nutrition Information Service, 1990). Content of AA from each food was manually entered into the database Version 3.5 of Nutritionist IV Diet Analysis (N² Computing, Salem OR).

**Whole blood aggregation.** Whole blood aggregation and the release of platelet ATP was measured simultaneously using the Chronolog Model 500 Whole Blood Lumi Aggregometer (Chronolog Corp. Havertown, PA) as described by Kwon et al. (1991) (Appendix A). Combination of 0.45 mL of citrated blood, 0.45 mL of 0.9 % freshly prepared saline solution and 0.1 mL
reconstituted Chrono-Lume Reagent (containing Luciferin-luciferase, Chronolog Corp.) was warmed at 37 °C. Either 2 μL of collagen (stock concentration 1μg/mL) or 5 μL reconstituted ADP (1 mM/mL) was added as aggregators. Impedance and luminescence was recorded for 6 minutes. The unit of measure for aggregation was expressed as the increase in impedance (Ohms) between two electrodes on which platelet aggregation occurs in the whole blood compared to a 20 Ohms internal standard. ATP release from sample platelets during aggregation was compared to 2 nmoles of ATP standard. The procedure was performed within 2 to 3 hours after blood was drawn. Impedance and ATP release were calculated by the formula in Appendix A. The remaining blood was used for preparation of platelet fatty acids.

Preparation of platelets. Platelets were harvested as described by Kwon et al. (1991). Citrated blood was centrifuged at 200 x g at 4°C for 10 min. The supernatant (platelet-rich-plasma) was recentrifuged at 1000 x g at 4 °C for 10 min (Appendix C). Five mL of ice-cold Tris-HCl/NaCl/EDTA buffer was used to wash platelets after discarding the platelet-poor-plasma. Following centrifugation at 1000 x g for 10 min at 4 °C, platelet pellets were resuspended in 1 mL of saline solution.

Lipid extraction. Lipids from serum or platelets were extracted according to the method of Folch et al. (1957). A mixture of 10 mL of chloroform/methanol (2:1, v/v) was added to 1 mL of fresh serum or platelets in a culture tube (Appendix B). Tubes were tightly capped with a Teflon™-coated cap and
vortexed for 1 min. The protein fraction was removed by paper filtration. The same procedure was repeated with 5 mL of the C: M mixture. After, 3 to 4 mL saline solution (0.9 %) was added and the extract was centrifuged at 2000 rpm for 10 min at room temperature. The extract was separated into two layers with the upper layer containing water soluble material in water-methanol-salt solution and the lower layer containing phospholipids in chloroform. The upper layer was discarded. One mL of chloroform/ methanol/ saline (3:47:48, v/v/v) was added to wash the surface of the chloroform layer, and then discarded. The extract of lipids in chloroform was transferred to a vial with a Teflon™-coated screw cap, flushed with nitrogen gas and stored at - 20 °C for further analysis.

**Thin layer chromatography separation.** Silica gel G Thin Layer Chromatography (TLC) plates (1000 microns) (Analtech, Newark, Delaware) were used to separate phospholipids, cholesteryl esters and triglycerides components as briefly described by Kwon et al. (1991) (Appendix E). Plates were scored to make 1/2 inch wide tracts and reactivated in a 110 °C oven for 2 hours. Fresh developing solvent (n-hexane : diethyl ether : glacial acetic acid = 80 : 20 : 1, v/v/v) was prepared in a TLC developing chamber and allowed to equilibrate for at least 1 hour until saturation was achieved. Extracts of lipid was obtained by evaporating the chloroform using a flash-evaporator (Buchler, Fort Lee, NJ). After applying spots of lipids, the TLC plates were developed in the saturated chamber, and then dried in the hood after the development was completed. Visualization of lipid spots were performed according to Nakamura et al. (1994). Each spot was visualized with 2',7'-dichlorofluorescein (0.2% in ethanol) under the UV-light, then scraped off and collected in culture tubes.
Methylation of fatty acids. The methylation process was based on the method of Morrison and Smith (1964), but borontrichloride methanol was used instead of borontrifluoride methanol as the methylation reagent. Two mL of borontrichloride methanol reagent (Sigma, St Louis, MO) and 50 mL of heptadecanoic acid (C17:0) (1.78 mg/mL) (Sigma, St Louis, MO) as an internal standard was added into each tube containing silica gel and the lipid fraction (Appendix F). After a 30 min hot water bath (85- 90 °C) for 30 min and cooling to room temperature, 5 mL of delonized-distilled water and 1.25 mL of hexane containing 0.05 % of BHT as an antioxidant were added. Following shaking in an electric shaker for 3 min, the culture tubes were centrifuged at 2000 rpm for 10 min. The top layer containing hexane and methylated phospholipids were then transferred to a clean culture tube and a small amount of sodium sulfate added to absorb the residual water. The methylated fatty acids in hexane were stored in a 4 mL glass vial filled with N₂ gas at -20 °C until analysis by gas chromatography.

Gas-liquid chromatography analysis. Methylated fatty acids were analyzed by gas chromatograph (GC) (Hewlett Packard, 5890A, Series II) using a capillary column (J&W Scientific, DB-23, 30 m x 0.53 mm I.D. x 0.5 microns, Varian, San Fernando, CA). Injector temperature was 220 °C and detector temperature was 230 °C. The temperature program began at 125 °C, increased 10 °C per min, and held at 175 °C for 5 min, then increased 5°C per min and held at 210 °C for 4 min. A multiple fatty acid standard (Matreya, Inc. Pleasant Gap, PA) and single fatty acid standards (Matreya, Inc. Pleasant Gap, PA) were used to determine peaks for each fatty acid. Fatty acid distribution
(wt %) was calculated by the formula shown in Appendix G. In this study, only the fatty acid substrates and products of Δ6- and Δ5-desaturase are presented.

**Serum glucose determination.** Serum glucose was determined by the glucose oxidase method initially developed by Keilin and Hartree (1948) and modified by Young et al. (1975). Enzymatic glucose kits (Stanbio, San Antonio, TX) were used for the analysis (Appendix R). Serum stored at -20 °C was used within 2 weeks of storage. Ten μL of serum or glucose standard in duplicate were added into each test tube containing 1 mL of enzymatic reagent. One tube containing 1 mL of enzymatic reagent was used as the blank. All tubes were mixed well and incubated at 37 °C for 5 minutes. Absorbance of standard and samples were read using a Spectronic 301 spectrophotometer (Milton Roy, Inc., Rochester, NY) at 500 nm wave length within 60 min. Concentration of glucose was calculated using the formula shown in Appendix R.

**Insulin determination.** Insulin was measured using radioimmunoassay (RIA) kits (Binex, Portland, ME) (Appendix S) following the method recommended in the instructions provided with the kit. After labeling all tubes in duplicate including non-specific binding (NSB), maximum binding (Bo), calibrators A through F, controls, and serum samples, different solutions were added to individual tubes to have a total volume of 200 μL in each tube. Detailed procedures are indicated in Appendix P. All tubes were capped, vortexed, and incubated at room temperature for 90 min. Then, 500 μL of cold
precipitating reagent was added to each tube followed by vortexing. After incubating at room temperature for 10 min and centrifuging for 15 min, the supernatant was removed by aspiration. $^{125}$I activity of each tube and the standard curve was determined using a 1470 Wizard ™ automatic γ-counter (Wallac, Inc., Gaithersburg, MD). Concentrations of insulin were calculated based on a standard curve (Appendix S).

**C-peptide determination.** $^{125}$I-labeled RIA kits (Diagnostic Systems Laboratories, Inc., Webster, TX) were used for the analysis of serum c-peptide (Appendix T). The analysis was performed by The Ohio State University General Clinical Research Center according to the procedures recommended on the instruction sheets along with the kit. All tubes were labeled in duplicate including non-specific binding (NSB), maximum binding (Bo), calibrators A through F, controls, and serum samples. After reagents and serums were brought to room temperature, different solutions were added to individual tubes to have a total volume of 250 μL in each tube. Detailed procedures are indicated in Appendix T. All tubes were capped, vortexed, and incubated at room temperature for 4 hrs. Then, 1mL of cold precipitating reagent was added to each tube and vortexed. After incubating at room temperature for 20 min and centrifuging for 5 min, the supernatant was removed by aspiration. $^{125}$I activity of each tube and the standard curve was determined using a 1470 Wizard ™ automatic γ-counter (Wallac, Inc., Gaithersburg, MD). Concentrations of c-peptide were calculated based on a standard curve (Appendix T).
Estradiol determination. Concentration of serum 17β-estradiol was determined using 125I-labeled RIA kits (ICN, Costa Mesa, CA) (Appendix U) according to the procedures recommended by the company. After all reagents and sera had been brought to room temperature, 500 μL of dilute buffer was added to tubes 1 and 2. Fifty μL of 0 pg/mL 17β-estradiol standard was added into tubes 1, 2, 3, and 4. Then, 50 μL each of 17β-estradiol standards (10, 30, 100, 300, 1000, 3000 pg/mL), controls (control 1, 2, and 3), and sera were added to respective tubes in duplicate. After the addition of 500 μL of 17β-estradiol-125I to tubes, 500 μL of anti-17β-estradiol was added to all tubes, except tubes 1 and 2. Following vortexing, all tubes were incubated at 37 °C for 90 minutes. Then, 500 μL of precipitant solution was added followed by vortexing. After centrifuged at 1000 x g for 20 minutes at 4 °C, the supernatants was aspirated and the activity of 125I in precipitates were counted using a 1470 Wizard™ automatic γ-counter (Wallac, Inc., Gaithersburg, MD). Concentration of 17β-estradiol was calculated (Appendix U).

Serum thromboxane B2 determination. The maximum production of TXB2, the stable metabolite of TXA2, was determined in serum using enzyme immunoassay (EIA) (Appendix V). TXB2 was extracted by solid phase extraction using Amprep C2 micro column (Amersham Corporation, Arlington Heights, IL). The extraction and determination were performed based on procedures provided by the company. One mL acidified serum (pH3) was
applied to the column, which had been conditioned with 2mL of methanol and 2 mL water. After washing the column with 5 mL water, 5mL 10% ethanol and 5mL hexane, TXB₂ was eluted using 5mL methyl formate, dried under N₂ gas and redissolved in assay buffer. All assay reagents and standards were equilibrated to room temperature. After pipetting specific amounts of assay reagents and/or serum samples into preassigned individual wells (refer to Appendix S for detail.), the microtitre plate was covered and incubated at room temp (15-30°C) for 1 hour while shaking with a Microplate Shaker EAS 2/4 (SLT Lab instruments, Austria). All wells were aspirated and washed four times with 400 μL wash buffer followed by the immediate addition of 150 μL of enzyme substrate. The plate was incubated for exactly 15 minutes at room temp (15-30°C) while shaking. Reactions were stopped by adding 100 μL of 1M sulfuric acid to each well. After mixing the contents of each well by shaking, the optical density was read in a Microplate Autoreader EL310 (Bio-Tek Instruments, Winooski, VT) at 450nm within 30 minutes. Concentrations of TXB₂ from each serum sample were determined based on a standard curve (Appendix V).

Statistical analysis. This study was a 2 x 2 factorial design with diabetic condition (with or without NIDDM) and menopausal status (pre- and post-menopausal) as independent variables. Serum and platelet fatty acids, measures of platelet aggregation, serum glucose, hormonal status (insulin and estrogen), serum c-peptide and TXB₂ production are dependent variables. Results were analyzed by Analysis of Variance (ANOVA), using the general linear model (GLM) procedure of the SAS software package (SAS Institute,
1988). Differences between means were assessed by the least significant differences (LSD) test. Difference of the diabetic duration between pre- and post-menopausal diabetic women was assessed by the t-test. ANOVA-derived P values were provided for menopausal status (pre- vs post-menopausal status), diabetic condition (with vs without NIDDM), and menopausal status x diabetic condition interaction. Correlation coefficients were calculated using the correlation procedure in SAS (SAS Institute, Inc., 1988). All significant differences and effects were declared when p<.05.

RESULTS

Forty-seven females were recruited for this study, including 10 premenopausal diabetic women (Pre-NIDDM), 14 premenopausal controls (Pre-Control), 11 postmenopausal diabetic (Post-NIDDM) and 12 postmenopausal controls (Post-Control). However, two premenopausal controls were excluded due to high BMI (greater than 30) and hyperlipidemia. One postmenopausal control subject was eliminated because she took aspirin the night before blood collection. Age of premenopausal and postmenopausal groups differed significantly, as expected (Table 4.1). There was a significant menopausal and diabetic interaction on age (p<.05). Post-Control women were significantly older than Post-NIDDM women. Premenopausal women were taller than postmenopausal women (p<.05). Both diabetic groups were heavier than the control groups (p<0.05) based on the recruiting criteria specifying that NIDDM subjects should have a BMI over 30; whereas, controls subjects had BMI less
than 30 (Table 4.1). Overall, subjects were normotensive with or without medication based on the recruiting criteria (Eaker et al., 1989). However, there were significant menopausal and diabetic effects on systolic and diastolic pressure (p<0.05). Both measures were higher in diabetic and/or older subjects. Post-NIDDM women had significantly longer duration of diabetes than Pre-NIDDM women (6.20 ± 1.54 vs 11.18 ± 2.51; p<0.05) (Table 4.1).

Diabetic condition was evaluated based on fasting serum glucose and insulin levels (Table 4.2). Since five post-menopausal and four pre-menopausal diabetic subjects were receiving insulin injections, c-peptide in serum was determined to verify the actual secretion of endogenous insulin. As expected, there was a significant diabetic effect on fasting serum glucose, insulin and c-peptide levels (p<.05) (Table 4.2). Pre-NIDDM and Post-NIDDM women had greater values than controls. This implies that obese diabetic women were insulin resistant.

There was a significant menopausal effect on serum estrogen concentrations (p<.05). Premenopausal women had greater estrogen levels than postmenopausal subjects, as expected. (Table 4.2).

Table 4.3 shows the recent dietary intake of diabetic and control subjects. There were significant differences between diabetic and control women for intake of total fat, saturated fatty acids (SFA) and linolenic acid (p<.05). Diabetic women tended to consume more of these nutrients than controls during the week before blood collection. Post-menopausal women had higher intake of AA than pre-menopausal subjects during this time period (p<.05). However, when dietary intake was compared based on per Kg of body weight (Table 4.4), no significant differences of nutrients and fatty acids between
diabetic and control women were found, except for energy intake. Control subjects had higher relative calorie intake (per Kg of body weight) than diabetic women (p<.05). Long-term dietary pattern did not show significant differences in the major nutrients and fatty acids between diabetic and control subjects (Table 4.5), except for protein. After adjusting for body weight (Table 4.6), only energy intake was different between diabetic and control women (p<.05). No differences were noted in other nutrients and fatty acids.

Table 4.7 contains data on substrate and product fatty acids of Δ6- and Δ5-desaturase from serum (PL), respectively. There was a significant menopausal and diabetic interaction for 18:2ω6 in serum PL (p<.05). Pre-Control subjects had significantly greater 18:2ω6 than Post-Control. There was a significant diabetic effect on AA from serum PL (p<.05). Diabetic women had greater AA than controls, regardless of menopausal status (p<.05). Ratios of γ-linolenic acid (18:3ω6)/ linoleic acid (18:2ω6) and arachidonic acid (AA) (20:4ω6)/ dihomo-γ-linolenic acid (20:3ω6) were calculated as the indices of Δ6 (Δ6D) and Δ5 desaturase (Δ5D) activity, respectively. The index of ω6 pathway activity (20:4ω6/18:2ω6) was used as an indicator of overall desaturase activity for both Δ6D and Δ5D. Index of ω6 pathway activity had the same pattern as AA in serum PL. Overall, diabetic women had greater index of ω6 pathway activity than control subjects (p<.05). No menopausal effect was noted in fatty acids or indices of Δ6D and Δ5D activity from serum PL.
In platelet PL (Table 4.8), no significant effect of diabetic condition on fatty acids was observed. Only 20:3\(\omega6\) and 20:4\(\omega6\) had significant menopausal and diabetic interaction. Pre-NIDDM women had significantly greater 20:3\(\omega6\) than Pre-Control or Post-NIDDM subjects. Pre-Control had lower AA than other groups (p<.05). There was no diabetic effect on indices of \(\Delta6D\) and \(\Delta5D\) activity (p >.05). No menopausal effect was noted in fatty acids or indices of \(\Delta6D\) and \(\Delta5D\) activity from platelet PL.

AA content and indices of \(\Delta6\) and \(\Delta5\) desaturase activity from serum and platelet PL did not significantly correlate with insulin, c-peptide and glucose levels (p>.05) (Table 4.9). Only the index of \(\omega6\) pathway activity calculated from serum PL had a significant positive correlation with serum insulin and c-peptide concentrations (p<.05). Estrogen level did not correlate with AA and indices of desaturase activity from serum and platelet PL (p>.05).

There was a significant menopausal and diabetic interaction on collagen-induced aggregation (p<.05) (Table 4.10). Pre-Control and Post-diabetic women had significantly greater platelet aggregation than Post-Control. A diabetic effect was found in ADP-induced aggregation indicating greater aggregation in diabetic women than controls (p<.05), regardless of menopausal status. No significant menopausal effect was observed in measures of platelet aggregation induced by either collagen or ADP. The time between the addition of agonists and the commencement of platelet aggregation did not differ between all groups (p>.05). A significant menopausal effect was found for thromboxane B\(_2\) (TXB\(_2\)) production (p<.05). Post-menopausal women in both
NIDDM and control groups produced greater amounts of TXB₂ than the younger groups (p<.05). There was no significant diabetic effect on TXB₂ production (p>.05).

No correlations were found between collagen-induced platelet aggregation and measures of NIDDM conditions. Only ADP-induced platelet aggregation had a significant positive correlation with serum c-peptide level (p<.05). There were no correlations between ATP release from platelet granules and measures of diabetes (p>.05), nor for lag time (p>.05). Serum estrogen did not correlate with measures of platelet aggregation (p>.05).

Production of TXB₂ in serum was positively correlated with insulin, duration of diabetes, and ADP-induced ATP release (p<.05) (Table 4.12). Platelet aggregation induced by both collagen and ADP showed positive, but not significant, correlation with TXA₂ (p>.05). There were no correlations between the production of TXA₂ and indices of Δ6 and Δ5 desaturase activity from serum and platelet PL and AA in platelet PL.
<table>
<thead>
<tr>
<th></th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
<th>ANOVA probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=12)</td>
<td>NIDDM (n=10)</td>
<td>Control (n=11)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>35.58 ± 2.13c</td>
<td>40.90 ± 1.56c</td>
<td>70.18 ± 3.65a</td>
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<tr>
<td>Height (m)</td>
<td>1.63 ± 0.02</td>
<td>1.63 ± 0.01</td>
<td>1.57 ± 0.02</td>
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<tr>
<td>Weight (Kg)</td>
<td>62.70 ± 2.58</td>
<td>94.19 ± 7.24</td>
<td>60.55 ± 2.91</td>
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<td>BMI² (Kg/M²)</td>
<td>24.63 ± 1.05</td>
<td>35.82 ± 1.84</td>
<td>25.22 ± 1.10</td>
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<td>Systolic (mm-Hg)</td>
<td>109.42 ± 2.57</td>
<td>130.00 ± 4.03</td>
<td>132.82 ± 3.41</td>
</tr>
<tr>
<td>Diastolic (mm-Hg)</td>
<td>72.17 ± 2.14</td>
<td>83.70 ± 4.08</td>
<td>80.27 ± 2.13</td>
</tr>
</tbody>
</table>

1 SEM = Standard Error of Mean.
2 Abbreviation: Meno = Pre- and Post-menopausal status; DM = Type II diabetes; Meno x DM = menopausal and diabetic interaction.
3 BMI (Body Mass Index) = Weight (Kg)/(Height (m))^2.

Within rows, means followed by different letters had significant menopausal and diabetic interaction (p<0.05).

Table 4.1: Description of diabetic and control subjects. (mean ± SEM ¹)
<table>
<thead>
<tr>
<th></th>
<th>Pre-menopausal</th>
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<th>Post-menopausal</th>
<th></th>
<th>ANOVA probability level</th>
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<tr>
<td></td>
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<td>NIDDM (10)</td>
<td>Control (11)</td>
<td>NIDDM (11)</td>
<td>Meno²</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>94.38±2.40</td>
<td>145.10±12.60</td>
<td>99.05±3.79</td>
<td>157.64±10.12</td>
<td>0.2874</td>
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<tr>
<td>Insulin (μU/mL)</td>
<td>13.65±0.67</td>
<td>24.22±5.19</td>
<td>20.28±1.55</td>
<td>46.90±14.53</td>
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<td>C-peptide (ng/mL)</td>
<td>0.75±0.07</td>
<td>1.56±0.27</td>
<td>1.12±0.13</td>
<td>1.49±0.18</td>
<td>0.3879</td>
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<tr>
<td>Estrogen (pg/mL)</td>
<td>68.41±7.14</td>
<td>64.35±6.45</td>
<td>45.84±4.22</td>
<td>51.35±3.06</td>
<td>0.0037</td>
</tr>
</tbody>
</table>

1 SEM = Standard Error of Mean.
2 Abbreviation: Meno = Pre- and Post-menopausal status; DM = Type II diabetes; Meno x DM = menopausal and diabetic interaction.

Table 4.2: Indicators of diabetic condition and menopausal status (mean ± SEM).
<table>
<thead>
<tr>
<th></th>
<th>Pre-menopausal</th>
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<td>Control (n=12)</td>
<td>NIIDDM (n=10)</td>
</tr>
<tr>
<td>Calories (Kcal)</td>
<td>1562.75±93.03</td>
<td>1773.00±159.48</td>
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<td>Protein (gm)</td>
<td>62.09±5.77</td>
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<td>Fat (gm) a</td>
<td>48.18±5.48</td>
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<tr>
<td>Cholesterol (gm)</td>
<td>168.08±24.31</td>
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</tr>
<tr>
<td>SFA 2 (gm) a</td>
<td>17.85±2.59</td>
<td>24.70±2.75</td>
</tr>
<tr>
<td>MUFA 3 (gm)</td>
<td>14.23±1.92</td>
<td>22.82±2.83</td>
</tr>
<tr>
<td>PUFA 4 (gm)</td>
<td>8.25±1.13</td>
<td>12.33±0.98</td>
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<tr>
<td>18:2 ω6 (mg)</td>
<td>4.61±0.68</td>
<td>8.62±1.09</td>
</tr>
<tr>
<td>20:4 ω6 (mg) b</td>
<td>0.06±0.01</td>
<td>0.10±0.03</td>
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<tr>
<td>18:3 ω3 (mg) a</td>
<td>0.32±0.05</td>
<td>0.51±0.09</td>
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<tr>
<td>20:5 ω3 (mg)</td>
<td>0.59±0.58</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>22:6 ω3 (mg)</td>
<td>0.01±0.00</td>
<td>0.06±0.06</td>
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</table>

1 SEM = Standard Error of Mean.
2 SFA = Saturated fatty acid.
3 MUFA = Monounsaturated fatty acid.
4 PUFA = Polyunsaturated acid.
a significant diabetic effect (p<.05).
b significant menopausal effect (p<.05).

Table 4.3: Recent dietary intake of diabetic and control subjects. (mean ± SEM 1)
<table>
<thead>
<tr>
<th></th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
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<tbody>
<tr>
<td></td>
<td>Control (n=12)</td>
<td>NIDDM (n=10)</td>
</tr>
<tr>
<td>Calories (Kcal/Kg)*</td>
<td>25.08± 1.48</td>
<td>18.90± 1.57</td>
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<tr>
<td>Protein (gm/Kg)</td>
<td>0.99± 0.08</td>
<td>0.89± 0.20</td>
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<tr>
<td>Fat (gm/Kg)</td>
<td>0.76± 0.08</td>
<td>0.76± 0.07</td>
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<tr>
<td>Cholesterol (gm/Kg)</td>
<td>2.64± 0.33</td>
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<td>SFA 2 (gm/Kg)</td>
<td>0.28± 0.04</td>
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<td>MUFA 3 (gm/Kg)</td>
<td>0.23± 0.00</td>
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<td>PUFA 4 (gm/Kg)</td>
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<td>0.13± 0.01</td>
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<td>18:2 ω6 (ng/Kg)</td>
<td>80.00± 10.00</td>
<td>90.00± 10.00</td>
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<td>20:4 ω6 (ng/Kg)</td>
<td>1.00± 0.21</td>
<td>1.06± 0.31</td>
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<td>18:3 ω3 (ng/Kg)</td>
<td>5.00± 0.80</td>
<td>5.64± 1.23</td>
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<td>20:5 ω3 (ng/Kg)</td>
<td>7.00± 7.20</td>
<td>0.00± 0.00</td>
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<tr>
<td>22:6 ω3 (ng/Kg)</td>
<td>0.10± 0.07</td>
<td>0.61± 0.58</td>
</tr>
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</table>

1 SEM = Standard Error of Mean
2 SFA = Saturated fatty acid.
3 MUFA = Monounsaturated fatty acid.
4 PUFA = Polyunsaturated acid.
* significant diabetic effect (p<.05).

Table 4.4: Recent dietary intake of diabetic and control subjects (per Kg of body weight). (mean ± SEM 1)
<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=12)</td>
<td>NIDDM (n=10)</td>
</tr>
<tr>
<td>Calories (Kcal)</td>
<td>2209.12 ± 254.23</td>
<td>2043.13 ± 205.34</td>
</tr>
<tr>
<td>Protein (gm)</td>
<td>82.24 ± 7.23</td>
<td>104.35 ± 12.43</td>
</tr>
<tr>
<td>Fat (gm)</td>
<td>91.32 ± 23.21</td>
<td>85.53 ± 15.34</td>
</tr>
<tr>
<td>Cholesterol (gm)</td>
<td>245.16 ± 27.14</td>
<td>278.24 ± 53.23</td>
</tr>
<tr>
<td>SFA (gm)</td>
<td>25.26 ± 4.21</td>
<td>30.43 ± 6.34</td>
</tr>
<tr>
<td>MUFA (gm)</td>
<td>33.33 ± 9.43</td>
<td>27.57 ± 5.67</td>
</tr>
<tr>
<td>PUFA (gm)</td>
<td>23.00 ± 8.32</td>
<td>19.12 ± 7.45</td>
</tr>
<tr>
<td>18:2 ω6 (mg)</td>
<td>20.17 ± 8.03</td>
<td>18.20 ± 6.58</td>
</tr>
<tr>
<td>20:4 ω6 (mg)</td>
<td>0.11 ± 0.01</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>18:3 ω3 (mg)</td>
<td>0.93 ± 0.46</td>
<td>0.87 ± 0.33</td>
</tr>
<tr>
<td>20:5 ω3 (mg)</td>
<td>0.01 ± 0.01</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>22:6 ω3 (mg)</td>
<td>0.02 ± 0.01</td>
<td>0.13 ± 0.09</td>
</tr>
</tbody>
</table>

1 SEM = Standard Error of Mean.
2 SFA = Saturated fatty acid.
3 MUFA = Monounsaturated fatty acid.
4 PUFA = Polyunsaturated acid.
* significant diabetic effect (p<.05).

Table 4.5: Long-term dietary intake of diabetic and control subjects. (mean ± SEM 1)
<table>
<thead>
<tr>
<th></th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NIDDM</td>
</tr>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Calorie (Kcal/Kg)</td>
<td>36.24± 4.83</td>
<td>21.91± 2.32</td>
</tr>
<tr>
<td>Protein (gm/Kg)</td>
<td>1.32± 0.13</td>
<td>1.13± 0.14</td>
</tr>
<tr>
<td>Fat (gm/Kg)</td>
<td>1.50± 0.41</td>
<td>0.90± 0.16</td>
</tr>
<tr>
<td>Cholesterol (gm/Kg)</td>
<td>3.94± 0.44</td>
<td>3.08± 0.66</td>
</tr>
<tr>
<td>SFA ² (gm/Kg)</td>
<td>0.41± 0.07</td>
<td>0.32± 0.05</td>
</tr>
<tr>
<td>MUFA ³ (gm/Kg)</td>
<td>0.54± 0.17</td>
<td>0.30± 0.05</td>
</tr>
<tr>
<td>PUFA ⁴ (gm/Kg)</td>
<td>0.39± 0.15</td>
<td>0.20± 0.07</td>
</tr>
<tr>
<td>18:2 ω6 (ng/Kg)</td>
<td>340.00± 140.00</td>
<td>180.00± 60.00</td>
</tr>
<tr>
<td>20:4 ω6 (ng/Kg)</td>
<td>2.00± 0.23</td>
<td>1.79± 0.41</td>
</tr>
<tr>
<td>18:3 ω3 (ng/Kg)</td>
<td>20.00± 8.61</td>
<td>8.69± 2.67</td>
</tr>
<tr>
<td>20:5 ω3 (ng/Kg)</td>
<td>0.20± 0.08</td>
<td>0.34± 0.22</td>
</tr>
<tr>
<td>22:6 ω3 (ng/Kg)</td>
<td>0.30± 0.11</td>
<td>1.48± 1.16</td>
</tr>
</tbody>
</table>

¹ SEM = Standard Error of Mean.
² SFA = Saturated fatty acid.
³ MUFA = Monounsaturated fatty acid.
⁴ PUFA = Polyunsaturated acid.

* significant diabetic effect (p<.05).

Table 4.6: Long-term dietary intake of diabetic and control subjects (per Kg of body weight). (mean ± SEM ¹)
### Table 4.7: Fatty acid composition (wt %) of serum phospholipids from diabetic and control subjects (mean ± SEM)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
<th>ANOVA probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=12)</td>
<td>NIDDM (n=10)</td>
<td>Control (n=11)</td>
</tr>
</tbody>
</table>

**Polyunsaturated (ω6)**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2ω6</td>
<td>23.08 ± 0.47 a</td>
<td>19.16 ± 0.20 ab</td>
<td>19.01 ± 1.09 b</td>
<td>20.78 ± 2.27 ab</td>
<td>0.3687</td>
</tr>
<tr>
<td>18:3ω6</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>20:3ω6</td>
<td>1.37 ± 0.06</td>
<td>4.90 ± 2.82</td>
<td>1.67 ± 0.19</td>
<td>2.14 ± 0.50</td>
<td>0.3519</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>8.42 ± 0.39</td>
<td>10.84 ± 0.68</td>
<td>9.35 ± 0.73</td>
<td>10.41 ± 0.80</td>
<td>0.7056</td>
</tr>
</tbody>
</table>

**Indices of desaturase activity**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>18:3ω6/18:2ω6</td>
<td>5.23 ± 0.83</td>
<td></td>
<td>9.32 ± 4.26</td>
<td>8.69 ± 3.28</td>
<td>0.2424</td>
</tr>
<tr>
<td>20:4ω6/18:2ω6</td>
<td>0.59 ± 0.06</td>
<td></td>
<td>0.51 ± 0.05</td>
<td>0.57 ± 0.08</td>
<td>0.2376</td>
</tr>
</tbody>
</table>

**Indices of desaturase activity**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>18:3ω6/18:2ω6</td>
<td>5.23 ± 0.83</td>
<td></td>
<td>9.32 ± 4.26</td>
<td>8.69 ± 3.28</td>
<td>0.2424</td>
</tr>
<tr>
<td>20:4ω6/18:2ω6</td>
<td>0.59 ± 0.06</td>
<td></td>
<td>0.51 ± 0.05</td>
<td>0.57 ± 0.08</td>
<td>0.2376</td>
</tr>
</tbody>
</table>

1 SEM = Standard Error of Mean
2 Abbreviation: Meno = Pre- and Post-menopausal status; DM= Type II diabetes; Meno x DM = menopausal and diabetic interaction.
3 Index of Δ6D activity = 18:3 ω6/ 18:2 ω6; index of Δ5D activity = 20:4 ω6/ 20:3 ω6. ω6 path = 20:4 ω6/ 18:2 ω6; represents overall activity of Δ6D and Δ5D.

Within rows, means followed by different letters had significant menopausal and diabetic interaction (p<0.05).

Table 4.7: Fatty acid composition (wt %) of serum phospholipids from diabetic and control subjects (mean ± SEM)
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Pre-menopausal Control (n=12)</th>
<th>NIDDM (n=10)</th>
<th>Post-menopausal Control (n=11)</th>
<th>NIDDM (n=11)</th>
<th>ANOVA probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyunsaturated (ω6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2ω6</td>
<td>4.91 ± 0.62</td>
<td>4.41 ± 0.28</td>
<td>4.90 ± 0.25</td>
<td>4.49 ± 0.30</td>
<td>0.9738</td>
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<td>0.7481</td>
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<tr>
<td>18:3ω6</td>
<td>0.16 ± 0.12</td>
<td>0.20 ± 0.09</td>
<td>0.14 ± 0.09</td>
<td>0.24 ± 0.16</td>
<td>0.8420</td>
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<td>20:3ω6</td>
<td>0.57 ± 0.12</td>
<td>0.87 ± 0.11</td>
<td>0.73 ± 0.12 b</td>
<td>0.55 ± 0.11 b</td>
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<tr>
<td>20:4ω6</td>
<td>17.70 ± 1.81 b</td>
<td>21.63 ± 1.58</td>
<td>22.03 ± 0.53 a b</td>
<td>21.33 ± 0.19</td>
<td>0.3322</td>
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<td>0.0254</td>
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<tr>
<td>Indices of desaturase activity³</td>
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<td></td>
<td></td>
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<tr>
<td>Δ6D</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.03</td>
<td>0.8882</td>
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<td>0.6238</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.3517</td>
</tr>
<tr>
<td>Δ5D</td>
<td>23.44 ± 2.56</td>
<td>34.18 ± 9.64</td>
<td>26.55 ± 2.28</td>
<td>28.72 ± 2.05</td>
<td>0.8753</td>
</tr>
<tr>
<td></td>
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<td>0.2147</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.4341</td>
</tr>
<tr>
<td>ω6 path</td>
<td>3.94 ± 0.40</td>
<td>5.06 ± 0.46</td>
<td>4.59 ± 0.28</td>
<td>4.98 ± 0.42</td>
<td>0.4644</td>
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<td>0.3517</td>
</tr>
</tbody>
</table>

1 SEM = Standard Error of Mean
2 Abbreviation: Meno = Pre- and Post-menopausal status; DM = Type II diabetes; Meno x DM = menopausal and diabetic interaction.
3 Index of Δ6D activity = 18:3 ω6/18:2 ω6; index of Δ5D activity = 20:4 ω6/20:3 ω6; ω6 path = 20:4 ω6/18:2 ω6, it represents overall activity of Δ6D and Δ5D.

* b Within rows, means followed by different letters had significant menopausal and diabetic interaction (p<0.05).

Table 4.8: Fatty acid composition(wt%) of platelet phospholipids from diabetic and control subjects. (mean ± SEM ¹)
<table>
<thead>
<tr>
<th>Indices</th>
<th>Insulin</th>
<th>c-peptide</th>
<th>Glucose</th>
<th>Estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>.28</td>
<td>.18</td>
<td>.22</td>
<td>.00</td>
</tr>
<tr>
<td>Δ6D index1</td>
<td>-.12</td>
<td>.20</td>
<td>.19</td>
<td>-.07</td>
</tr>
<tr>
<td>Δ5D index2</td>
<td>-.01</td>
<td>-.07</td>
<td>-.11</td>
<td>-.04</td>
</tr>
<tr>
<td>ω6 path</td>
<td>.37*</td>
<td>.38*</td>
<td>.24</td>
<td>-.09</td>
</tr>
<tr>
<td><strong>Platelets</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>.16</td>
<td>.24</td>
<td>.11</td>
<td>-.12</td>
</tr>
<tr>
<td>Δ6D index1</td>
<td>.08</td>
<td>.10</td>
<td>-.04</td>
<td>-.10</td>
</tr>
<tr>
<td>Δ5D index2</td>
<td>.23</td>
<td>.20</td>
<td>.05</td>
<td>.24</td>
</tr>
<tr>
<td>ω6 path</td>
<td>.25</td>
<td>.19</td>
<td>.22</td>
<td>.10</td>
</tr>
</tbody>
</table>

1. Index of Δ6D activity = 18:3 ω6/18:2 ω6.
3. ω6 path = 20:4 ω6/18:2 ω6; represents overall activity of Δ6D and Δ5D.

*p < .05.

Table 4.9: Correlation coefficients (r) for indices of Δ6D and Δ5D activity, arachidonic acid (AA) from serum and platelet phospholipids and measures of diabetic and menopausal status.
<table>
<thead>
<tr>
<th>Platelet Function</th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
<th>ANOVA probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=12)</td>
<td>NIDDM (n=10)</td>
<td>Control (n=11)</td>
</tr>
<tr>
<td>Aggregation using Collagen</td>
<td>Impedance (Ohms)</td>
<td>37.17± 1.09 a</td>
<td>32.02± 2.70 b</td>
</tr>
<tr>
<td></td>
<td>ATP release (nmol/mL)</td>
<td>1.05± 0.11</td>
<td>1.06± 0.24</td>
</tr>
<tr>
<td></td>
<td>Lag time (min)</td>
<td>0.62± 0.04</td>
<td>0.64± 0.04</td>
</tr>
<tr>
<td>Aggregation using ADP</td>
<td>Impedance (Ohms)</td>
<td>17.51± 2.66</td>
<td>23.24± 2.49</td>
</tr>
<tr>
<td></td>
<td>ATP release (nmol/mL)</td>
<td>0.58± 0.12</td>
<td>0.41± 0.12</td>
</tr>
<tr>
<td></td>
<td>Lag time (min)</td>
<td>0.58± 0.06</td>
<td>0.44± 0.06</td>
</tr>
<tr>
<td>Hormone production by platelets</td>
<td>Thromboxane B2 (ng/mL)</td>
<td>0.90± 0.27</td>
<td>0.75± 0.24</td>
</tr>
</tbody>
</table>

1 SEM = Standard Error of Mean
2 Abbreviation: Meno = Pre- and Post-menopausal status; DM = Type II diabetes; Meno x DM = menopausal and diabetic interaction.

Within rows, means followed by different letters had significant menopausal and diabetic interaction (p<05).

Table 4.10: Platelet function in diabetic and control subjects. (mean ± SEM ¹)
<table>
<thead>
<tr>
<th>Aggregation</th>
<th>Insulin</th>
<th>c-peptide</th>
<th>Glucose</th>
<th>Estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (2 μg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impedance (Ohms)</td>
<td>.21</td>
<td>-.07</td>
<td>.07</td>
<td>.19</td>
</tr>
<tr>
<td>ATP release (nmol/L)</td>
<td>-.05</td>
<td>-.23</td>
<td>.17</td>
<td>-.11</td>
</tr>
<tr>
<td>Lag time 1 (min)</td>
<td>.01</td>
<td>-.03</td>
<td>-.04</td>
<td>.02</td>
</tr>
<tr>
<td>ADP (5 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impedance (Ohms)</td>
<td>.12</td>
<td>.31 *</td>
<td>.21</td>
<td>.01</td>
</tr>
<tr>
<td>ATP release (nmol/L)</td>
<td>.16</td>
<td>-.18</td>
<td>-.06</td>
<td>.08</td>
</tr>
<tr>
<td>Lag time 1 (min)</td>
<td>-.03</td>
<td>-.18</td>
<td>-.02</td>
<td>.06</td>
</tr>
</tbody>
</table>

1 The time between the addition of agonist and when blood coagulation commenced.
* p<.05.

Table 4.11: Correlation coefficients (r) for measures of platelet aggregation and measures of diabetic and menopausal status.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Thromboxane B&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Variable</th>
<th>Thromboxane B&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diabetic status</strong></td>
<td></td>
<td><strong>A&lt;sub&gt;6&lt;/sub&gt; desaturase</strong></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>.06</td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>.42 *</td>
<td>Phospholipids</td>
<td>.01</td>
</tr>
<tr>
<td>c-peptide</td>
<td>-.11</td>
<td>Platelets</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>.57 *</td>
<td>Phospholipids</td>
<td>-.04</td>
</tr>
<tr>
<td><strong>Menopausal status</strong></td>
<td></td>
<td><strong>A&lt;sub&gt;5&lt;/sub&gt; desaturase</strong></td>
<td></td>
</tr>
<tr>
<td>Estrogen</td>
<td>-.04</td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td><strong>Platelet aggregation</strong></td>
<td></td>
<td>Phospholipids</td>
<td>.20</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
<td>Platelets</td>
<td>Phospholipids .05</td>
</tr>
<tr>
<td>Impedance (Ohm)</td>
<td>.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP release (nmol/mL)</td>
<td>-.07</td>
<td><strong>ω&lt;sub&gt;6&lt;/sub&gt; pathway</strong></td>
<td></td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>-.06</td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td>Phospholipids</td>
<td>.14</td>
</tr>
<tr>
<td>Impedance (Ohm)</td>
<td>.17</td>
<td>Platelets</td>
<td>Phospholipids .12</td>
</tr>
<tr>
<td>ATP release (nmol/mL)</td>
<td>.43 *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>.04</td>
<td>Platelet Phospholipids</td>
<td>AA (wt %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<sup>1</sup> Index of A<sub>6</sub>D activity = 18:3 ω<sub>6</sub>/ 18:2 ω<sub>6</sub>; Index of A<sub>5</sub>D activity = 20:4 ω<sub>6</sub>/ 18:3ω<sub>6</sub>; Index of ω<sub>6</sub> path = 20:4 ω<sub>6</sub>/ 18:2 ω<sub>6</sub>; ω<sub>6</sub> path represents overall activity of A<sub>6</sub>D and A<sub>5</sub>D.

* p<.05.

Table 4.12: Correlation coefficients (r) between thromboxane B<sub>2</sub> production and diabetic and menopausal conditions, measures of platelet aggregation, indices of desaturase activity<sup>1</sup>, and arachidonic acid (AA) from platelet phospholipids.
DISCUSSION

Obesity is often associated with NIDDM. Both are highly related to insulin resistance as Syndrome X, or Reaven's Syndrome (Reaven, 1988). The characteristics of insulin resistance are hyperglycemia and hyperinsulinemia. In this study, obese diabetic female subjects had elevated fasting serum glucose, c-peptide, and insulin levels (p<.05) compared to controls, regardless of menopausal status, indicating insulin resistance in diabetic subjects. Higher systolic and diastolic pressures were also observed in diabetic women (p<.05) further confirming that diabetic subjects had typical Syndrome X. Despite the fact that postmenopausal diabetic women had significantly longer duration of NIDDM than premenopausal diabetic women (p<.05), their serum c-peptide did not differ (p>.05). This indicates that older diabetic subjects had not lost their ability to endogenously produce insulin.

Many studies reported that insulin enhances the Δ6D (Mimouni and Poisson, 1992) and Δ5D (El Boustani et al., 1989; Brenner, 1990) activities. To assess the effect of insulin on female subjects, ratios of enzyme products and substrates from serum and platelets were calculated as the indices of desaturase activity because direct measures of desaturase activity from the liver is unfeasible in human subjects. This method has been used by other researchers (Jones et al., 1986; Siguel and Maclure, 1987; Borkman et al., 1993). There was a significant diabetic effect on AA and the index of ω6 pathway activity from serum PL (p<.05). Both were higher in the diabetic groups regardless of menopausal status. This indicated greater overall
desaturase activity in diabetic groups compared to controls (p<.05), which confirmed the hypothesis. In platelet PL (Table 4.8), there was no significant diabetic effect on fatty acids. Only 20:3ω6 and 20:4ω6 had significant menopausal and diabetic interaction with Pre-Control group showing the lowest content of AA. Pre- and post-menopausal diabetic subjects in this study had greater serum insulin levels than controls. Positive correlations were only noted between ω6 pathway index from serum PL and insulin or c-peptide (p<.05) confirming the hypothesis that indices of desaturase is positively correlated with measures of diabetic condition. The correlations were not as strong in platelet PL (p>.05). Serum fatty acid ratios appeared to be more sensitive than platelet fatty acid ratios in response to changes of desaturase activity. This study agreed with the findings from other studies that insulin has a positive effect on desaturase activity (El Boustani et al., 1989; Brenner, 1990; Mimouni and Poisson, 1992); however, the effect can only be confirmed in overall desaturase activity, not as significant in either Δ6D or Δ5D activity individually.

Estrogen has been reported to suppress Δ6D and Δ5D activity (Brenner, 1981; Gonzalez et al., 1986; Marra et al., 1988). This has not been verified in humans. Premenopausal women were recruited to compare with postmenopausal women who did not receive any estrogen-containing drugs. Since the production of estrogen varies dramatically throughout the menstrual cycle, samples were collected on either the second or third day of menstruation to determine a basal level of estrogen to minimize variation in serum estrogen.
level among the subjects. Estrogen levels from both premenopausal groups were greater than the postmenopausal groups. However, there was no significant impact of estrogen on desaturase activity, based on the observation that indices of desaturase activity and product fatty acids from serum and platelet phospholipids did not have consistent and significant differences for menopausal status. In control groups, postmenopausal status seemed to increase indices of Δ6D and Δ5D activity from serum and platelet PL indicating that loss of estrogen might enhance desaturase activity. However, when diabetic conditions were involved, the correlation was neither significant nor consistent. Insulin and estrogen may interact as part of desaturase enzyme activity regulation.

Dietary intake could influence the fatty acid compositions of serum and platelets (Hornstra et al., 1983; Socini et al., 1983; Weaver et al., 1985; Vericel et al., 1987; McDonald et al., 1989; Agren et al., 1990; Phinney et al., 1990; Piche and Mahadevappa, 1990; Agren et al., 1991; Kwon et al., 1991; Hansen et al., 1993). To clarify the potential effect of diet, our laboratory used a semi-quantitative food frequency questionnaire and three day dietary record to represent the long-term and recent influence of diet. There was a consistent pattern in the recent dietary intake. Diabetic women tended to consume more total fat, saturated fatty acids, and linolenic acid as a total per person than controls during the week before blood collection. This may be due to the fact that heavier people (diabetic subjects) ate more to maintain body weight, since dietary intake adjusted with body weight did not have the difference on major nutrients and fatty acids. However, lean controls tended to consume more calories per Kg of body weight. This may be explained that lean subjects had
relatively greater proportion of lean body mass, which has a higher metabolic rate. Long-term dietary intake before or after adjusting for body weight did not show any significant differences in the major nutrients and fatty acids. Therefore, differences in fatty acids between groups cannot be explained by dietary differences. AA from the dietary sources was much lower than linoleic acid (LA) (Tables 4.3 and 4.5). However, the AA content (wt %) in serum and platelet phospholipids was relatively high, especially platelet AA was higher than LA (Table 4.8). This may indicate that the part of AA in serum and platelet phospholipids was from endogenous synthesis in addition to the dietary sources. The high content of AA in platelet phospholipids may be mainly from endogenous synthesis.

AA is the metabolic precursor for thromboxane A₂ production in platelets (Mayes, 1990a). It is released from platelet membrane phospholipids by phospholipase A₂ and converted into TXA₂ by cyclooxygenase, endoperoxidase and thromboxane synthase (Longenecker, 1985; Mayes, 1990b). TXA₂ is a strong aggregator and vasoconstrictor. The content of AA in tissue PL, especially in serum and platelets, may play an important role in platelet functions. Studies found that diabetic patients synthesize more TXA₂ than healthy individuals (Lagarde, 1980; Halushka, 1981; Davi et al., 1982). However, the female diabetic subjects in this study did not have greater amounts of TXB₂, the stable metabolite of TXA₂, compared to controls (p>0.05). Despite that, TXB₂ had a significant positive correlation with insulin levels and duration of diabetes (p<.05). Postmenopausal women, both diabetic and control groups, had significantly greater TXB₂ than premenopausal subjects, but TXB₂ did not correlate with estrogen level (p>.05). Production of TXB₂ has
been noted to increase with age (Chang et al., 1980; Kent et al., 1981; Murota, 1983; Vericel et al., 1985; Moncada, 1986; Meydani et al., 1992). This is consistent with our findings.

There was a significant diabetic effect on ADP-induced platelet aggregation. Diabetic women had greater aggregation than control subjects. Even though the positive correlation between ADP-induced platelet aggregation and insulin was not significant (p>.05), it had a significant positive correlation with c-peptide (p<.05). This implies a positive effect of insulin on platelet aggregation. TXB$_2$ production also had a significant correlation with ATP release when platelets were stimulated with ADP (p<.05). Despite greater production of TXB$_2$ in postmenopausal women, collagen or ADP-induced platelet aggregation did not differ significantly between pre- and post-menopausal groups. Neither estrogen nor menopausal status had a significant correlation with aggregation.

The purpose of this study is to establish the link between aging, hormonal status (including insulin and estrogen), and metabolism of arachidonic acid including platelet aggregation as the pathological index. Insulin may enhance desaturase activity and the content of AA in serum and platelets. Insulin also had positive correlation with TXB$_2$ production, which was not correlated with AA availability in platelet phospholipids, however. Estrogen did not have as strong an effect on indices of desaturase activities or aggregation as insulin but postmenopausal women did show greater TXB$_2$ production than younger subjects.
In conclusion, diabetic condition had a significant positive effect on desaturase activities, leading to greater AA contents in serum and platelet phospholipids. However, estrogen levels did not show as significant effect on fatty acid desaturation nor platelet aggregation in this study. It appears that platelet aggregation was not a very sensitive functional indicator of desaturase activity for the effect of insulin and estrogen, especially for estrogen.
CHAPTER 5

SUMMARY AND CONCLUSIONS

The impact of insulin and age on Δ6 and Δ5 desaturase activities has received much attention by investigators. However, most studies were done with either male subjects only or mixed genders. Data from female humans and animals were insufficient. Estrogen was thought to have a negative effect on Δ6D activity, and responses of Δ6D activity seemed to differ between male and female rats. A previous study using three generations of female human subjects indicated a different response of Δ6D and Δ5D activity from other studies. These two studies used SHHF/Mcc-faCP female rats and female humans with non-insulin-dependent diabetes mellitus (NIDDM) to investigate the effects of insulin and estrogen, with aging, on Δ6 and Δ5 desaturase enzyme activities and to seek a link between age, hormonal status, desaturase activity and arachidonic acid metabolism. The functional impact as indicated by platelet aggregation due to alteration of desaturase activity was also evaluated.

In the first study using SHHF/Mcc-faCP female rats, a direct measure of Δ6 and Δ5 desaturase activity using 14C-labeled substrates was conducted.
Dietary influence was minimized by feeding the same diet to all rats. A significant genotype effect on both average and total Δ6D activities as well as total Δ5D activities was observed, with obese animals having the greater activities. Age did not have as significant an influence on Δ6 desaturase activity as genotype. However, the activity of Δ5 desaturase in the female rats consistently decreased with age. Product fatty acids of desaturase enzymes in liver, serum, and platelet phospholipids (PL) were consistent and correlated with changes of desaturase activities, but these fatty acids were not as consistent with platelet aggregation. Platelet aggregation induced by collagen did not differ with age or genotype. No correlations were found between platelet aggregation and desaturase activities. Platelet aggregation was positively correlated, but not significantly, with AA in serum and platelet PL.

Despite the significant genotype effect on Δ6D activity, total Δ5D activity, and AA content in phospholipids from serum, platelets, and liver, platelet aggregation did not show a consistent pattern. There was no genotype or age effect for lag time. It appears that platelet aggregation was not a very sensitive functional indicator of pathology in desaturase activity for the genotype and age effects.

The influence of insulin and estrogen could not be determined in the first study, and the link between AA content in tissue lipid fractions and platelet aggregation was not clearly established. Also, whether or not the observations found in the rat model can be applied to humans was not certain. Therefore, the second study was conducted using pre- or post-menopausal female humans with or without NIDDM to investigate the insulin and estrogen effects on
desaturase activity and platelet aggregation. Serum TXB2 was determined to clarify the link between AA content in platelet PL and platelet aggregation.

In this study, diabetic condition had a significant positive effect on AA from serum PL and overall desaturase activity using indices of desaturase activity. There were significant positive correlations between overall desaturase activity and insulin or c-peptide. Platelet aggregation and ATP release representing secondary aggregation were higher, but not significant in diabetic women. However, the correlation with diabetic condition was only observed in ADP-induced aggregation. Production of thromboxane (TXB2) in diabetic subjects was not different from controls, but a correlation was found between insulin and TXB2. Estrogen did not have a significant effect on any of the measures in this study.

Both studies indicated the significant diabetic and/or genotype effect on Δ6 and Δ5 desaturase activities and fatty acid compositions of tissues, but the effect on platelet aggregation was not as consistent. Estrogen and age did not have as strong an influence on Δ6 desaturase activity as did insulin, but Δ5D activity decreased with age in the rat study. There was a link between insulin, desaturase activity and fatty acid compositions in tissue fractions, but the link did not extend to platelet aggregation as a functional indicator of desaturase activity and pathology in these female rats and humans.
BIBLIOGRAPHY


APPENDIX A

WHOLE BLOOD PLATELET AGGREGATION
WHOLE BLOOD PLATELET AGGREGATION
(Ref.: Chronolog Corp. Manual; Kwon et al., 1991)

Reagents & Chemicals:
1. Sterile physiological saline (SPS)
2. ADP reagent
3. Collagen
4. Chrono-Lume Reagent
5. Chrono-Lume ATP Standard
6. NaCl

Equipment & Supplies:
1. Vacutainer with 3.8% Sodium citrate
2. Aggregometer
3. Stir bars
4. Micropipette (1 μL - 10 μL)
5. Pipette tips
6. Pipette (100 μL - 1000 μL)
7. Freezer (-70 °C)
8. Plastic cuvettes
9. Wash bottle
10. Volumetric flask (250 mL)

Preparation of Reagents:
(1) Sterile physiological saline (0.9%)
   2.25 g NaCl in 250 mL
   ➞ + fresh distilled water to 250 mL
   ➞ Prepare fresh daily

(2) Reconstituted ADP reagent (1 mM)
   Tap the vial of ADP gently to get contents to the bottom
   ➞ + 5 mL SPS
   ➞ Mix well
   ➞ Divide reconstituted ADP into different vials for storage in -70 °C freezer
   ➞ Thaw one vial each time
(3) Chrono-Lume Reagent
   Tap the vial gently
   → +1.25 mL deionized distilled water
   → Mix well
   → Stand for 20 min
   → Store at 2-5 °C and use within 8 hours or at -70 °C for 6 mon and thaw only once

(4) Chrono-Lume ATP Standard (2 umol)
   Tap the vial gently
   → + 5 mL SPS
   → Mix well
   → Store at 2-5 °C and use within 24 hour or -20 °C for two weeks or -70 °C for 6 mon

Procedure:
   Warm the aggregometer to 37 °C
   → Put one cuvette with 1 mL of saline into the incubation wells
   → Place 5 empty plastic cuvettes into the incubation wells
   → Add a stir bar into each cuvette
   → + 450 μL SPS to each cuvette
   → + 450 μL whole blood into each cuvette
   → Swirl gently to mix
   → + 100 μL reconstituted Chrono-Lume Reagent
   → Swirl gently to mix
   → Plug the Electrode Probe Assembly jack into the receptacle in the side of the reaction well
   → Insert Electrode Probe Assembly firmly into a cuvette containing 1 mL of saline
   → Turn on 'Chart' switch
   → Insert the Electrode Probe into the cuvette with saline solution and close the cover
   → Adjust red impedance pen toward the left at the 10 (or 90) mark using 'Impedance Zero' knob on the aggregometer
   → Wait until the red pen stops moving, readjust the red pen to 10 (or 90) mark
   → Transfer the first cuvette with blood into the aggregation well
   → Rinse the Electrode Probe with saline solution and dry the Probe
   → Insert into the first cuvette and close the cover

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→ Adjust the 'Gain' knob to 'x0.02' mark
→ Adjust the red pen to the left at the 10 (or 90) mark and green luminescence pen to the right at 90 (or 10) mark using the 'Zero' knob on the Chart Recorder
→ Wait for stable baselines for both red and green pens
→ +5 μL reconstituted Chrono-Lume ATP Standard quickly and close the cover
→ Label the chart with gain setting
→ Repeat the procedure once with the second cuvette
→ Remove the cuvette and replace with the third cuvette
→ Rinse the Electrode Prove with water and SPS, dry gently with a paper tissue
→ Insert the Electrode Probe into the cuvette
→ Set the 'Gain' knob at 'x0.05' mark
→ Adjust both red and green pens
→ Wait for stable baselines for both red and green pens
→ Press and hold the 'Calibrate' button on the Aggregometer and adjust the red pen to the 50 mark using 'Impedance Gain' knob on the Aggregometer
→ +2 μL collagen quickly and close the cover
→ Wait for 6 min
→ Label the gain setting
→ Repeat the procedure with the forth cuvette
→ After the forth cuvette, put the fifth cuvette
→ Repeat the procedure but set the 'Gain' at 'x0.1' mark
→ +5 μL reconstituted ADP quickly and close the cover
→ Wait for 6 min and label the gain setting
→ Repeat the whole procedure once

**Calculation:**

1. Impedance:

\[
\text{Impedance (Ohms)} = \frac{\text{deviation of impedance curve for sample (cm)}}{\text{deviation of internal standard (cm)}} \times 20
\]
(2) ATP release:

\[
\text{ATP release (nmol/L)} = \frac{\text{deviation of luminescence curve for sample (cm)}}{\text{Gain setting for sample}} \times \frac{\text{deviation of luminescence curve for standard (cm)}}{\text{Gain setting for ATP standard}} \times 2 \text{ nmol/L}
\]
APPENDIX B

LIPID EXTRACTION FROM SERUM
LIPID EXTRACTION FROM SERUM
(Ref.: Folch et al., 1957)

Reagents & Chemicals:
1. Deionized distilled water (DDW)
2. NaCl
3. Chloroform
4. Methanol

Equipments: (**Do not use plastic supplies for lipid extraction.)
1. Vacutainers (non-anticoagulant)
2. Culture tubes w/ Teflon™-coated screw cap (15 mm x 200 mm)
3. Filter paper (pleated)
4. Glass funnels
5. Centrifuge
6. Electrical shaker
7. Culture tube rack
8. Test tube rack
9. Pasteur pipettes
10. Nitrogen gas
11. 250 mL volumetric flask
12. Graduated cylinder (50 mL and 100 mL)
13. Containers for reagents
14. Eppendorf pipette(100 μL - 1000 μL)
15. Macropipette (5 mL)
16. Test tubes (12 x 75 mm) w/ plastic caps
17. Glass vials w/ caps
18. -20 °C freezer

Preparation of reagents:
(1) C:M solvent (2:1, v/v)
   2 volumes of chloroform + 1 volumes of methanol

(2) Saline solution (0.9%)
   2.25 gm NaCl to 250 mL volumetric flask
   → + DDW to 250 mL

(3) C:M:S solvent (3:47:48, v/v)
   3 volumes of chloroform + 47 volumes of methanol + 48 volumes of saline solution
Procedure:

(1) Serum preparation: (** This should be done right after blood collection.)
- Blood collected in vacutainers (non-anticoagulant)
  → Stand for 20 - 30 min at room temp
  → Centrifuge at 400 xg (1465 rpm) for 20 min, at room temp
  → Transfer 1 mL supernatant (serum) into one culture tube for lipid extraction

(2) Extraction of fresh serum phospholipid:
- 1 mL serum in screw-capped culture tubes
  → + 10 mL C:M solvent (2:1, v/v)
  → Close all tubes tightly, and put all tubes in a test tube rack
  → Use electronic shaker to vortex all tubes for 1 min
  → Filter w/ pleated filter paper (remove protein fraction) into a new culture tube
  → Wash w/ 3 mL then 2 mL of C:M solvent (2:1, v/v) and filter through the filter paper
  → + 3 to 4 mL saline solution to each new tube, cap and shake vigorously
  → Centrifuge at 2000 rpm for 10 min
  → Use pasteur pipettes to discard upper part (water-methanol-salt solution)
  → + 1 mL C:M:S solvent (3:47:48) carefully (** Do not shake.**)
  → Discard the upper layer using a pasteur pipette (* May suck some chloroform out to make sure no water left.)*
  → Transfer the bottom layer to a large vial, fill with N2 gas
  → Cap and store at -20 °C for further procedure.
APPENDIX C

PREPARATION OF PLATELETS
PREPARATION OF PLATELETS
(Ref. : Kwon et al., 1991)

**Reagents & Chemicals:**
1. NaCl
2. Ethylenediamine Tetracetic Acid (EDTA)
3. Tris base
4. Concentrated HCl
5. Saline solution
6. Deionized distilled water (DDW)

**Equipments and Supplies:**
1. Centrifuge
2. Pasteur pipettes
3. N2 gas
4. Freezer (- 70 °C)
5. pH meter
6. Test tubes (12 x 75 mm)
7. Macropipette (5 mL)
8. Pipette tips
9. Volumetric flasks (250 mL and 50 mL)
10. Beaker

**Preparation of Reagents:**

1. **Saline solution (0.9 %)**
   
   2.25 mg NaCl in 250 mL volumetric flask
   
   → + DDW to 250 mL

2. **Tris-HCl (154 mM, pH 7.5)** M.W. of Tris-base = 121.1 units
   
   Prepare this buffer in the ice-bath
   
   → 0.9325 mg Tris-base to 50 mL volumetric flask
   
   → +DDW to 2/3 of the volume
   
   → Transfer to a small beaker and place the beaker in the ice
   
   → Adjust to pH 7.5 by adding HCl
   
   → Transfer the buffer back to the volumetric flask and rinse the beaker with DDW
   
   → +DDW to 50 mL, check the pH again
   
   → Keep the buffer in the refrigerator
(3) EDTA (77 mM) M.W. = 368.4 units 
0.7092 g to 25 mL volumetric flask

+DDW to 25 mL

(4) Mixture of Tris-HCl : NaCl : EDTA = 4 : 45 : 1, v/v/v

Add 4 parts of Tris-HCl, 45 parts of NaCl, and 1 part of EDTA together

Procedure:

Blood collected in a vacutainer with sodium citrate

- Mix well by gentle inversion 5 times

- Centrifuge 200 xg (1032 rpm) for 10 min at 4 °C

- Remove supernatant (Platelet rich plasma) to another test tube

- Centrifuge supernatant at 5000 xg (5196 rpm) for 10 min at 4 °C

- Discard the supernatant (platelet poor plasma)

- +5 mL ice-cold mixture of Tris-HCl/NaCl/EDTA

- Suck the platelet pellet in and out through the tip of pasteur pipette to resuspend

- Centrifuge 5000 xg (5196 rpm) for 10 min at 4 °C

- Discard the supernatant and resuspend the platelet pellet with 1 mL saline solution

- Transfer the platelet solution to a culture tube for lipid extraction

- Extract lipids when platelets were fresh
APPENDIX D

EXTRACTION OF LIPIDS FROM TISSUES
EXTRACTION OF LIPIDS FROM TISSUES
(Ref.: Christie, 1982)

Reagents & Chemicals:
1. Methanol
2. Chloroform
3. KCl
4. Deionized distilled water (DDW)

Equipments: (***) Do not use plastic supplies for lipid extraction.)
1. Homogenizing tubes
2. Homogenizer
3. Scalpel
4. Cutting board
5. Macropipette (5mL)
6. Pipette tips (5mL)
7. Buchner funnel
8. Flask for aspiration
9. Filter paper
10. Pasteur pipettes
11. Erlenmeyer flask
12. Beakers
13. Graduated cylinder (100 mL)
14. Volumetric flask (100 mL)
15. Glass vials (12mL) w/ caps

Preparation of reagents:
(1) C:M solvent (2:1, v/v)
   2 volumes of chloroform + 1 volume of methanol
   (can prepare and store in the container for less than one week use.)

(2) KCl solution (0.88%)
   0.88 g of KCl into 100 mL volumetric flask
   → Add DDW until 100 mL
   → Shake well and transfer to a glass container

(3) M:W solvent (1:1,v/v)
   1 volume of methanol + 1 volume of DDW
   (can prepare and store for future use)

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Procedure:

- Weigh 1 g of tissue and freshly chop on the cutting board
- Transfer to the homogenizing tube
- + 10 mL methanol, homogenize for 1 min.
- + 20 mL chloroform, homogenize for 2 min.
- Filter through Buchner funnel
- Transfer the solid residue to the homogenizing tube
- + 30 mL C:M solvent, homogenize for 3 min.
- Filter
- Use 20 mL chloroform to wash the tube and solid residue, filter
- Use 10 mL methanol to wash the tube and solid residue, filter
- Transfer the filtrates to a 100 mL graduated cylinder to measure the volume
- Transfer the filtrates to an Erlenmeyer flask
- Add 1/4 of the filtrate volume of KCl solution
- Shake and allow to settle
- Remove the top layer using aspiration
- Transfer the bottom layer to the graduated cylinder
- Transfer back to the Erlenmeyer flask
- Add 1/4 of the lower layer volume of M:W solution
- Shake and allow to settle
- Remove the top layer using aspiration
- The bottom phase is stored -- lipids in chloroform, transfer it to the vials and store in the -20 °C freezer for further procedure
APPENDIX E

SEPARATION OF SERUM LIPIDS BY THIN LAYER CHROMATOGRAPHY (TLC)
SEPARATION OF SERUM LIPIDS BY THIN LAYER CHROMATOGRAPHY
(Ref.: Christie, 1982)

**Reagents & Chemicals:**
1. n-Hexane
2. Diethyl ether
3. Acetic acid (glacial)
4. Acetone
5. Chloroform

**Equipments & Supplies:**
1. Flash-evaporator
2. TLC plates
3. Chamber & glass cover
4. Pasteur pipettes
5. Hair dryer
6. Scraper
7. Culture tubes w/ Teflon-coated screw cap (15 x 200 mm)
8. Cylinder of nitrogen
9. Adaptors and evaporation tubes for flash-evaporator

**Preparation of reagents:**
(1) TLC developing solvent: (**Prepare this reagent 2 hr beforehand.**)
   160 mL of n-hexane + 40 mL of diethyl ether + 2 mL of glacial acetic acid
   (**TLC developing solvent should be made fresh daily.**)

(2) 2', 7'-dichlorofluorescein (0.2% in ethanol)
   0.2 gm of 2', 7'-dichlorofluorescein in 100 mL volumetric flask
   → Add ethanol to 100 mL
   → Mix well and transfer to a spray bottle

**Procedure:**
→ Make 1/2 inch wide tracts on TLC plates
→ Reactivate TLC plates in 100 °C oven for at least 3 hrs
→ Prepare the TLC developing solvent and pour it into the chamber 2 hr beforehand
→ Cover the chamber with glass cover, may put something heavy on the top of the glass cover
Transfer samples into evaporation flasks from the vials
Wash the vials w/ a little amount of chloroform
Evaporate Chloroform using a flash-evaporator, control the temperature of water between 50 °C and 60 °C
Wash the evaporation flasks w/ 500 μL of Chloroform, make sure to flush down all of the lipid on the wall
Transfer the sample to a test tube
Cool the TLC plate in the dessicator (** cooling the TLC plate and spotting the samples on the plate, should be done within one and half hour.)
Spot lipid extract on the plate around 1 inch above the edge of the TLC plate using a pasteur pipette
Record the position, sample #, and date of each sample
Carefully put the plate on a rack and into the developing chamber (**The sample spots should not be soaked under the solvent, but should be above the surface of the liquid. Solvents will be pulled up to the spot by capillary action.)
Quickly cover the chamber w/ glass cover
When the solvent front reaches about 1 cm from the top edge of TLC plate, take the plate out carefully (It will take around 35 min to 45 min for developing.)
Let the plate dry in the hood
Spray the plate with 2',7'-dichlorofluorescein and visualize spots under a UV light (Fig. E.1)
Scrape off each lipid spot including the phospholipid, free fatty acid, triglyceride cholesteryl ester (based on the order from the original spot and up)
Transfer the individual spot to a culture tube
Fill the tube w/ N₂ gas, cap and freeze at -20 °C
Fig. E.1: Location of lipid fractions on TLC plate.
APPENDIX F

METHYLATION OF FATTY ACIDS
METHYLATION OF FATTY ACIDS
(Ref.: Morrison and Smith, 1964)

Reagent & Chemicals:
1. Borontrichloride (BCl₃) methanol
2. Hexane
3. Heptadecanoic acid (C₁₇:₀)
4. BHT
5. Sodium sulfate

Equipment & Supplies:
1. Heating block
2. Culture tubes (15 mm x 200 mm)
3. Test tube rack
4. Glass vials (4 mL)
5. Centrifuge
6. Pasteur pipettes
7. Freezer (-20 °C)
8. Macropipette (5 mL)
9. Beaker (1000 mL)
10. Thermometer
11. Nitrogen (N₂) gas

Preparation of Reagents:
(1) Hexane-BHT (5%)
   Add 0.05 g BHT for every 100 mL Hexane

(2) Heptadecanoic acid (C₁₇:₀) internal standard (1.78 mg/mL)
   Measure 17.8 mg heptadecanoic acid in the 10 mL volumetric flask
   → + n-hexane to 10 mL
   → Mix well
   → Transfer to 3 different vials to prevent repeated freezing and thawing
   → store in the - 70 °C freezer

Procedure:
This procedure is conducted after the lipids have either been previously
extracted and/or separated into lipid fractions via Thin Layer
Chromatography (TLC)
→ Preheat tap water to 80-90 °C
→ + 2 mL of BCl₃ methanol reagent into each culture tube containing lipid
   spots from TLC
+ 50 µL C17:0 internal standard (89 µg)
Cap the tubes as tight as possible
Place tubes in hot water bath (80 - 90 °C) for 30 min
Vortex periodically through out the 30 min period
Check the volume of methylation reagent, make sure at least 1 mL of reagent remaining
Cool the tubes in cold water until reaching room temp.
+ 5 mL deionized distilled water
+ 1.25 mL of hexane-BHT
Vortex tubes for 30 sec.
Centrifuge at 2000 rpm for 10 min
Transfer the top layer to a clean culture tube
Add a small amount of sodium sulfate (to absorb the residual water)
Shake vigorously
Centrifuge at 2000 rpm for 5 min
Transfer the top layer to a clean glass vial (4 mL)
Fill with N2 gas
Cap and store in the freezer for gas chromatography analysis
APPENDIX G

FORMULAS AND CALCULATIONS
FORMULAS AND CALCULATIONS

(1) wt % of fatty acids after GC separation:

Total weight of lipids (\(\mu g\)) = \(\frac{\% \text{ area of total fatty acids}}{\% \text{ area of internal standard}}\) \(\times 89 \mu g\)

Weight of a specific fatty acid (\(\mu g\)) = \(\frac{\% \text{ area of total fatty acids}}{\% \text{ area of internal standard}}\) \(\times 89 \mu g\)

Wt % of specific fatty acid (wt %) = \(\frac{\text{Weight of a specific fatty acid (\(\mu g\)) }}{\text{Total weight of lipids (\(\mu g\))}} \times 100\%\)

(2) Body Mass Index (BMI):

\[ \text{BMI} = \frac{\text{Weight (Kg)}}{(\text{Height (m)})^2} \]
APPENDIX H

ISOLATION OF LIVER MICROSONES
ISOLATION OF LIVER MICROSONES
(Ref.: Nakamura et al., 1994)

Reagent & Chemicals:
1. Sucrose
2. Tris Base
3. Saline solution
4. KCl
5. Sodium phosphate
6. n-acetylcysteine
7. KOH
8. Deionized distilled water (DDW)

Equipments & Supplies:
1. Ice bucket
2. Glass plate (20 x 20)
3. Scalpel
4. Glass-glass homogenizer
5. Ultracentrifuge tubes
6. Ultracentrifuge (Beckman)
7. Pasteur Pipette
8. Vials with caps
9. Measuring cylinder (10 mL)
10. Volumetric flasks (500 mL and 1000 mL)
11. Freezer (-70 °C and -20 °C)
12. Beaker (500 mL and 1000 mL)

Preparation of Reagents:
(1) Homogenization buffer (0.25 M sucrose, 10 mM Tris-base, pH 7.5)
- Measure 42.788 g of sucrose (M.W. = 342.30 units) and 0.606 g of Tris-base (M.W. = 121.1 units) into a 500 mL beaker
- Add deionized distilled water (DDW) to about 300 mL
- Mix well and transfer to a beaker
- Place the beaker in the ice
- Adjust pH to 7.5 using HCl
- Transfer back to 500 ml volumetric flask
- Add DDW to 500 mL
(2) Incubation buffer (0.25 M sucrose, 0.15 M KCl, 0.04 M phosphate buffer, pH 7.4, 0.70 mM n-acetylcysteine)

Measure 4.8 g of NaH₂PO₄, 85.6 g sucrose, 11.18 g of KCl, 114.24 mg of n-acetylcysteine into a 1000 ml beaker

→ Add about 500 mL DDW
→ Adjust pH to 7.4 with KOH (prepared in DDW)
→ Transfer to 1000 mL volumetric flask
→ Add DDW to 1000 mL, recheck pH value
→ Divide the buffer into several vials, cap and store in the -20 °C freezer

Procedure:

All processes must be carried out under ice cold conditions

→ Weigh the whole liver and record
→ Cut and weigh 1 g of liver sample in a plastic weigh boat, record the weight
→ Transfer the minced liver to a glass-glass homogenizer
→ Add 12 mL of ice-cold homogenizing solution and homogenize
→ Transfer the homogenate to two 10 mL plastic centrifuge tubes
→ Balance tubes with homogenization buffer
→ Centrifuge in a refrigerated ultracentrifuge at 12000 xg (9000 rpm) at 4 °C for 40 min
→ Transfer the homogenate to two 10 mL clean ultracentrifuge tubes with pasteur pipette
→ Discard the pellet, balance the tubes with homogenization buffer
→ Centrifuge at 150,000 xg (34,000 rpm) at 4 °C for 120 min
→ Discard the supernatant
→ Resuspend the pellet which contains microsomes with 3 mL of incubation buffer using pasteur pipettes and vortex
→ Divide the content into three vials
→ Flush with N₂ gas, cap and parafilm around the cap
→ Store in the -70 °C freezer
APPENDIX I

QUANTIFICATION OF MICROSOMAL PROTEINS
QUANTIFICATION OF MICROSONAL PROTEINS

Reagents & Chemicals:
1. Deionized distilled water (DDW)
2. Bovine serum albumin
3. Cupric sulfate (CuSO₄)
4. Potassium tartrate
5. Sodium hydroxide (NaOH)
6. Sodium carbonate (Na₂CO₃)
7. Folin-phenol reagent

Equipments & Supplies:
1. Volumetric flasks (10 mL and 500 mL)
2. Culture tubes w/ Teflon™-coated screw cap (15 mm x 200 mm)
3. Culture tube rack
4. Eppendorf pipette (100 µL - 1000 µL)
5. Repeatable pipette
6. Pipette tips
7. Quartz cuvette for spectrophotometer
8. Spectrophotometer

Preparation of reagents:
(1) Bovine serum albumin (BSA) working standard solution (1 mg/mL)
   10 mg of BSA to a 10 mL volumetric flask
   → DDW to 10 mL

(2) Alkaline copper reagent (0.01% CuSO₄·5H₂O, 0.02% K tartrate, 2% Na₂CO₃, and 0.1N NaOH)
   Dissolve 0.05 g of CuSO₄ in a 500 mL volumetric flask with a small amount of DDW
   → + 0.1 g of K tartrate to the flask, dissolve
   → + 2 g of NaOH to the flask, dissolve
   → + 10 g of Na₂CO₃ to the flask, dissolve
   → DDW to 500 mL, mix well
   → Discard after 1 day

(3) Folin-phenol reagent (1N)
   Dilute the reagent from original concentration (shown on bottle label) to 1 N
Procedure:

(1) Determination:
   Label all tubes in duplicate.
   → 0.1 mL of microsomal suspension
   → + 4.9 mL of DDW to dilute
   → Pipette 1 mL diluted sample to a culture tube in duplicate and add 5 mL of DDW
   → Prepare standards in duplicate according to the table below

<table>
<thead>
<tr>
<th>Tube</th>
<th>BSA standard (mL)</th>
<th>Water (mL)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>6.0</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>5.9</td>
<td>0.1 mg/mL</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>5.8</td>
<td>0.2 mg/mL</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>5.7</td>
<td>0.3 mg/mL</td>
</tr>
<tr>
<td>4</td>
<td>0.4</td>
<td>5.6</td>
<td>0.4 mg/mL</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>5.5</td>
<td>0.5 mg/mL</td>
</tr>
</tbody>
</table>

   → + 5 mL of alkaline copper reagent to all tubes, mix well
   → Stand at room temperature for 15 min
   → + 0.5 mL of Folin-phenol reagent rapidly and mix immediately
   → Stand at room temperature for 30 min
   → Read absorbance in a spectrophotometer at 500 nm

(2) Calculation:
   a. Plot a standard curve and determine the linear recreation of standard concentrations
   b. Apply the average absorbance of unknown to the linear recreation for diluted concentration of microsomal protein
   c. Concentration of microsomal protein = diluted concentration of microsomal protein x 50
APPENDIX J

DETERMINATION OF DESATURASE ENZYME ACTIVITY
DETERMINATION OF DESATURASE ENZYME ACTIVITY
(Ref.: Nakamura et al., 1994)

Reagents & Chemicals:
1. Incubation buffer (Appendix G)
2. KOH
3. Methanol
4. HCl (38%)
5. Linoleic acid (Sigma)
6. 1-14C-linoleic acid (New England Nuclear, about 50 μCi/mmol in ethanol, 1.9 μmol/mL)
7. Linoleyl methyl ester
8. γ-linolenyl methyl ester
9. Petroleum ether
10. Dihomo-γ-linolenic acid
11. 1-14C-dihomo-γ-linolenic acid (New England Nuclear, about 50 μCi/mmol in ethanol, 1.9 μmol/mL)
12. Dihomo-γ-linolenic methyl ester
13. Arachidonic methyl ester
14. Propylene glycol
15. NaF
16. ATP-Na2
17. CoA
18. NADH
19. MgCl2.6H2O
20. Nicotinamide
21. BHT
22. ScintiLene cocktail
23. 2',7'-dichlorofluorescein

Equipments & Supplies:
1. Volumetric flask (5 mL, 10 mL and 100 mL)
2. Pipettes (10 μL - 100 μL and 100 μL to 1000 μL)
3. Pipette tips
4. Pasteur pipettes
5. Vials with caps (4 mL)
6. Propylene glycol
7. N2 gas
8. Culture tubes
9. Scintillation vial (7 mL and 20 mL)
10. Water bath with shaker
11. Scintillation vial rack
12. Scraper
13. UV light
14. Sprayer

Preparation of Reagents:
(1) KOH (2N in methanol)
   Weigh 11.2 g of KOH (MW=56 units) into 1000 mL volumetric flask
   → Add methanol to 100 mL

(2) HCl (6N)
   Pour 57.6 mL 38% HCl into a 100 mL volumetric flask
   → Add deionized distilled water to 100 mL

(3) γ-linolenyl methyl ester stock solution (20 mg/mL)
   Transfer 100 mg of γ-linolenyl methyl ester from an ampule to a 5 mL
   volumetric flask
   → Rinse the ampule with 2-3 mL of pet ether
   → Add pet ether into the flask until 5 mL
   → Transfer the content to 2-3 vials
   → Flush with N₂, cap and store in -20 °C freezer

(4) γ-linolenyl methyl ester working standard (1 μg/μL)
   Transfer 100 μL of stock solution (20 mg/mL) to a clean vial
   → Add 1.9 mL of pet ether
   → Flush with N₂, cap and store in freezer

(5) Linoleyl methyl ester stock (20 mg/mL) and working standard (1 μg/μL)
   Same way as γ-linolenyl methyl ester

(6) Dihomo-γ-linolenic methyl ester stock (20 mg/mL) and working standard (1
    μg/μL) Same way as γ-linolenyl methyl ester

(7) Arachidonic methyl ester stock (20 mg/mL) and working standard (1 μg/μL)
    Same way as γ-linolenyl methyl ester
(8) Cold stock solution of linoleic acid as substrate (10 nmol/μL)
Weigh 100 mg of linoleic acid into 10 mL volumetric flask
→ Add methanol to 10 mL (10 mg/mL)
→ Mix well
→ Transfer 2.8 mL of this stock solution to 10 mL volumetric flask
→ Add propylene glycol to 10 mL

(9) Cold stock solution of dihomo-γ-linolenic acid substrate (10 nmol/μL)
Transfer 50 mg of dihomo-γ-linolenic acid to a culture tube
→ Add 5 mL methanol, flush with N2 and mix
→ Transfer 3.065 mL of this solution to a 10 mL volumetric flask
→ Add propylene glycol until 10 mL
→ Divide into two culture tubes
→ Flush with N2 and store in a -20 °C freezer

(10) Cofactor mixture (0.04 M NaF, 1.3 mM ATP, 0.06 mM CoA, 0.87 mM NADH, 5 mM MgCl2 and 0.33 mM nicotinamide)
Prepare this mixture fresh everyday
→ Adjust the amounts of chemicals based on the number of assay
The amounts for one assay are:

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>MW (units)</th>
<th>mg/assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA</td>
<td>918</td>
<td>0.09</td>
</tr>
<tr>
<td>ATP</td>
<td>608</td>
<td>1.20</td>
</tr>
<tr>
<td>MgCl2</td>
<td>203</td>
<td>1.69</td>
</tr>
<tr>
<td>NADH</td>
<td>783.6</td>
<td>1.04</td>
</tr>
<tr>
<td>NaF</td>
<td>42</td>
<td>2.55</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>122.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Incubation buffer</td>
<td>---</td>
<td>1.25 (mL)</td>
</tr>
</tbody>
</table>

→ Weigh and transfer cofactors to a 20 mL scintillation vial using 4-8 mL of incubation buffer
→ Add incubation to the amount required
→ Mix well

(11) BHT in methanol (2%)
Weigh 2 g of BHT to 100 mL volumetric flask
→ Add methanol to 100 mL
→ Mix well
Procedure:

(1) Incubation:
- Depending on the amount of assay, pipette 1 μL per assay 1-14C-linoleic acid (for Δ6 desaturase) or 1-14C-dihomo-γ-linolenic acid (for Δ5 desaturase) into a 7 mL scintillation vial using a syringe.
  - Add cold linoleic acid (for Δ6D) or dihomo-γ-linolenic acid (for Δ5D) (10 nmol/μL) in a 1:5 ratio of hot to cold substrate.
  - Add incubation buffer in a 1 to 100 ratio of hot substrate to incubation buffer.
  - Cap and vortex briefly to prepare substrate suspension.
  - Set water bath at 37 °C.
  - Label culture tubes, duplicate tube for each rat (15 min and 30 min) and 1 blank tube for each two rats.
  - Add 1 mL of the cofactor mixture to each culture tube.
  - Add 100 μL of the substrate suspension to each tube.
  - Vortex briefly and place all tubes into water bath with the shaking speed at 90 cycle/min.
  - Add 400 μL (for Δ6D) or 200 μL (for Δ5D) microsomal protein to tubes and incubate 15 min and 30 min (for Δ6D) or 10 min (for Δ5D).
  - Incubate the blank tubes at 30 min (for Δ6D) or 10 min (for Δ5D).
  - Stop the reaction by adding 2 mL of 2N KOH in methanol.

(2) Saponification:
- Add 50 μL BHT to each tube.
  - Flush with N2, cap and vortex.
  - Cook in 75 °C water bath for 1 hour.
  - After cooling, add 1 mL of 6N-HCl to acidify.
  - Flush with N2, cap and vortex briefly.
  - +2 mL of pet ether.
  - Flush with N2, cap and vortex for 30 sec.
  - Centrifuge at 2000 rpm for 5 min.
  - Transfer the top layer to a new culture tube.
  - Flush with N2, cap and store in a -20 °C freezer.

(3) Methylation:
- Refer to Appendix F except that there is no addition of internal standard.
  - Transfer the final top layer containing fatty acid methyl esters (FAME) to a 7 mL scintillation vial.

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(4) **Separation of Fatty acid methyl esters:**
Reactivate the AgNO₃ TLC plates in an oven at 110 °C for at least 2 hours
→ Add 60 ml pet ether and 40 mL diethyl ether (for Δ6D) or 30 mL pet ether and 70 mL diethyl ether (for Δ5D) in a developing chamber
→ Cover the top glass and allow the reagents to saturate for one and a half hours
→ + 50 μL γ-linolenyl methyl ester (for Δ6D) or arachidonic methyl ester (for Δ5D) to all tubes
→ + 50 μL linoleyl methyl ester (for Δ6D) or dihomo-γ-linolenic methyl ester (for Δ5D) to the background tubes
→ Blow dry the fatty acid methyl esters with N₂
→ Dissolve with 100 μL pet ether and apply the FAME to TLC plate in the hood
→ Rinse the vial with 100 μL pet ether and transfer the washing to the plate
→ Repeat the washing two more times
→ Place the plates on a rack and develop the plates in the chambers until the front reaches to 1 inch below the top
→ Air dry the plate in the hood for about 15 min
→ Spray 0.2% 2',7'-dichlorofluorescein (see Appendix E) and visualize under the UV light (Fig. 1.1 for Δ6D and Fig. 1.2 for Δ5D)
→ Properly mark areas that contain different FAME
→ Scrape areas of FAME from the plate and transfer to different scintillation vials

(5) **Quantification:**
Add 5 mL of ScintiLene to each vial
→ Cap and vortex
→ Count the radioactivity with a scintillation counter

(6) **Calculation:**
\[
\text{DPM of a product FAME} \quad \frac{\text{DPM of total FAME}}{\text{DPM of a product FAME}} \times 100\%
\]

a. % conversion of product = _____________________ x 100%

b. corrected total % conversion of products
   = (sum of % conversion of all product FAME from a sample)
   - (sum of % conversion of the blank)

c. average desaturase activity (pmol/min/mg of liver microsomal proteins)
   = 1900 pmol * (corrected total % conversion of products/100)/incubation time (min)/mg of liver microsomal proteins

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d. total desaturase activity (nmol/min/total liver microsomal protein)
   \[= 1.9 \text{ nmol} \times \left(\frac{\text{corrected total \% conversion of products/100}}{\text{incubation time (min)}}\right) / \text{total liver microsomal proteins}\]

(7) Location of FAME on a TLC plate:

Fig. J.1: Location of fatty acid methyl esters on AgNO₃-impregnated TLC plate for \(\Delta 6\)D activity determination.

Fig. J.2: Location of fatty acid methyl esters on AgNO₃-impregnated TLC plate for \(\Delta 5\)D activity determination.
APPENDIX K

PREPARATION OF SILVER NITRATE IMPREGNATED TLC PLATES
PREPARATION OF SILVER NITRATE IMPREGNATED TLC PLATES

Reagents & Chemicals:
1. Silica Gel G
2. AgNO3
3. Deionized distilled water (DDW)

Equipments & Supplies:
1. Five glass plates (20 x 20 cm)
2. Two side glass plates (5 x 20 cm)
3. Plate guide
4. Spreader
5. Beaker (500 mL)
6. Weigh boat
7. Spatula
8. Oven

Procedure:
The preparation processes should be carried out in a dark room
► Thoroughly clean and dry five glass plates and two side glass plates
► Place glass plates on the guide
► Make sure the spreader is moving smoothly
► Weigh 45 g of Silica Gel G and 2.25 g of AgNO3 into a beaker
► Add 150 mL DDW and mix gently using a spatula
► Pour the solution into the spreader and spread in a constant speed within 5 seconds
► Leave the plates for about one and a half hour in the dark room
► Wash spreader thoroughly to prevent from corroding
► Stack plates into the rack with at least 1 inch interval
► Leave overnight in the desiccator
► Dry the plates in an oven at 110 °C for at least 3 hours
► Store the plates in a dark desiccator
► Reactivate the plates in the oven at 110 °C for at least 2 hours before using
APPENDIX L

FLIER FOR RECRUITMENT OF SUBJECTS
ADVERTISEMENT FOR SUBJECTS

RESEARCH STUDY NEEDS VOLUNTEERS

Volunteers will be Paid $50.00 for Participation

Dr. Lydia Medeiros is doing a research study to learn how diabetic women and non-diabetic women use fat as they age. This study will investigate some factors that may explain why diabetes increases the risk for heart disease. Dr. Medeiros is a Faculty member in the Department of Human Nutrition and Food Management at The Ohio State University.

Women between ages 18-45 and 55-80 are needed as participants. All participants should be non-smokers, non-vegetarians and not hypertensive (with or without medication).

If you are interested in helping us learn more about the risk for heart disease in older diabetic women contact:

Dr. Lydia Medeiros
Dept. of Human Nutrition and Food Management
1787 Neil Avenue
Columbus, OH 43210-1295
(614) 292-2699
APPENDIX M

PRE-SCREENING FORM
INSULIN AND ESTROGEN CONTROL OF ESSENTIAL FATTY ACID DESATURATION
PRE-SCREENING FORM

Office Use Code # ________________

Name ________________________________
Address ________________________________

City __________________________________
State _____________________________
Zip Code ________________

Telephone number Day time ( )
Night time ( )

Your age ________________

Do you use tobacco products of any kind? Yes ____ No ____
If yes, specify type: _____________________________
Amount used daily: _____________________________

Do you drink alcoholic beverages? Yes ____ No ____
If yes, number of drinks/day: _____________________________

What is your height? ________________ (ft,in) ________________ (cm)
What is your weight? ________________ (lbs) ________________ (kg)
[Calculate BMI: ________________ (wt/lft^2)]

Do you have diabetes? Yes ____ No ____
If yes, at what age were you first diagnosed? ________________ (yr)
Do you take insulin injections daily? Yes ____ No ____
Do you take oral hypoglycemic medications? Yes ____ No ____

Is there any chance that you are pregnant or could become pregnant in the next 90 days?
Yes ____ No ____
Do you have a monthly menstrual period? Yes ____ No ____
If yes, are you experiencing any abnormalities? Yes ____ No ____
If yes, please describe briefly: _____________________________

Are you taking oral contraceptives? Yes ____ No ____
If no, are you post menopausal? Yes ____ No ____
Are you taking estrogen replacement medications? Yes ____ No ____

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Do your principles or religious beliefs prevent you from eating:

- Red meats (beef, pork, lamb, wild game)?
- Poultry (chicken, turkey, etc)?
- Fish or seafood?
- Dairy products?
- Eggs?

Do you have high blood pressure? Yes _____ No _____
Do you take medication for high blood pressure? Yes _____ No _____
If yes, what type of medication do you take? __________________________
Dosage __________ per day.
What is your blood pressure? __________________________ (mmHg)

[Note: The blood pressure asked for on this form is for screening purposes only, since it is a self-declared value. If the subject is found to be hypertensive when BP is actually measured at the first visit, they will not be able to continue the study. Please make this clear to the potential subject during pre-screening]

Do you take aspirin or aspirin-based pain relievers? Yes _____ No _____
If yes, dosage ______ per day
Type: Adult aspirin _______ Baby aspirin _______
Brand name: _________________________________________________________

Do you take any other medications for pain? Yes _____ No _____
If yes, what is the name of the medication: ___________________________________________

[Check the list of aspirin, aspirin-based and NSAID drugs to see if the medications used by the subject disqualifies them from participation in the study.]

Accepted for participation: Yes _____ No _____
APPENDIX N

CONSENT TO INVESTIGATIONAL TREATMENT OR PROCEDURE
CONSENT TO INVESTIGATIONAL TREATMENT OR PROCEDURE

I, ___________________________, hereby authorize or direct Dr. Lydia Medeiros and associated of her choosing, to perform the following treatment or procedure (describe in general terms),

You are part of a study in which two groups of women (aged 18-45 or aged 55-80) will be compared. You should be a non-smokers, non-vegetarian and normotensive with or without medication. One group at each age is normal weight and non-diabetic, and the other group at each age is diabetic and perhaps overweight or obese. You should not be taking aspirin, aspirin-based or anti-inflammatory steroid drugs. If you are a young woman, you should not be pregnant or using oral contraceptives. Older women should be past menopause and not taking estrogen. As part of this study I will be asked to do these things:
1. I will complete a diet history and 3-day dietary record to learn HISTORICAL and USUAL daily food intake.
2. My height (without shoes), weight (light, in-door clothing) and my blood pressure will be measured.
3. I will provide a medical history to learn about chronic medical conditions and medications, including nutrient supplements, that could effect the study.
4. I will provide two samples of urine to test how my kidneys are working.
5. I will have a blood sample drawn from a vein in my arm. The total amount of blood drawn at each of two visits will be 21 cc or approximately 1 oz. (about 5 teaspoons).
6. I will make three visits to the metabolic laboratory of the Dept, of Human Nutrition and Food Management on the 3rd floor of Campbell Hall on the OSU campus. The building is handicapped accessed.

The experimental (research) portion of the treatment or procedure is:

Diabetes can lead to heart disease in later life. Young and older women with or without diabetes will be compared to learn the influence of gender and aging on fat production and function in the blood. Serum and platelet fatty acids, serum triglycerides, cholesterol, and lipoprotein cholesterol, insulin and estrogen hormones, and platelet aggregation will be measured to study the effects of aging and diabetes on these measurements, and to learn more about the reason why diabetic women have different risk for heart disease.
This is done as part of an investigation entitled:

Insulin and Estrogen Control of Essential Fatty Acid Desaturation?

1. Purpose of the procedure or treatment:

Information learned from this study will help us learn why diabetes can increase the risk for heart disease.

2. Possible appropriate alternative procedures or treatment (not to participate in the study is always an option):

I may choose not to participate in this study.

3. Discomforts and risks reasonably to be expected:

There should be no risk or discomforts caused by the collection of diet history, medical history, height, weight, urine sample or blood pressure. There could be minimal risk and discomfort caused by the collection of the blood sample. Bruising and/or fainting could occur. A trained, experienced medical technologist will draw the blood sample. The risks and discomforts of blood drawing would include pain from the needle, light-headedness, fainting, bruising, and rarely, infection. Sterile, disposable blood collecting supplies will be used. I may feel light-headed because you will be asking me to not eat or drink anything more than water after 9:00 pm the night before blood collection. Breakfast will be provided as soon as blood has been collected. I will be asked to not take aspirin, aspirin-based or anti-inflammatory steroid drugs two weeks prior to blood collection because these drugs interfere with the blood chemical tests being done as part of this study.

4. Possible benefits for subjects/society:

I will receive $50.00 for participation at the completion of all data collection from me. Also, by participating in this study, I will have the satisfaction of knowing that the nutritional needs of aging women will be better understood.

5. Anticipated duration of subject's participation (including number of visits):

I will be asked to come to the metabolic laboratory of the Endocrinology Clinic on the 5th floor of McCampbell Hall three times. This visit can occur at any time during the day since blood will not be collected and I will not be fasting. Instructions will be given on the collection of a three-day dietary intake record that I will complete at home on the days the investigator assigns to me. This will require approximately 5 minutes of recording after each meal for three days. I will provide a small urine sample so that we can be sure there are no problems with my kidneys. A medical conditions history, height, weight, and blood pressure
will be recorded during this visit. The first visit will require approximately one hour to complete the various forms. On the second visit, I will be fasting and breakfast will be served after blood collection. Another urine sample will be collected. This will require approximately one hour, including the time spent eating breakfast. Blood will be collected again and breakfast served on the third visit. No other data will be collected so the visit will require approximately 30 minutes.

I hereby acknowledge that Dr. Lydia Medeiros has provided information about the procedure described above, about my rights as a subject, and she answered all questions to my satisfaction. I understand that I may contact her at Phone No. 292-2699 should I have additional questions. She has explained the risks described above and I understand them; she has also offered to explain all possible risks or complications.

I understand that, where appropriate, the U.S. Food and Drug Administration may inspect records pertaining to this study. I understand further that records obtained during my participation in this study that may contain my name or other personal identifiers may be made available to the sponsor of this study. Beyond this, I understand that my participation will remain confidential.

I understand that I am free to withdraw my consent and participation in this project at any time after notifying the project director without prejudicing future care. No guarantee has been given to me concerning this treatment or procedure.

I understand in signing this form that, beyond giving consent, I am not waiving any legal rights that I might otherwise have, and I am not releasing the investigator, the sponsor, the institution, or its agents from any legal liability for damages that they might otherwise have.

In the event of injury resulting from participation in this study, I also understand that immediate medical treatment is available at University Hospitals of the Ohio State University and that the costs of such treatment will be at my expense; financial compensation beyond that required by law is not available. Questions about this should be directed to the Office of Research Risks at 292-5958.

I have read and fully understand the consent form. I sign it freely and voluntarily. A copy has been given to me.

Date: ___________ Time _______ pm Signed ____________________________ (subject)

Witness (es) ________________________________
If Required ________________________________ (Person Authorized to Consent for Subject if Required)
I certify that I have personally completed all blanks in this form and explained them to the subject or his/her representative before requesting the subject or his/her representative to sign it.

Date: ___________________ Signed: _____________________________________

(Signature of Project Director or her Authorized Representative)
APPENDIX O

MEDICAL CONDITIONS QUESTIONNAIRE
INSULIN AND ESTROGEN CONTROL OF ESSENTIAL FATTY
ACID DESATURATION
MEDICAL CONDITIONS QUESTIONNAIRE

Office Use Code # ___________________

Do you consider your residence: Rural ______ Suburban ______ Urban ______

Marital status ______

Circle the highest grade or year of regular school you have attended?

Never attended school; or attended kindergarten only ______
Elementary 1 2 3 4 5 6 7 8
High school 9 10 11 12
College 1 2 3 4 5+

Did you complete the year marked above? Yes ______ No ______

If you are post-menopausal:
did you experience surgical menopause? Yes ______ No ______ If yes, at what age ______
did you experience natural menopause? Yes ______ No ______ If yes, at what age ______

Have you had any recent weight changes? Yes ______ No ______

If yes, please describe ____________________________

Does your work or daily activity primarily involve the following?

______ sitting
______ standing
______ walking or other active exercise
______ heavy labor (such as heavy lifting, etc.)

Outside of your normal work or daily responsibilities, how often do you engage in exercise of 20 minutes or more which markedly increases your breathing (such as rigorous walking, cycling, running, swimming)?

______ seldom or never
______ less than one time per week
______ one or two times per week
______ three to five times per week
______ six or more times per week

Are you on any type of special diet? Yes ______ No ______

If yes, please specify: ____________________________

How long have you been on this diet? ____________________________

Food Allergies, or foods disliked or avoided: ____________________________

251
Other factors interfering with eating or digestion of food:  Yes _____ No _____
If yes, please specify: ________________________________________________________________

Check-off the following health conditions that you have continued to experience in the past years or had diagnosed by a health practitioner or physician in the last year?

<table>
<thead>
<tr>
<th>Conditions</th>
<th>In Past (before '91):</th>
<th>Present ('93 or '94):</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other allergy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuberculosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other respiratory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid goiter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (without insulin)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (with insulin)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hernia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histiocytosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gall bladder disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary tract infections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheumatic fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

List other disease conditions: ____________________________________________________________


Please list any drugs or medications you are currently taking:

<table>
<thead>
<tr>
<th>Name of Drug:</th>
<th>Taken how often:</th>
<th>Amount taken:</th>
<th>Why prescribed:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

List additional drugs on back of page:

Do you take vitamins/minerals and/or nutritional supplements? Yes ______ No ______
If yes, how frequently? Regularly ______ Occasionally _____
Taken within the last week? Yes ______ No ______

Please indicate the extent to which you personally have taken any of the following combinations and/or single supplements within the last week. If you are not taking any particular brand supplement, leave the space blank.

<table>
<thead>
<tr>
<th>Type:</th>
<th>Brand/type:</th>
<th># Months taken:</th>
<th>Dosage/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple vitamins/ minerals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple vitamins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple vitamins w/ iron</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geritol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple vitamins w/ minerals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish oil pills</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others not listed above</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single supplements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rose hips</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dolomite</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone meal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oyster shell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tums</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other calcium supp.</td>
<td>(specify)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evening primrose oil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others not listed:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Weight: ________ (lb) ________ (kg) Height: ________ (ft.in) ________ (cm)

BMI (wt/ht<sup>2</sup>): __________________________________________

Fasting glucose: __________________________________________

Blood Pressure:

<table>
<thead>
<tr>
<th></th>
<th>1st visit:</th>
<th>2nd visit:</th>
<th>3rd visit:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulse pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
For pre-menopausal subjects only:
Date of first day of your last menstrual period: ________________________________

Date of first blood collection: ____________________ Time: ________________

Date of second blood collection: ____________________ Time: ________________

DO NOT EAT OR DRINK ANYTHING AFTER 9:00 PM THE NIGHT BEFORE BLOOD COLLECTION VISITS
APPENDIX P

ONE-YEAR FOOD FREQUENCY QUESTIONNAIRE
The purpose of this questionnaire is to investigate your usual intake of the food items in the following tables. We want to know how much and how often you ate the foods listed during the last year. For example, if you usually drank 2 cups of skim milk every day in the past year, just put 2 in the "Day" column in the example table 1:

Example Table 1

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>2</td>
<td>1 cup</td>
<td>skim/nouf fat milk</td>
</tr>
</tbody>
</table>

Example 2. If you usually ate 2 oz of round steak every week in the past year, write down 2.3 in the "Week" column in the example table 2: (7 oz / 3 oz = 2.3 or 2 1/3 servings per week, because 1 serving of round steak for us is 3 oz.)

Example Table 2

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4255</td>
<td>2.3</td>
<td>3 oz</td>
<td>round steak-choice grade (full cut)</td>
</tr>
</tbody>
</table>

There is no need to write in the "code" column. All you need to do is to pay attention to the serving size and food name and to put the proper number of servings that you usually ate in a day, a week, or a month. If you did not eat the food or you ate the food less than one serving size per month in the last year, simply leave the item blank.

For seasonal foods (like fresh berries in the summer), just estimate how much you ate in the months they were available and then spread that serving number out over 12 months. Write in how many servings you ate per month.

Dairy and Nondairy Foods

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td></td>
<td>1 cup</td>
<td>skim/nouf fat milk</td>
</tr>
<tr>
<td>51</td>
<td></td>
<td>1 cup</td>
<td>2% fat/lowfat milk</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>1 cup</td>
<td>3.3% fat/whole milk</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>1 tbsp</td>
<td>cream-Half &amp; Half (fluid)</td>
</tr>
<tr>
<td>Code</td>
<td>Servings per:</td>
<td>Serving Size</td>
<td>Food Name/Description</td>
</tr>
<tr>
<td>------</td>
<td>---------------</td>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>1 tbsp</td>
<td>cream-nondairy (powder)</td>
</tr>
<tr>
<td>3670</td>
<td></td>
<td>1 tbsp</td>
<td>sour cream</td>
</tr>
<tr>
<td>85</td>
<td></td>
<td>1 cup</td>
<td>sherbet</td>
</tr>
<tr>
<td>78</td>
<td></td>
<td>1 cup</td>
<td>ice cream</td>
</tr>
<tr>
<td>3811</td>
<td></td>
<td>6 oz</td>
<td>plainfruit yogurt</td>
</tr>
<tr>
<td>5244</td>
<td></td>
<td>1 cup</td>
<td>frozen yogurt</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>1 oz</td>
<td>cream cheese</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>1 oz or 1 slice</td>
<td>cheese (processed or aged) e.g. American, cheddar, plain, others, or as a part of a dish)</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>1 cup</td>
<td>fresh cheese (cottage or ricotta)</td>
</tr>
</tbody>
</table>

**Fats and Oils**

* Vegetable oil is followed by different kinds of oil sources. Please circle the one that you use the most frequently in the last year.

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td></td>
<td>1 tbsp</td>
<td>vegetable oil* e.g. corn, canola, sunflower, safflower, olive, soybean, peanut, etc.</td>
</tr>
<tr>
<td>824</td>
<td></td>
<td>1 pat</td>
<td>stick margarine</td>
</tr>
<tr>
<td>5434</td>
<td></td>
<td>1 tbsp</td>
<td>tub margarine</td>
</tr>
<tr>
<td>110</td>
<td></td>
<td>1 tbsp</td>
<td>diet margarine</td>
</tr>
<tr>
<td>105</td>
<td></td>
<td>1 pat</td>
<td>butter</td>
</tr>
<tr>
<td>7045</td>
<td></td>
<td>1 tbsp</td>
<td>peanut butter - chunky / creamy</td>
</tr>
<tr>
<td>2806</td>
<td></td>
<td>1 oz</td>
<td>nuts</td>
</tr>
<tr>
<td>1980</td>
<td></td>
<td>1 tbsp</td>
<td>wheat germ</td>
</tr>
<tr>
<td>708</td>
<td></td>
<td>1 cup</td>
<td>cream soup/ chowder-prepared with milk</td>
</tr>
<tr>
<td>715</td>
<td></td>
<td>1 cup</td>
<td>cream soup/ chowder-prepared with water</td>
</tr>
<tr>
<td>138</td>
<td></td>
<td>1 tbsp</td>
<td>clear salad dressings, e.g. Italian, Caesar, or red wine vinegar</td>
</tr>
</tbody>
</table>

258
<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>137</td>
<td></td>
<td>1 tbsp</td>
<td>clear salad dressings, light, e.g. light Italian, Caesar, or red wine vinegar</td>
</tr>
<tr>
<td>1205</td>
<td></td>
<td>1 tbsp</td>
<td>clear salad dressings, fat-free, e.g. nonfat Italian, French, or red wine vinegar</td>
</tr>
<tr>
<td>139</td>
<td></td>
<td>1 tbsp</td>
<td>mayonnaise/creamy salad dressings, e.g. Thousand Island, French, blue cheese, Ranch, Catalina, honey Dijon</td>
</tr>
<tr>
<td>140</td>
<td></td>
<td>1 tbsp</td>
<td>mayonnaise/creamy salad dressings, light, e.g. Thousand Island, French, blue cheese, Ranch, Catalina, honey Dijon</td>
</tr>
<tr>
<td>1208</td>
<td></td>
<td>1 tbsp</td>
<td>mayonnaise/creamy salad dressings, nonfat, e.g. Thousand Island, French, blue cheese, Ranch, Catalina, honey Dijon</td>
</tr>
</tbody>
</table>

### Fruits and Fruit Juices

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2501</td>
<td></td>
<td>1 oz</td>
<td>raisins-seedless-natural</td>
</tr>
<tr>
<td>2556</td>
<td></td>
<td>10</td>
<td>grapes-fresh</td>
</tr>
<tr>
<td>304</td>
<td></td>
<td>10</td>
<td>prunes-dried-uncooked</td>
</tr>
<tr>
<td>235</td>
<td></td>
<td>1</td>
<td>bananas-fresh-peeled</td>
</tr>
<tr>
<td>5633</td>
<td></td>
<td>1 cup</td>
<td>peaches-canned- halves/slices</td>
</tr>
<tr>
<td>283</td>
<td></td>
<td>1</td>
<td>peaches-fresh-whole</td>
</tr>
<tr>
<td>6422</td>
<td></td>
<td>1</td>
<td>plums-fresh</td>
</tr>
<tr>
<td>223</td>
<td></td>
<td>1</td>
<td>apples-fresh-with skin- 2 3/4 inch diameter</td>
</tr>
<tr>
<td>3049</td>
<td></td>
<td>1 cup</td>
<td>pears-canned-4 syrup pack</td>
</tr>
<tr>
<td>259</td>
<td></td>
<td>1</td>
<td>pears-fresh</td>
</tr>
<tr>
<td>225</td>
<td></td>
<td>1 cup</td>
<td>apple juice or cider</td>
</tr>
<tr>
<td>273</td>
<td></td>
<td>1</td>
<td>oranges-fresh-whole</td>
</tr>
<tr>
<td>Code</td>
<td>Servings per:</td>
<td>Serving Size</td>
<td>Food Name/Description</td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>--------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>275</td>
<td></td>
<td>1 cup</td>
<td>orange juice-fresh</td>
</tr>
<tr>
<td>248</td>
<td></td>
<td>1/2</td>
<td>grapefruit-white, pink &amp; red-fresh</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td>1 cup</td>
<td>grapefruit juice-unsweetened</td>
</tr>
<tr>
<td>6010</td>
<td></td>
<td>1 cup</td>
<td>other fruit juice</td>
</tr>
<tr>
<td>1035</td>
<td></td>
<td>1 cup</td>
<td>strawberries-canned</td>
</tr>
<tr>
<td>313</td>
<td></td>
<td>1 cup</td>
<td>strawberries-fresh/frozen-whole</td>
</tr>
<tr>
<td>238</td>
<td></td>
<td>1 cup</td>
<td>blueberries/raspberries-fresh/frozen</td>
</tr>
<tr>
<td>271</td>
<td></td>
<td>1 cup</td>
<td>cantaloupe-fresh-cubed pieces</td>
</tr>
<tr>
<td>5640</td>
<td></td>
<td>1 cup</td>
<td>pineapple-canned</td>
</tr>
<tr>
<td>318</td>
<td></td>
<td>1 cup</td>
<td>watermelon-fresh</td>
</tr>
</tbody>
</table>

### Vegetables

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>588</td>
<td></td>
<td>1/2 cup</td>
<td>broccoli-raw-boiled-drained</td>
</tr>
<tr>
<td>594</td>
<td></td>
<td>1/2 cup</td>
<td>cabbage or cole slaw</td>
</tr>
<tr>
<td>608</td>
<td></td>
<td>1/2 cup</td>
<td>cauliflower-raw-boiled-drained</td>
</tr>
<tr>
<td>591</td>
<td></td>
<td>1/2 cup</td>
<td>brussels sprouts-raw-boiled</td>
</tr>
<tr>
<td>613</td>
<td></td>
<td>1 ear</td>
<td>corn on the cob</td>
</tr>
<tr>
<td>617</td>
<td></td>
<td>1/2 cup</td>
<td>corn-canned-drained</td>
</tr>
<tr>
<td>641</td>
<td></td>
<td>1/2 cup</td>
<td>peas-frozen-boiled-drained</td>
</tr>
<tr>
<td>8601</td>
<td></td>
<td>1</td>
<td>carrots-raw-2 3/4&quot;</td>
</tr>
<tr>
<td>602</td>
<td></td>
<td>1/2 cup</td>
<td>carrots-boiled-drained-sliced</td>
</tr>
<tr>
<td>619</td>
<td></td>
<td>1/2 cup</td>
<td>cucumber-raw-sliced</td>
</tr>
<tr>
<td>569</td>
<td></td>
<td>1/2 cup</td>
<td>green/yellow beans-boiled-drained</td>
</tr>
<tr>
<td>7056</td>
<td></td>
<td>1/2 cup</td>
<td>mixed vegetable-frozen-boiled</td>
</tr>
<tr>
<td>630</td>
<td></td>
<td>1/2 cup</td>
<td>mushrooms-raw-chopped</td>
</tr>
<tr>
<td>633</td>
<td></td>
<td>1/2 cup</td>
<td>onion-raw-chopped</td>
</tr>
<tr>
<td>643</td>
<td></td>
<td>1/2</td>
<td>sweet pepper-raw</td>
</tr>
</tbody>
</table>

260
<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per Day</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>665</td>
<td></td>
<td>1/2 cup</td>
<td>winter squash-bake</td>
</tr>
<tr>
<td>1066</td>
<td></td>
<td>1/2 cup</td>
<td>eggplant, zucchini, summer squash</td>
</tr>
<tr>
<td>1136</td>
<td></td>
<td>1</td>
<td>yams or sweet potatoes-boiled or baked</td>
</tr>
<tr>
<td>660</td>
<td></td>
<td>1/2 cup</td>
<td>spinach-boiled-drained</td>
</tr>
<tr>
<td>859</td>
<td></td>
<td>1 cup</td>
<td>spinach-raw-chopped</td>
</tr>
<tr>
<td>628</td>
<td></td>
<td>1 cup</td>
<td>iceberg lettuce-raw-chopped</td>
</tr>
<tr>
<td>1865</td>
<td></td>
<td>1 cup</td>
<td>romaine lettuce-raw-shredded</td>
</tr>
<tr>
<td>671</td>
<td></td>
<td>1</td>
<td>tomatoes-raw</td>
</tr>
<tr>
<td>675</td>
<td></td>
<td>1 cup</td>
<td>tomato juice-canned</td>
</tr>
<tr>
<td>1899</td>
<td></td>
<td>1 cup</td>
<td>tomato sauce-canned</td>
</tr>
<tr>
<td>522</td>
<td></td>
<td>1 cup</td>
<td>lentils-whole-cooked</td>
</tr>
</tbody>
</table>

Eggs and Egg Products

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per Day</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td></td>
<td>1</td>
<td>egg white</td>
</tr>
<tr>
<td>99</td>
<td></td>
<td>1</td>
<td>egg yolk</td>
</tr>
<tr>
<td>99</td>
<td></td>
<td>1</td>
<td>egg-fried in butter/margarine-whole</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>1</td>
<td>egg hard cooked/ poached</td>
</tr>
<tr>
<td>102</td>
<td></td>
<td>1</td>
<td>egg-scrambled with milk and butter/margarine</td>
</tr>
<tr>
<td>5228</td>
<td></td>
<td>1</td>
<td>egg-whole-omelet - buttern/margarine and salt</td>
</tr>
</tbody>
</table>

Beef

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per Day</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4255</td>
<td></td>
<td>3 oz</td>
<td>round steak- choice grade (full cut, bottom, or round)- braised/roasted</td>
</tr>
<tr>
<td>Code</td>
<td>Servings per:</td>
<td>Serving Size</td>
<td>Food Name/Description</td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>--------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>4424</td>
<td></td>
<td>3 oz</td>
<td>hamburger patty-medium fat-broiled-well done</td>
</tr>
<tr>
<td>4184</td>
<td></td>
<td>3 oz</td>
<td>chuck roast-choice grade-blade cut-braised-fat trimmed</td>
</tr>
<tr>
<td>4386</td>
<td></td>
<td>3 oz</td>
<td>top sirloin or strip steak-choice grade-broiled-fat trimmed</td>
</tr>
</tbody>
</table>

**Pork**

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1319</td>
<td></td>
<td>3 oz</td>
<td>ham-whole-fully cooked-roasted</td>
</tr>
<tr>
<td>5679</td>
<td></td>
<td>3 oz</td>
<td>pork chop(center loin chop)-broiled-lean-fat trimmed</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>1 patty or link</td>
<td>sausage patty or link (fresh pork)</td>
</tr>
<tr>
<td>161</td>
<td></td>
<td>1 slice</td>
<td>bacon-broiled, pen fried or roasted</td>
</tr>
</tbody>
</table>

**Luncheon Meat**

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>202</td>
<td></td>
<td>1</td>
<td>frankfurter(hot dog)-meat only, no bun</td>
</tr>
<tr>
<td>168</td>
<td></td>
<td>1 slice</td>
<td>bologna-cured pork</td>
</tr>
</tbody>
</table>

**Poultry**

(Light meat includes meat from back and breast. Dark meat includes meat from leg, thigh, and wing.)

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3180</td>
<td></td>
<td>3 oz</td>
<td>chicken-light meat with skin-flour coated-fried</td>
</tr>
<tr>
<td>3181</td>
<td></td>
<td>3 oz</td>
<td>chicken-light meat with skin-roasted</td>
</tr>
<tr>
<td>Code</td>
<td>Servings per</td>
<td>Serving Size</td>
<td>Food Name/Description</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
<td>--------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>3185</td>
<td></td>
<td>3 oz</td>
<td>chicken-dark meat with skin-flour coated-fried</td>
</tr>
<tr>
<td>3186</td>
<td></td>
<td>3 oz</td>
<td>chicken-dark meat with skin-roasted</td>
</tr>
<tr>
<td>3188</td>
<td></td>
<td>3 oz</td>
<td>chicken-light meat without skin-fried</td>
</tr>
<tr>
<td>3189</td>
<td></td>
<td>3 oz</td>
<td>chicken-light meat without skin-roasted</td>
</tr>
<tr>
<td>3191</td>
<td></td>
<td>3 oz</td>
<td>chicken-dark meat without skin-fried</td>
</tr>
<tr>
<td>3192</td>
<td></td>
<td>3 oz</td>
<td>chicken-dark meat without skin-roasted</td>
</tr>
<tr>
<td>3246</td>
<td></td>
<td>3 oz</td>
<td>turkey-light meat with skin-roasted</td>
</tr>
<tr>
<td>3247</td>
<td></td>
<td>3 oz</td>
<td>turkey-dark meat with skin-roasted</td>
</tr>
</tbody>
</table>

**Lamb and Veal**

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>184</td>
<td></td>
<td>3 oz</td>
<td>lamb-leg-lean and fat-roasted</td>
</tr>
<tr>
<td>3275</td>
<td></td>
<td>3 oz</td>
<td>lamb-loin-lean and fat-broiled</td>
</tr>
<tr>
<td>3308</td>
<td></td>
<td>3 oz</td>
<td>veal-leg-lean and fat-roasted</td>
</tr>
<tr>
<td>3314</td>
<td></td>
<td>3 oz</td>
<td>veal-loin-lean and fat-roasted</td>
</tr>
</tbody>
</table>

**Seafood**

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2942</td>
<td></td>
<td>3 oz</td>
<td>salmon-Atlantic-cooked-dry heat</td>
</tr>
<tr>
<td>1833</td>
<td></td>
<td>3 oz</td>
<td>pollock-Atlantic-cooked-dry heat/breaded(e.g.,Gorton's®/Van de Kempe® fish sticks and patties)</td>
</tr>
<tr>
<td>1847</td>
<td></td>
<td>3 oz</td>
<td>whiting-cooked-dry heat/breaded(e.g., Mrs. Paul's® fish sticks and patties)</td>
</tr>
</tbody>
</table>
### Food Name/Description

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1573</td>
<td>3 oz</td>
<td>3 oz</td>
<td>cod-Atlantic-cooked-dry heat/breaded (e.g. Kroger® fish sticks and patties)</td>
</tr>
<tr>
<td>1820</td>
<td>3 oz</td>
<td>3 oz</td>
<td>catfish-breaded-fried</td>
</tr>
<tr>
<td>159</td>
<td>3 oz</td>
<td>3 oz</td>
<td>tuna-canned in oil-chunk/solid</td>
</tr>
<tr>
<td>3900</td>
<td>3 oz</td>
<td>3 oz</td>
<td>tuna-canned in water-chunk/solid</td>
</tr>
<tr>
<td>2962</td>
<td>3 oz</td>
<td>3 oz</td>
<td>tuna-yellowfin-fresh</td>
</tr>
<tr>
<td>8071</td>
<td>3 oz</td>
<td>3 oz</td>
<td>shrimp-mixed species</td>
</tr>
<tr>
<td>158</td>
<td>3 oz</td>
<td>3 oz</td>
<td>shrimp-breaded-fried/fried/popped/bifteny</td>
</tr>
<tr>
<td>1844</td>
<td>3 oz</td>
<td>3 oz</td>
<td>trout-rainbow-cooked-dry heat</td>
</tr>
<tr>
<td>1831</td>
<td>3 oz</td>
<td>3 oz</td>
<td>perch-cooked-dry heat</td>
</tr>
<tr>
<td>2909</td>
<td>3 oz</td>
<td>3 oz</td>
<td>haddock</td>
</tr>
<tr>
<td>6089</td>
<td>1 cup</td>
<td>1 cup</td>
<td>scallops-mixed species</td>
</tr>
<tr>
<td>155</td>
<td>1</td>
<td>1</td>
<td>scallop-frozen-breaded-fried</td>
</tr>
<tr>
<td>2931</td>
<td>3 oz</td>
<td>3 oz</td>
<td>roughy-orange</td>
</tr>
<tr>
<td>2976</td>
<td>1</td>
<td>1</td>
<td>lobster-raw-all varieties</td>
</tr>
<tr>
<td>1880</td>
<td>1</td>
<td>1</td>
<td>clams-cooked-moist heat</td>
</tr>
<tr>
<td>1882</td>
<td>1</td>
<td>1</td>
<td>mussels-blue-cooked-moist heat</td>
</tr>
<tr>
<td>2971</td>
<td>1</td>
<td>1</td>
<td>crab-Alaska king</td>
</tr>
</tbody>
</table>

### Desserts

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>409</td>
<td>Day Week Month</td>
<td>1 slice = 1/6 of rectangular pound cake (35g=2 oz)</td>
<td>shortening cake, e.g. yellow cake, Devil's food cake, pound cake, marble cake, gingerbread, etc.</td>
</tr>
<tr>
<td>411</td>
<td>Day Week Month</td>
<td>1 slice = 1/12 of 10” cake (63 g=2 oz)</td>
<td>cakes; chiffon, sponge and Angel’s food cake</td>
</tr>
<tr>
<td>7882</td>
<td>Day Week Month</td>
<td>1 slice = 1/8 of 8” two-layer cake of 1/6 (60 g=3 oz)</td>
<td>cake with icing</td>
</tr>
<tr>
<td>7849</td>
<td>Day Week Month</td>
<td>1 slice = 1/4 of 7” cake (121 g=4 oz)</td>
<td>cheese cake</td>
</tr>
<tr>
<td>Code</td>
<td>Servings per:</td>
<td>Serving Size</td>
<td>Food Name/Description</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
<td>--------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>454</td>
<td></td>
<td>1 slice = 1/8 of 9&quot; pie (155g = 5 oz)</td>
<td>fruit pie</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 slice = 1/5 of 9&quot; pie (138g = 5 oz)</td>
<td>cream pie</td>
</tr>
<tr>
<td>4486</td>
<td></td>
<td>1 (39 g = 1 oz)</td>
<td>sweet roll or other pastry</td>
</tr>
<tr>
<td>7291</td>
<td></td>
<td>1 (16 g = 1 oz)</td>
<td>cookies-ready made</td>
</tr>
<tr>
<td>413</td>
<td></td>
<td>1 (28 g = 1 oz)</td>
<td>brownies - 1 3/4&quot; x 3/4&quot;</td>
</tr>
<tr>
<td>437</td>
<td></td>
<td>1 (50 g = 2 oz)</td>
<td>doughnuts-glazed</td>
</tr>
<tr>
<td>538</td>
<td></td>
<td>1 oz</td>
<td>small chocolate bar or 1 oz chocolate pieces (= 8 Hershey's Kisses®)</td>
</tr>
<tr>
<td>1783</td>
<td></td>
<td>1</td>
<td>candy bar-regular (e.g. Milky Way® etc)</td>
</tr>
<tr>
<td>544</td>
<td></td>
<td>1 oz</td>
<td>candy without chocolate</td>
</tr>
<tr>
<td>549</td>
<td></td>
<td>1 tbsp</td>
<td>jams, jellies, preserves, syrup or honey</td>
</tr>
</tbody>
</table>

**Breads, Cereals, and Snacks**

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>366</td>
<td>Day Week Month</td>
<td>1 cup</td>
<td>cooked breakfast cereal</td>
</tr>
<tr>
<td>372</td>
<td></td>
<td>1 cup</td>
<td>cold breakfast cereal/frosted</td>
</tr>
<tr>
<td>1211</td>
<td></td>
<td>1 cup</td>
<td>cold breakfast cereal-not frosted</td>
</tr>
<tr>
<td>341</td>
<td></td>
<td>1 slice</td>
<td>white bread</td>
</tr>
<tr>
<td>358</td>
<td></td>
<td>1 slice</td>
<td>dark bread (e.g. rye, pumpernickel, etc.)</td>
</tr>
<tr>
<td>489</td>
<td></td>
<td>1</td>
<td>roll hotdog bun or hamburger</td>
</tr>
<tr>
<td>1406</td>
<td></td>
<td>1 (60 g = 2 oz)</td>
<td>pita</td>
</tr>
<tr>
<td>1391</td>
<td></td>
<td>1 (25 g = 1 oz)</td>
<td>tortilla-corn</td>
</tr>
<tr>
<td>1689</td>
<td></td>
<td>1 (35 g = 1 oz)</td>
<td>tortilla-flour</td>
</tr>
<tr>
<td>319</td>
<td></td>
<td>1</td>
<td>English muffin or bagel</td>
</tr>
<tr>
<td>448</td>
<td></td>
<td>1</td>
<td>muffins or biscuits</td>
</tr>
<tr>
<td>2877</td>
<td></td>
<td>1 cup</td>
<td>pasta, spaghetti, egg noodles</td>
</tr>
</tbody>
</table>

265
### Cod* Sarvinoa

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>452</td>
<td>1</td>
<td>1</td>
<td>pancakes or waffles</td>
</tr>
<tr>
<td>129</td>
<td>1 cup</td>
<td>1 cup</td>
<td>rice-brown-cooked</td>
</tr>
<tr>
<td>484</td>
<td>1 cup</td>
<td>1 cup</td>
<td>rice-white-cooked</td>
</tr>
<tr>
<td>1144</td>
<td>1 (202 g = 7 oz)</td>
<td>1 cup</td>
<td>potatoes-baked-flesh &amp; skin-whole</td>
</tr>
<tr>
<td>5798</td>
<td>1 cup</td>
<td>1 cup</td>
<td>potatoes-French fried</td>
</tr>
<tr>
<td>853</td>
<td>1 cup</td>
<td>1 cup</td>
<td>potatoes-mashed</td>
</tr>
<tr>
<td>654</td>
<td>10 chip</td>
<td>10 chip</td>
<td>potato tortilla or corn chips</td>
</tr>
<tr>
<td>7305</td>
<td>4</td>
<td>4</td>
<td>large crackers, e.g. Keebler® saltine Zesta</td>
</tr>
<tr>
<td>1651</td>
<td>6</td>
<td>6</td>
<td>small crackers, e.g. WheatThin®</td>
</tr>
<tr>
<td>1083</td>
<td>1 slice (≈140g)</td>
<td>1 slice</td>
<td>pizza* (add meat on pizza to individual meat name)</td>
</tr>
<tr>
<td>1081</td>
<td>1 ring</td>
<td>1 ring</td>
<td>onion rings</td>
</tr>
<tr>
<td>478</td>
<td>1 cup</td>
<td>1 cup</td>
<td>popcorn-plain popped</td>
</tr>
<tr>
<td>477</td>
<td>1 cup</td>
<td>1 cup</td>
<td>popcorn-popped with oil &amp; salt</td>
</tr>
</tbody>
</table>

### Beverages

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2391</td>
<td>Day</td>
<td>1 can(12oz)</td>
<td>diet carbonated soft drinks - caffeine free</td>
</tr>
<tr>
<td>693</td>
<td>Week</td>
<td>1 can(12oz)</td>
<td>regular carbonated soft drinks - caffeine free</td>
</tr>
<tr>
<td>1718</td>
<td>Month</td>
<td>1 can(12oz)</td>
<td>sweetened noncarbonated fruit drinks, e.g. Hawaiian Punch® Kool-Aid® etc</td>
</tr>
<tr>
<td>887</td>
<td></td>
<td>1 fl.oz</td>
<td>liquor purchased at supermarket</td>
</tr>
<tr>
<td>867</td>
<td></td>
<td>1 fl.oz</td>
<td>liquor, e.g. Whiskey, Gin, Rum, Vodka, etc</td>
</tr>
<tr>
<td>891</td>
<td></td>
<td>3 fl.oz</td>
<td>red wine</td>
</tr>
<tr>
<td>1481</td>
<td></td>
<td>3 fl.oz</td>
<td>white wine</td>
</tr>
<tr>
<td>888</td>
<td></td>
<td>1 can or 1 bottle</td>
<td>beer</td>
</tr>
<tr>
<td>871</td>
<td></td>
<td>1 can or 1 bottle</td>
<td>light beer</td>
</tr>
</tbody>
</table>
The following table is for you to fill out if you have some foods that you usually ate in the past year, but they are not listed on the questionnaire. Leave the "code" column blank.

Still, there are foods, such as sugar, whipped cream, salt, that you put in or on top of the food before your first bite. Please write down the food name, the usual serving size, and the number of serving size you usually ate in a day, a week, or a month.

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>731</td>
<td></td>
<td>1 cup</td>
<td>coffee</td>
</tr>
<tr>
<td>3154</td>
<td></td>
<td>1 tbsp</td>
<td>coffee-decaffeinated-instant powder</td>
</tr>
<tr>
<td>733</td>
<td></td>
<td>1 cup</td>
<td>tea</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Code</th>
<th>Servings per:</th>
<th>Serving Size</th>
<th>Food Name/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5153</td>
<td></td>
<td>1 tap or 1 pack (≈ 4 g)</td>
<td>sugar-white or brown-granulated</td>
</tr>
<tr>
<td>5218</td>
<td></td>
<td>1 tbsp</td>
<td>whipped cream, e.g. Reddi-Whip®</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>1 tbsp</td>
<td>non-dairy whipped cream, e.g. Cool-Whip®</td>
</tr>
</tbody>
</table>
APPENDIX Q

THREE-DAY DIETARY RECORD
Diet Record or Recall

Code number ____________________

Name __________________________ Day of Week/Weekend ____________

Instructions: Please write down all of the items you ate or drank either yesterday or for the last 3 days. Please include the way the food was prepared. Any sauces, gravies or other condiments that were eaten should be listed as a separate food item. Estimate the quantity of food consumed. Be as accurate as possible. If the food is an unusual food that we may not be familiar with please describe in the comment line.

<table>
<thead>
<tr>
<th>Food Item</th>
<th>How Prepared</th>
<th>Amount Eaten</th>
<th>Comments</th>
<th>Food Code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX R

GLUCOSE OXIDASE ENZYMATIC-COLORIMETRIC DETERMINATION
GLUCOSE OXIDASE ENZYMATIC-COLORIMETRIC DETERMINATION
(Ref.: Stanbio glucose kit insert instruction)

Equipments & supplies:
1. Eppendorf pipetter (1mL and 10μL)
2. Pipette tips (1mL and 10μL)
3. Disposable test tubes (12x75mm)
4. Spectrophotometer
5. Vortexer
6. Water bath
7. Cuvette
8. Timer

Procedures:

(1) Serum:
Serum should be collected within 30 min of blood collection to prevent glycolysis.

→ Glucose in serum is stable:
   (a) When stored at 2-8°C, 48 hours.
   (b) When stored at -20°C, no more than 2 weeks.
   (c) When stored at -70°C, no more than 4 months.

→ Do not thaw serum more than two times.

(2) Determination:
Bring enzymatic reagent and standard (concentration 100 mg/dL) to the room temperature. Turn on the spectrophotometer.

→ Prepare test tubes, 1 blank and duplicates for standard and samples.
→ Properly mark tubes.
→ Pipette 1mL of enzymatic reagent to each tube including the blank.
   (* Pour the reagent into a larger vial and pipette from that vial. Do not pipette directly from the reagent bottle to avoid contamination.)
→ Pipette 10 μL of glucose standard to the standard tubes and vortex immediately.
→ Pipette 10 μL of sample to duplicate tubes. Vortex immediately.
→ Incubate in 37 °C water bath for 5 min or in room temp for 10 min.
→ Set spectrophotometer at 500 nm.
→ Use the “Blank” tube to set zero. Read absorbances of standard and samples within 60 min.
Calculate glucose using the formula:

\[
\text{Glucose (mg/dL)} = \frac{\text{Absorbance (Unknown)}}{\text{Absorbance (Standard)}} \times 100 \text{ mg/dL}
\]

\[
\text{Glucose (mmol/L)} = \text{Glucose (mg/dL)} \times 0.0556
\]

** Normal range: 70-105 mg/dL (3.9-5.8 mmol/L)
APPENDIX S
INSULIN DOUBLE ANTIBODY RADIOIMMUNOASSAY (RIA)
INSULIN DOUBLE ANTIBODY RADIOIMMUNOASSAY (RIA)
(Ref.: Binex RIA kit insert instruction)

Equipments and supplies:
1. Precision pipets: 50 μL, 100 μL, 200 μL
2. Repeating pipet: 1 mL
3. Pipet tips
4. Refrigerated centrifuge
5. Borosilicate glass tubes or polystyrene test tubes (12x75 mm)
6. Gamma counter
7. Deionized distilled water (DDW)
8. Vortex mixer

Reagent and sample preparation:
(1) Insulin Controls:
   Add 2 mL DDW to each vial of control.
   → Wait for 20 min, invert each vial slowly a few times (*** Avoid vigorous agitation and foaming).
   → Allow the vials to stand for a few more min.
   → Visually check for complete reconstitution. Swirl each vial gently, if necessary.

(2) Precipitating Reagent:
   Mix well before using.

(3) Serum or plasma:
   If some samples are from obese subjects, centrifuge all serum or plasma samples at 400 x g (1465 rpm) after thawing to eliminate the triglyceride, which may interfere with insulin determination.
   (** Bring all reagent vials and bottles to room temperature prior to use.)
**Procedure:**

Label tubes as follows:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>Total counts (TC)</td>
</tr>
<tr>
<td>3,4</td>
<td>Non-specific binding (NSB)</td>
</tr>
<tr>
<td>5,6</td>
<td>Maximum binding (Bo)</td>
</tr>
<tr>
<td>7,8</td>
<td>Calibrator A (5 μU/mL)*</td>
</tr>
<tr>
<td>9,10</td>
<td>Calibrator B (15 μU/mL)*</td>
</tr>
<tr>
<td>11,12</td>
<td>Calibrator C (35 μU/mL)*</td>
</tr>
<tr>
<td>13,14</td>
<td>Calibrator D (75 μU/mL)*</td>
</tr>
<tr>
<td>15,16</td>
<td>Calibrator E (150 μU/mL)*</td>
</tr>
<tr>
<td>17,18</td>
<td>Calibrator F (300 μU/mL)*</td>
</tr>
<tr>
<td>19,20</td>
<td>Low control (16 - 29 μU/mL)*</td>
</tr>
<tr>
<td>21,22</td>
<td>High control (45-74 μU/mL)*</td>
</tr>
<tr>
<td>23,24</td>
<td>Unknown (samples)</td>
</tr>
</tbody>
</table>

No more than 100 tubes.

* Approximate concentrations. Refer to labels on vials for exact values.

- Pipet 200 μL Assay Buffer into tube 3 and 4 (NSB).
- Pipet 100 μL Assay Buffer into tube 5 and 6 (Bo).
- Pipet 100 μL Calibrators (A to F), controls and samples to individual tubes.
  (** For samples from obese subjects, pipet only 50 μL serum into duplicate tubes and add 50 μL assay buffer to dilute.)
- Pipet 100 μL Insulin Tracer into all tubes.
- Pipet 100 μL Insulin Antiserum into all tubes except TC and NSB tubes.
- Vortex all tube gently, cover and incubate for 90 min at room temp.
- Shake precipitating Reagent prior to use. Add 1 mL of Precipitating Reagent to all tubes except TC.
- Vortex tubes and incubate for 10 min at room temp.
- Centrifuge at 1500 x g (2840 rpm) for 15 min at 2-8°C.
  (** If the pellet is not completely formed, centrifuge for another 10 min.)
- Carefully aspirate the supernatant of each tube (except TC) into a radioactive waste container.
- Count radioactivity in all tubes for one min.
- Calculate the concentration based on the standard curve.
Flow Chart

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th>NSB</th>
<th>B0</th>
<th>Calibrator</th>
<th>Unknowns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Buffer (μL)</td>
<td>----</td>
<td>200</td>
<td>100</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Calibrators (μL)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>100(A-F)</td>
<td>----</td>
</tr>
<tr>
<td>Unknowns (μL)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>100 (or 50)</td>
<td>----</td>
</tr>
<tr>
<td>Controls (μL)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>100 (control tubes only)</td>
<td>----</td>
</tr>
<tr>
<td>Insulin Tracer (μL)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100 *</td>
</tr>
<tr>
<td>Antiserum (μL)</td>
<td>----</td>
<td>----</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

** Vortex and Incubate, 90 min, room temp.

Precipitating Reagent (μL) 1000 1000 1000 1000 ** Vortex and incubate, 10 min, room temp.  
** Centrifuge 1500 x g (2840 rpm), 15 min, 2-8 °C, and decant.  
** Count activity for one minutes.

Calculation:
Concentrations of insulin:

\[
\text{CPM (sample) - CPM (NSB) \over CPM (Bo) - CPM (NSB)} \times 100
\]

(a) \( \% \text{B/Bo} = \frac{\text{CPM (sample) - CPM (NSB)}}{\text{CPM (Bo) - CPM (NSB)}} \times 100 \)

CPM = Average counts of a duplicate sample  
NSB = Particular serum or standard being calculated  
Bo = Non-specific binding tube (blank)  

(b) Construct a standard curve on a semi-log paper:
\( x\)-axis = log of concentrations of insulin standards  
\( (5,15,35,75,150,300 \text{ μU/mL}) \)
\( y\)-axis = percent bound (\% B/Bo)

(c) Determine the insulin concentrations (μU/mL) of each sample based on the standard curve.
APPENDIX T

C-PEPTIDE OF INSULIN RADIOIMMUNOASSAY (RIA)
C-PEPTIDE OF INSULIN RADIOIMMUNOASSAY (RIA)
(Ref.: Diagnostic Systems Lab. RIA kit insert instruction)

**Equipments and supplies:**
1. Precision pipets: 50 µL, 100 µL, 200 µL
2. Repeating pipet: 1 mL
3. Pipet tips
4. Refrigerated centrifuge
5. Borosilicate glass tubes or polystyrene test tubes (12x75 mm)
6. Gamma counter
7. Deionized distilled water (DDW)
8. Vortex mixer
9. Tube rack

**Reagent and sample preparation:**

(1) C-peptide Controls (Level I and II):
   Add 1 mL DDW to each vial of control.
   → Swirl and allow the vials to stand for a few more min.
   → Visually check for complete reconstitution. Swirl each vial gently if necessary.

(2) Precipitating Reagent:
   Mix well before using.

(3) Serum or plasma:
   Serum may be stored at 2-8 °C for up to 24 hours, and should be frozen at -20 °C or lower for longer periods.
   If some samples are from obese subjects, centrifuge all serum or plasma samples at 400 x g (1465 rpm) after thawing to eliminate the triglyceride, which may interfere with c-peptide determination.

(4) C-peptide Antiserum (lyophilized):
   Add 10 mL DDW to the vial
   → Swirl and allow the vials to stand for a few more min.
   → Visually check for complete reconstitution. Swirl each vial gently if necessary.

(5) C-peptide Standards A to F (lyophilized):
   Add 5 mL DDW to the vial labeled A (0 ng/mL), and 1 mL DDW to vials B - F
   → Swirl and allow the vials to stand for a few more min.
   → Visually check for complete reconstitution. Swirl each vial gently if necessary.
C-peptide [I-125] Reagent (lyophilized)
Add 11 mL DDW to the vial
→ Swirl and allow the vials to stand for a few more min.
→ Visually check for complete reconstitution. Swirl each vial gently if necessary.

*** Bring all reagent vials and bottles to room temperature prior to use.***

**Procedure:**
Label tubes as follows:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>Total counts (TC)</td>
</tr>
<tr>
<td>3,4</td>
<td>Non-specific binding (NSB)</td>
</tr>
<tr>
<td>5,6</td>
<td>Maximum binding (Bo) (Standard A)</td>
</tr>
<tr>
<td>7,8</td>
<td>Standard B (0.1 ng/mL)*</td>
</tr>
<tr>
<td>9,10</td>
<td>Standard C (0.5 ng/mL)*</td>
</tr>
<tr>
<td>11,12</td>
<td>Standard D (1.5 ng/mL)*</td>
</tr>
<tr>
<td>13,14</td>
<td>Standard E (5 ng/mL)*</td>
</tr>
<tr>
<td>15,16</td>
<td>Standard F (20 ng/mL)*</td>
</tr>
<tr>
<td>17,18</td>
<td>Low control *</td>
</tr>
<tr>
<td>19,20</td>
<td>High control *</td>
</tr>
<tr>
<td>21,22</td>
<td>Unknown (samples)</td>
</tr>
</tbody>
</table>

(No more than 100 tubes.)
* Refer to labels on vials for exact values.

→ Pipet 150 µL Standard A into tube 3 and 4 (NSB).
→ Pipet 50 µL Standard A into tube 5 and 6 (Bo).
→ Pipet 50 µL Standard (A to F), controls and samples to individual tubes.
→ Pipet 100 µL c-peptide Antiserum into all tubes except TC and NSB tubes.
→ Pipet 100 µL c-peptide [I-125] Reagent into all tubes.
→ Vortex all tube gently, cover and incubate at room temp for 4 hours.
→ Shake precipitating Reagent prior to use. Add 1 mL of Precipitating Reagent to all tubes except TC.
→ Vortex tubes and incubate at room temp for 20 min.
→ Centrifuge at 1500 x g (2840 rpm) for 20 min at 2-8°C.
(*** If the pellet is not completely formed, centrifuge for another 10 min.)
→ Carefully aspirate the supernatant of each tube (except TC) into a radioactive waste container.
→ Count radioactivity in all tubes for one min.
→ Calculate the concentration based on the standard curve

**Flow Chart**

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th>NSB</th>
<th>B₀</th>
<th>Calibrator</th>
<th>Unknowns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard A (µL)</td>
<td>----</td>
<td>150</td>
<td>50</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Standards (µL)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>50 (B-F)</td>
<td>----</td>
</tr>
<tr>
<td>Unknowns (µL)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>50</td>
</tr>
<tr>
<td>Controls (µL)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>50</td>
<td>50 (control tubes only)</td>
</tr>
<tr>
<td>Antiserum (µL)</td>
<td>----</td>
<td>----</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>c-peptide [I-125] (µL)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

** Vortex and incubate, room temp, 4 hours.

Precipitating Reagent (µL) ---- 1000 1000 1000 1000

** Vortex and incubate, room temp, 20 min.
** Centrifuge 1500 x g (2840 rpm), 20 min, 2-8 °C, and decant.
** Count activity for one minute.

**Calculation:**

Concentrations of insulin:

\[
\text{CPM (sample)} - \text{CPM (NSB)}
\]

\[
\% B/B₀ = \frac{\text{CPM (sample)} - \text{CPM (NSB)}}{\text{CPM (B₀)} - \text{CPM (NSB)}} \times 100
\]

- CPM = Average counts of a duplicate sample
- Bo = Particular serum or standard being calculated
- NSB = Non-specific binding tube (blank)
- B₀ = maximum binding tube

(b) Construct a standard curve on a semi-log paper:
- x-axis = log of concentrations of c-peptide standards (0.1, 0.5, 1.5, 5, 20 ng/mL)
- y-axis = percent bound (% B/B₀)

(c) Determine the c-peptide concentrations (ng/mL) of each sample based on the standard curve.
APPENDIX U

17β-ESTRADIOL DOUBLE ANTIBODY RADIOIMMUNOASSAY (RIA)
17β-ESTRADIOL DOUBLE ANTIBODY RADIOIMMUNOASSAY (RIA)
(Ref.: ICN RIA kit insert instruction)

Equipments and supplies:
1. Precision pipets: 50 μL and 500 μL
2. Repeating pipet: 500 μL
3. Pipet tips
4. Refrigerated centrifuge
5. Borosilicate glass tubes or polystyrene test tubes (12x75 mm)
6. Gamma counter
7. Deionized distilled water (DDW)
8. Vortex mixer
9. Test tube racks
10. Water bath 37 °C
11. Aspirator

Reagent and sample preparation:
Serum or plasma:
If some samples are from obese subjects, centrifuge all serum or plasma samples in microcentrifuge for 4-5 min after thawing to eliminate the triglyceride, which may interfere with insulin determination.
(*** The use of grossly hemolyzed or lipemic samples should be avoided.)
(*** Bring all reagent vials, bottles and samples to room temperature prior to use.)

Procedure:
Label tubes as follows:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>Total count (TC)</td>
</tr>
<tr>
<td>3,4</td>
<td>Non-specific binding (NSB)</td>
</tr>
<tr>
<td>5,6</td>
<td>Total binding (0 pg/mL); Bo</td>
</tr>
<tr>
<td>7,8</td>
<td>Standard A (10 pg/mL)</td>
</tr>
<tr>
<td>9,10</td>
<td>Standard B (30 pg/mL)</td>
</tr>
<tr>
<td>11,12</td>
<td>Standard C (100 pg/mL)</td>
</tr>
<tr>
<td>13,14</td>
<td>Standard D (300 pg/mL)</td>
</tr>
<tr>
<td>15,16</td>
<td>Standard E (1000 pg/mL)</td>
</tr>
<tr>
<td>17,18</td>
<td>Standard F (3000 pg/mL)</td>
</tr>
<tr>
<td>19,20</td>
<td>unknown (samples)</td>
</tr>
</tbody>
</table>

(No more than 100 tubes.)
→ Pipet 500 μL DILUENT BUFFER into tube 3 and 4 (NSB).
→ Pipet 50 μL 0 pg/mL into tube 3, 4, 5 and 6.
→ Pipet 50 μL Standard (A to F) and samples to individual tubes.
→ Pipet 500 μL ESTRADIOL 125I TRACER into all tubes.
→ Pipet 500 μL ANTI-ESTRADIOL into all tubes except total count (tube 1 and 2) and NSB (tube 3 and 4) tubes.
→ Vortex all tubes thoroughly, incubate for 90 min at 37°C.
→ Add 1 mL of PRECIPITANT SOLUTION to all tubes.
→ Vortex tubes thoroughly.
→ Centrifuge at 1500 x g (2840 rpm) for 20 min at 2-8°C.
   (*** If the pellet is not completely formed, centrifuge for another 10 min.)
→ Aspirate the supernatant of each tube (except TC) into a radioactive waste container.
→ Count radioactivity in all tubes for one min.

Flow Chart

<table>
<thead>
<tr>
<th></th>
<th>NSB</th>
<th>B0</th>
<th>Standard</th>
<th>Unknowns</th>
</tr>
</thead>
<tbody>
<tr>
<td>DILUENT BUFFER (μL)</td>
<td>500</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>0 pg/ mL (μL)</td>
<td>50</td>
<td>50</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>STANDARD A-F (μL)</td>
<td>----</td>
<td>----</td>
<td>50</td>
<td>----</td>
</tr>
<tr>
<td>SAMPLES (μL)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>50</td>
</tr>
<tr>
<td>ESTRADIOL TRACER (μL)</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>ANTI-ESTRADIOL (μL)</td>
<td>----</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>

** Vortex and incubate, 90 min, 37 °C.

PRECIPITANT SOLUTION 500 500 500 500 (μL)

** Vortex.

** Centrifuge 1500 x g (2840 rpm), 20 min, 2-8 °C, and aspirate.
** Count activity for one minutes.
Calculation:
Concentrations of 17 β-estradiol:

\[ \frac{\text{CPM (sample)} - \text{CPM (NSB)}}{\text{CPM (0 pg/mL)} - \text{CPM (NSB)}} \times 100 \]

(a) \( \% B/B_0 = \) ____________________________ x 100

CPM = Average counts of a duplicate sample
NSB = Non-specific binding tube (blank)
0 pg/mL = total binding tube (Bo tube)

(b) Construct a standard curve on a semi-log paper:
x-axis = log of concentrations of 17 β-estradiol standards (10, 30, 100, 300, 1000, 3000 pg/mL)
y-axis = percent bound (\% B/B_0)

(c) Determine the 17 β-estradiol concentrations (pg/mL) of each sample based on the standard curve.
APPENDIX V

THROMBOXANE B₂ ENZYME IMMUNOASSAY (EIA)
THROMBOXANE B2 ENZYME IMMUNOASSAY (EIA)
(Ref.: Amersham EIA kit insert instruction)

Reagents & Chemicals:
1. Sulfuric acid
2. Methanol
3. Hydrochloric acid
4. Ethanol
5. Hexane
6. Methyl formate
7. Assay buffer
8. TXB2 standard
9. TXB2 peroxidase conjugate
10. Antiserum
11. Wash buffer

Equipments and supplies:
1. Repeatable dispenser
2. Pipette tips for repeatable dispenser (25 μL, 50 μL, 100 μL, 250 μL, 1mL)
3. Polypropylene tubes
4. Volumetric flasks (50 mL and 500 mL)
5. Spectrophotometer plate reader
6. Microtitre plate shaker
7. Amprep C2 100 mg columns
8. Nitrogen gas

Reagent preparation:
(*** Allow all samples and reagents to room temp prior to use.)
(*** All reagents should be stored at 2-8 °C. Once opened, reuse them within 7 days.)

(1) Ethanol (10%)
   Transfer 50 mL of absolute ethanol to 500 mL volumetric flask
   → Add delonized distilled water to 500 mL

(2) Assay buffer
   Transfer the contents to a 50 mL volumetric flask and flush with DDW several times.
   → Add DDW to 50 mL

286
(3) TXB₂ Stock Standard (1.28 ng/mL)
   Add 2 mL diluted assay buffer into the vial containing standard
   → Mix the content until completely dissolved

(4) TXB₂ working standards (** Prepare within one hour of performing the EIA)
   Label 7 polypropylene tubes 0.5 pg, 1 pg, 2 pg, 4 pg, 8 pg, 16 pg, and 32 pg
   → Pipette 500 μL assay buffer into all tubes
   → Pipette 500 μL stock standard into the 32 pg tube and mix thoroughly
   → Transfer 500 μL from the 32 pg tube to the 16 pg tube and mix thoroughly
   → Repeat the dilution procedure successively with the remaining tubes

(5) TXB₂ peroxidase conjugate
   Add 6 mL diluted assay buffer into the vial
   → Mix the content until completely dissolved

(6) Antiserum
   Add 6 mL diluted assay buffer
   → Gently mix the contents by inversion and swirling until completely dissolved

(7) Wash buffer
   Transfer the contents of the bottle to a 500 mL volumetric flask and flush with distilled water several times
   → Add distilled water to 500 mL
   → Mix thoroughly

(8) Sulfuric acid (1 M)
   Transfer 5.57 mL H₂SO₄ (95.9%) to a 100 mL volumetric flask
   → Add distilled water to 100 mL

Procedure:
(1) Extraction of TXB₂:
   Rinse the Amprep C₂ 100 mg column with 2 mL methanol
   → Rinse the column with 2 mL water
   → Acidify 1 mL serum to pH 3
   → Apply serum to the column
   → Wash column with 5 mL water
   → Wash column with 5 mL 10% ethanol
   → Wash column with 5 mL hexane
→ Elute TXB2 with 5 mL methyl formate
→ Evaporate methyl formate using nitrogen gas
→ Redissolve the extract in 1 mL assay buffer

(2) Assay protocol:
Assign each well of the microtitre plate to blank (B), non-specific binding (NSB)
→ standards 0-64, and samples in duplicate
→ Pipette 100 µL assay buffer into NSB wells
→ Pipette 50 µL assay buffer to the zero standard wells (Bo)
→ Starting with the most dilute, pipette 50 µL of each standard or unknown sample into the appropriate wells
→ Pipette 50 µL of antiserum to all wells except the B and NSB wells
→ Pipette 50 µL TXB2 peroxidase conjugate into all wells except the B wells
→ Cover the plate with the lid and incubate at room temperature (15-30 °C) by shaking at 500 rpm for 1 hour on a microtitre plate shaker
→ Aspirate and wash all wells four times with 400 µL wash buffer
→ Immediately dispense 150 µL enzyme substrate into all wells
→ Cover the plate and mix on the shaker for exactly 15 min at room temperature
→ Pipette 100 µL 1M sulfuric acid into each well
→ Mix the contents of the plate on the shaker
→ Determine the optical density in the Microplate autoreader at 450 nm within 30 minutes
→ Determine the concentration of TXB2 based on the following calculations

**Calculation:**
Concentrations of TXB2:

\[
\text{OD (sample)} - \text{OD (NSB)}
\]
\[
\frac{\text{OD (Bo)} - \text{OD (NSB)}}{\text{OD (Bo)} - \text{OD (NSB)}} \times 100
\]

(a) \( \% \) B/Bo = \[
\frac{\text{OD (sample)} - \text{OD (NSB)}}{\text{OD (Bo)} - \text{OD (NSB)}} \times 100
\]

<table>
<thead>
<tr>
<th>OD</th>
<th>= Average optical density sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB</td>
<td>= Non-specific binding tube (blank)</td>
</tr>
<tr>
<td>Bo</td>
<td>= maximum binding tube</td>
</tr>
</tbody>
</table>

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(b) Construct a standard curve on a semi-log paper:
    x-axis = log of concentrations of TXB2 standards
          (0.5, 1, 2, 4, 8, 16, 32, 64 pg/well)
    y-axis = percent bound (% B/Bo)

(c) Determine the amount of TXB2 (pg/well) of each sample based on the standard curve.

(d) Calculation of TXB2 concentration:

    Concentration of TXB2 (ng/mL) = amount of TXB2 (pg/well) \times \frac{1\text{ well}}{50\text{ µL}} \times \frac{1000\text{ µL}}{1\text{ mL}} \times \frac{1\text{ ng}}{1000\text{ pg}}