University of Alberta

Is palmitic acid in the *sn*-1 position of dietary fat less cholesterolemic than palmitic acid in the *sn*-2 position?

By

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DEDICATION

I dedicate this thesis in loving memory of my best friend and cat, Candy Candace Precious Forsythe, August 1986- June 3, 2004. I love you and always will.

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LIST OF ABBREVIATIONS

%en	Percent total energy
^{2}H	Deuterium-labeled hydrogen
2-MG	2-monoglyceride
AHA	American Heart Association
ALA, C18:3n-3	α-linolenic acid
apo	Apolipoprotein
BBM	Brush border membrane
C8:0	Octanoic acid
C10:0	Decanoic acid
C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2n-6	Linoleic acid
CETP	Cholesterol ester transfer protein
CHD	Coronary Heart Disease
CoA	Coenzyme A
CRP	C-reactive protein
DHA, C22:6n-3	Docosahexanoic acid
EPA, C20:5n-3	Eicosapentanoic acid
FAs	Fatty acids
FER	Fractional esterification rate
FFAs	Free fatty acids
FSR	Fractional synthesis rate
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
IDL	Intermediate density lipoproteins
IRMS	Isotope ratio mass spectrometry

LCAT	Lecithin: cholesterol acyltransferase
LCFAs	Long-chain fatty acids
LDL	Low-density lipoprotein
LDL/HDL ratio	LDL cholesterol to HDL cholesterol ratio
LPL	Lipoprotein lipase
MIDA	Mass isotopomer distribution analysis
MUFA	Monounsaturated fat
PC	Phosphatidylcholine
PUFA	Polyunsaturated fat
RCT	Reverse cholesterol transport
SCFAs	Short chain fatty acids
SD	Standard deviation
SEM	Standard error mean
SFAs	Saturated fatty acids
sn	Stereospecific numbering
TC	Serum total cholesterol
TC/HDL ratio	Total cholesterol to HDL cholesterol ratio
TFAs	*Trans* fatty acids
TG	Triglycerides
Trans C18:1n-9	*Trans* elaidic acid
UWL	Unstirred water layer
VLDL	Very low density lipoprotein

CHAPTER 1-INTRODUCTION AND BACKGROUND

CORONARY HEART DISEASE

1. Introduction

Coronary Heart Disease (CHD) remains the leading cause of death and disability in North America. CHD is primarily caused by atherosclerosis, a process characterized by endothelial dysfunction and cholesterol deposition in macrophages and smooth muscle cells of arterial walls (1). It is widely appreciated that there exists a strong relationship between serum cholesterol, and the development of atherosclerosis. Hypercholesterolemia is critical; CHD is uncommon in societies with mean serum total cholesterol concentrations <4.6mmol/L (180 mg/dL) (1). Inflammation is also strongly related to progression of atheroma (2-5). Dietary fat influences serum cholesterol levels as demonstrated by numerous epidemiological and experimental studies (6-13). This first section is concerned with factors that increase the risk of atherosclerosis and CHD and how these factors can be modified.

2. Lipids, Lipoproteins and Risk Factors for CHD

Well-known risk factors for CHD include advanced age, male gender, high systolic blood pressure, high serum total cholesterol (TC) (reflecting high low-density lipoprotein (LDL) cholesterol), low high-density lipoprotein (HDL) cholesterol, and cigarette smoking. Investigations from the Framingham Heart Study (6;9;14-19) led to the development of equations that include these parameters to predict the development of coronary disease in healthy individuals. More recently, high concentrations of serum triglycerides (TG); the ratio of total cholesterol to HDL cholesterol (TC/HDL ratio); the ratio of LDL cholesterol to HDL cholesterol (LDL/HDL ratio); serum homocysteine levels; and fasting levels of C-reactive protein (CRP) have been incorporated into the assessment of cardiovascular disease risk.

Evidence for the relationship between serum cholesterol and CHD came from the Seven Countries Study, that demonstrated a strong relationship between blood cholesterol concentration and mortality from CHD (20;21). This relationship has been found for just about every population studied, from the Chinese at the lowest end of the cholesterol

spectrum to the British at the upper end. The Framingham Heart Study and several other cohort/prospective studies have shown that individuals with a high blood cholesterol concentration at baseline are more likely to develop CHD over the next 10-20 years than those with lower values (7;10;12;13;19;22-24).

Results of recent studies on HDL cholesterol strongly suggest that high concentrations of HDL in circulation help prevent CHD and other cardiovascular diseases (25-28). HDL concentrations >1.0 mmol/L are ideal. Based on data from the Framingham Heart Study and the Coronary Primary Prevention Trial, the TC/HDL ratio and LDL/HDL ratio appear to be better risk predictors than TC levels alone (23). Use of TC/HDL ratio includes the amount of cholesterol in triglyceride-rich very-low density lipoprotein (VLDL) fraction, which positively correlates with CHD (29).

C-reactive protein is an emerging independent risk factor for CHD (30;31). It is an acute phase reactant that markedly increases during an inflammatory response. New evidence has implicated the immune response and inflammation in the pathogenesis of atherogenesis and subsequent CHD (2-5;32;33). Atherothrombosis often occurs in the absence of hyperlipidemia; as such, CRP is important to measure to more comprehensively assess cardiovascular risk. CRP levels significantly correlate with calculated 10-year Framingham Coronary Heart Disease Risk Score in men and women not taking hormone replacement therapy. However, it correlates only minimally with most individual components of the Framingham Coronary Heart Disease Risk Score, including total cholesterol and HDL, because the presence of CRP indicates different aspects of CHD risk such as inflammation (34). Though they act independently of CRP, traditional cardiovascular risk factors often give more additive risks when combined with CRP (34-38). In comparison to LDL cholesterol, CRP has been shown to be a better predictor of CHD risk (39), but data from the Physicians Health Study (40) indicates that CRP only modestly predicts risk in subjects with low TC/HDL ratio.

Lipids (cholesterol and triglycerides) and lipoproteins (VLDL, LDL, HDL) are risk factors that have been shown for nearly a century to be modifiable by diet and lifestyle changes (6-13). More recently, the role of diet in the management of CRP levels has come under consideration (41-44). The most studied dietary component with respect to modifying serum cholesterol, triglycerides and lipoproteins has been dietary fat, while

research suggests that alcohol and a high glycemic-load carbohydrate diet are positively associated with CRP (41;42) and dietary fiber is negatively associated (43;44). The impact of saturated fat consumption is less significant (43). A recent cross-sectional examination of a nationally representative sample of 4,900 U.S. adult participants in the 1999 to 2000 National Health and Nutrition Examination Survey (NHANES 99-00), showed that saturated fat intake was only modestly associated with an increased likelihood of elevated CRP (43). Further research on the relationship between CRP and dietary fat is warranted.

3. The Lipid Hypothesis

The connections among dietary fat, serum lipids and atherosclerosis is classically known as the "lipid hypothesis". In research conducted from 1908-1914 (45), rabbits fed a diet high in cholesterol and saturated fatty acids (SFAs) developed atherosclerosis, and feeding cholesterol alone to rabbits produced identical vascular lesions (46). In the 1950's, controlled feeding studies demonstrated that SFAs, -and to a lesser extent dietary cholesterol-, increased serum total cholesterol concentrations in humans (47). Kinsell and colleagues demonstrated that feeding formula diets composed of exclusively protein and vegetable fat led to prompt reductions in serum total cholesterol while feeding similar amounts of animal fat did not have the same effect (48;49). The difference between animal and vegetable fat was the inclusion of cholesterol in animal fat and the increased amount of SFAs. This data provided evidence to postulate a primary role of dietary cholesterol and SFAs in the cause of atherosclerosis and CHD and suggested that source of dietary fat was important to consider (50).

The lipid hypothesis gained further support from ecological correlations relating SFA intake to rates of CHD in cohorts from different countries (e.g. the Seven Countries Study and the Japanese-Honolulu-San Francisco Study) (1;8;20;51;52) and from studies of immigrants from low to high-risk countries (22). Meanwhile the amount of monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) was suggested to be inversely related to CHD. Within-population studies such as the Ireland-Boston Diet-Heart Study (53) and the Honolulu Heart Program (54) demonstrated strong positive associations among SFAs and cholesterol consumption and CHD and a negative

association with consumption of MUFAs and PUFAs. Strong inverse associations between PUFA intake and CHD risk were reported in the Western Electric Study (55) and the Belgium Study (1;56).The low rate of CHD in France compared to other developed countries with similar dietary intake, (known as the French Paradox), has been attributed to the low consumption of animal fats and high consumption of plant fats (57-59). In some studies there was a positive association between MUFA intake and CHD (16;60;61); however, this association may be due to the collinearity between MUFA and SFA in the typical Western diet (61). Most animal fats consist of equal amounts of SFAs and MUFAs. Thus, when MUFA-rich plant oils are not part of the diet, a high MUFA intake is a good marker of a diet rich in animal fat (62). Collectively, this early work suggested that diets containing high contents of fat, SFAs and cholesterol leads to high blood cholesterol concentrations, resulting in high CHD-related morbidity and mortality.

Recently, using 14-year follow-up data from the Nurses' Health Study, Hu and colleagues (63) conducted detailed prospective analysis of dietary fat and CHD among 80, 082 women ages 34 to 59. The study was particularly powerful because of large sample sizes and repeated dietary assessments. A weak positive association was found between SFA intake and risk of CHD, but a significant and strong positive association was found with intake of *trans* fatty acids (TFAs). Five percent of energy from SFAs, compared with equivalent energy from carbohydrates, was associated with a 17 percent greater risk of CHD (relative risk = 1.17, 95 percent confidence interval 0.97-1.41, p = 0.10). Compared with equivalent energy from carbohydrates, the relative risk for 2%en from TFAs was 1.93 (1.43-2.61, $p < 0.001$); for 5%en from MUFAs, 0.81 (0.65-1.00, p = 0.05); and for 5%en from PUFAs, 0.62 (0.46-0.85, p = 0.002). Total fat intake was not significantly related to risk (for 5%en 1.02, 0.97-1.07, p = 0.55). It was estimated that replacement of 5%en from SFA by unsaturated fats would reduce risk by 42%, and replacement of 2%en from TFAs by non-hydrogenated unsaturated fats would reduce risk 53%. In suggesting that replacing some SFAs and all TFAs with non-hydrogenated unsaturated fats (MUFAs and PUFAs) is more effective in preventing CHD than reducing overall fat intake (64), these findings challenge the widely recommended low-fat, high-carbohydrate diets.

In summary, it appears that dietary cholesterol, SFAs and TFAs are associated with an increased risk of CHD, while PUFA intake is associated with greatest risk reduction, and MUFA intake is only associated with a small degree of risk reduction (1;9;13;46;53;65-72).

4. Effects of Dietary Lipids on Serum Lipids and Lipoproteins

Dietary fats, fatty acids and cholesterol influence risk of CHD predominantly via their effects on serum lipids and lipoproteins. In typical North America diets, SFAs, MUFAs, n-6 PUFAs and cholesterol, comprise the bulk of dietary fat intake whereas lipids of the n-3 PUFA class and *trans* fatty acids are consumed in smaller amounts. In this section, the effects of dietary lipids on serum lipids and lipoproteins will be discussed.

A. Effects of Saturated, Polyunsaturated and Monounsaturated Fatty Acids

Early investigations in humans of the relationship between dietary fat and serum cholesterol concentrations suggested that SFAs increase, and PUFAs decrease serum cholesterol, while MUFAs were considered neutral (73-75). Regression equations were developed from these studies that predicted changes in serum cholesterol concentrations in response to consumption of these classes of fatty acids. However, these investigations focused on the effects of these classes on serum total cholesterol concentrations rather than individual lipoproteins. This focus does not allow a complete assessment of the cholesterolemic effects because consideration of the individual components of total serum cholesterol (e.g. LDL, HDL) is just as important. LDL cholesterol is positively associated with CHD risk, while HDL is inversely associated (1;27;76-80). Therefore, subsequent research focused on how different fat classes influenced individual lipoproteins in humans. Furthermore, these early investigations were limited because of the difficulty distinguishing between classes of fatty acids within dietary lipids because fats and oils contain many combinations of different fatty acids. Interactions between fatty acids within different dietary fats and oils may influence the cholesterolemic effects more than effects of the individual fatty acids themselves. Additionally, the regression

equations developed did not include *trans* fatty acids; these fatty acids have been shown to significantly influence serum cholesterol concentrations (81).

Vega *et al* (82) demonstrated that a diet rich in n-6 PUFA lowered the cholesterol content of all lipoproteins (LDL, HDL, and VLDL) when compared to a diet rich in SFA. Mattson and Grundy (83) showed that MUFA and n-6 PUFA rich diets statistically and equally lowered LDL cholesterol in normotriglyceridemic men and women, and the n-6 PUFA rich diet lowered HDL cholesterol slightly more than the MUFA-rich diet. Wahrburg *et al.* (84) found that MUFA and n-6 PUFA rich diets led to significant reductions in total-, LDL- and HDL-cholesterol concentrations. When MUFA intake exceeded 1.5 times the amount of SFAs and PUFAs in the diet, the LDL/HDL ratio was significantly increased while serum TG levels also increased (85). These and other controlled feeding studies regarding the effects of fatty acid classes on serum total cholesterol and lipoprotein concentrations in humans have been summarized in several meta-analyses (8;75;86-89). All analyses confirm earlier human investigations that SFAs tend to increase and n-6 PUFAs tend to decrease total serum cholesterol while MUFA in moderate amounts can be considered neutral. However, SFAs increase levels of both LDL and HDL cholesterol while n-6 PUFAs decrease these levels (90). Therefore to obtain an optimal lipoprotein profile, a balance of SFA (to increase HDL) and n-6 PUFA (to decrease LDL) without very high levels of MUFAs is recommended (85;90-93).

When all 3 classes of fatty acids (SFA, MUFA and n-6 PUFA) replace carbohydrate in the diet, they elevate HDL cholesterol; this effect is slightly greater with SFAs (46;67). In general, a low-fat (<30%en), higher carbohydrate diet is not recommended because the lipoprotein profile is negatively affected and serum TG concentrations increase in many individuals (46;67;68;70;71). This data is supported with the recent meta-analysis conducted by Mensink *et al* (67), which found that the TC/HDL ratio did not change if SFAs were replaced with carbohydrates because total- and HDL-cholesterol decreased to a similar extent. The ratio decreased if SFAs or carbohydrates were replaced with unsaturated fatty acids (67) which agrees with observations of decreased CHD risk noted in the Nurses Health Study.

B. Effects of n-3 Polyunsaturated Fat

In typical North American diets, n-3 PUFA intake comprises about 0.5 –3% of total energy intake (%en) (94). The n-3 PUFA in plant oils is α-linolenic acid (C18:3n-3, ALA), while marine fish oils contain high amounts of the longer chain n-3 fatty acids eicosapentanoic acid (C20:5n-3, EPA) and docosahexanoic acid (C22:6n-3, DHA). The effects of n-3 PUFAs on lipids and lipoproteins seem to be dependant on chain length. In humans, C18:3n-3 does not differ from C18:2n-6 regarding the effect on serum cholesterol concentrations (94). The marine n-3s EPA and DHA do not affect total cholesterol, because these fatty acids only tend to increase LDL cholesterol by 5-10% and increase HDL cholesterol by 1-3% (95). However EPA and DHA have a significant serum TG-lowering effect, a benefit not seen with ALA intake (94).

C. Effects of Trans Fatty Acids

Trans fatty acids are produced when vegetable oils are partially hydrogenated to replace natural saturated fats in the diet (81;92;96). The TFA elaidic acid (*trans* C18:1n-9) significantly increases total- and LDL-cholesterol, but decreases HDL cholesterol concentrations compared with C18:1 in normocholesterolemic humans (81). *Trans* C18:1n-9 increases the TC/HDL ratio and LDL/HDL ratio even more than C12:0, C14:0 and C16:0 (97-107); thus TFAs are considered more detrimental for cardiovascular health than SFAs. Moreover, TFAs increase plasma levels of lipoprotein [a] (81;99) and may reduce endothelial function by impairing flow mediated dilation (105;108).

D. Effects of Individual Saturated Fatty Acids

Within the class of SFAs, many individual saturated fatty acids exist; the majority of the fatty acids range in chain length from 8 to 18 carbons atoms. Research investigating the effect of SFAs on serum cholesterol has tried to determine how these fatty acids differ in their metabolic influences on lipids and lipoproteins. The effect of individual SFAs on serum cholesterol concentrations is still being investigated. In 1965, Hegsted *et al* (75) was one of the first investigators to imply that individual fatty acids rather than classes of fats had different effects on serum total cholesterol concentrations. A total of 36 different test diets was fed to institutionalized men over a two year period

(75). Through regression analysis, 67% of the total variance in serum cholesterol was explained by changes in myristic acid (C14:0) alone, indicating that this SFA may be the most important determinant of serum total cholesterol concentrations. Diets rich in n-6 PUFAs lowered serum cholesterol, whereas no specific effects were detected for stearic (C18:0), lauric (C12:0), or short-chain SFAs. In a recent analysis from the Seven Countries Study (51), the average population intake of C12:0 and C14:0 was most strongly related to serum total cholesterol concentration ($r > 0.8$, $P < 0.001$). These studies only considered fatty acid influences on total cholesterol concentrations, but further research looked at how SFAs affect individual lipoproteins.

Compared with oleic acid (C18:1, the predominant MUFA) and carbohydrates, the SFAs C12:0, C14:0 and C16:0 have been reported to increase serum total-, LDL- and HDL-cholesterol concentrations (78;109;110). Among the cholesterol-elevating SFAs, C14:0 appears to be more potent than C12:0 or C16:0 (75;78;111;112), but in other studies, C12:0 elevates serum total cholesterol more than C16:0, partly due to a stronger rise in HDL cholesterol (67;113). Some research suggests that the effect of C14:0 and C16:0 on LDL cholesterol is more pronounced than on HDL cholesterol, resulting in an increased LDL/HDL cholesterol ratio (77;78;109). When dietary cholesterol intakes are less than 400mg/day, dietary combinations of C12:0 + C14:0 are more cholesterolemic than diets rich in C16:0 in both animals (103;114-116) and humans (111;117). In contrast, C18:0 the other major SFA, produces very little change in serum total cholesterol concentrations, eliciting an effect similar to C18:1 (118). The apparent neutral effect of C18:0 on lipoproteins has been suggested to be due to decreased absorption in some studies (86;119), but not in others (118;120).

5. Effects of Palmitic Acid on Serum Lipids and Lipoproteins

C16:0 accounts for > 60%en of the SFAs consumed in the North American diet. In this respect, this saturate is frequently regarded as the major contributor to SFA-induced hypercholesterolemia, but this may not be the case. In the 1990's Hayes *et al.* (121) challenged the cholesterol-elevating potential of all SFAs, by focusing on the fact that the assumed effect of any fatty acid is dependent both on the metabolic status of the individual as well as the other fatty acids present in the diet. There appear to be certain

dietary interactions that modify the cholesterolemic response to certain SFAs. First, SFAs interact with dietary cholesterol (122) and secondly, SFAs interact with PUFAs (mainly C18:2n-6) (121).

A. Palmitic Acid and Animal Research

Hayes *et al* (121) showed that in normocholesterolemic cebus monkeys consuming very-low cholesterol-containing or cholesterol-free diets, C16:0 did not impact serum total- or LDL-cholesterol levels. Only when LDL receptors were suppressed (as when 0.3%en cholesterol was fed) did C16:0 show a hypercholesterolemic effect (122). Cebus monkeys were used as the primates of choice because their plasma cholesterol responds in the same manner as humans; however, the level of cholesterol given is not representative of normal monkey or human consumption.

In low cholesterol diets, serum total- and LDL-cholesterol concentrations were dependant mostly on content of C14:0 and C18:2n-6. Myristic acid is cholesterol-elevating and C18:2n-6 is cholesterol-lowering (121). Above a threshold level of C18:2n-6 of 5-6%en, the composition of additional dietary fatty acids other than C14:0 may not negatively affect lipoprotein balance (103).

Gupta and Khosla (123) compared the effects of diets enriched in C16:0 with those enriched with C18:0 in normocholesterolemic cynomolgus monkeys. Rationale for using the cynomolgus monkey is that it has been widely used for investigating the effects of dietary fat and cholesterol on plasma lipoprotein metabolism and is considered one of the desirable animal models for evaluating the effects of dietary fats on plasma lipids (124). Both diets in this study met the conditions outlined in Hayes' threshold hypothesis; total fat intake was restricted to ~30%en, SFA was ~10%en, C18:2n-6 was ~10%en, and dietary cholesterol was low. Diets resulted in similar levels of serum lipids and lipoproteins; these results were attributed to low levels of dietary cholesterol and high levels of C18:2n-6 in both diets.

More recently, another study conducted in normocholesterolemic cynomolgus monkeys (103) compared diets containing ~32%en as fat differing dramatically in their fatty acid composition with or without the addition of cholesterol (0.1%en). Results showed that AHA (American Heart Association) Step-1 diets (containing an equal

proportion of SFA, MUFA, and PUFA) were not statistically different than diets rich in C16:0 (12%en as C16:0 from palmolein) containing 14%en SFA and only 3.5%en C18:2n-6 in concentrations of serum total-, LDL- or HDL-cholesterol and serum TGs with both high and low levels of dietary cholesterol. The AHA Step-1 diets and the high C16:0 diets also resulted in significantly lower serum total- and LDL-cholesterol and higher HDL cholesterol compared to diets high in *trans* fatty acids (7.4%en as *trans* C18:1n-9; and 5.6%en as C18:2n-6) and diets high in C12:0 + C14:0 (13%en as C12:0 + C14:0; and 4.8%en as C18:2n-6) (103). These observations support previous observations that diets rich in C12:0 + C14:0 and/or rich in TFAs are more cholesterolemic than diets rich in C16:0 (115;116;121;122;125).

B. Palmitic Acid and Human Research

Hayes *et al* (121) suggested that neutrality of C16:0 may also apply to humans, especially when dietary fat intake is moderate (~30-33%) and dietary cholesterol intakes are below 400mg/d. Numerous human studies have reported similar results; suggesting C16:0 is equivalent to C18:1 (81;117;122;126;127) and C18:0 (128) within moderate fat and moderate cholesterol diets. Khosla and Sundram (116) conducted a meta-analysis of more than 30 studies and also found that C16:0 may be considered neutral with respects to its influence on serum cholesterol concentrations. Analyses of accumulating data in humans show that the best predictor of observed variations in serum cholesterol are dietary levels of C14:0 and C18:2n-6 (81;96;104;111;121;126).

In controlled human feeding studies, Clandinin *et al* (129;130) found that normocholesterolemic subjects fed C16:0 (10%en) with high levels of C18:2n-6 (10%en), and moderate dietary cholesterol, did not experience negative effects on serum total- and LDL-cholesterol. Recently, French *et al* (104) showed that when dietary fat and C16:0 levels were held constant (30%en and 10%en respectively) and cholesterol intake was low, increasing intakes of C18:2n-6 from ~2%en to 10%en caused concentrations of serum total- and LDL-cholesterol to significantly decrease. At least ~4.5%en from C18:2n-6 was required to see a cholesterol lowering effect in the presence of high C16:0 (~10%en). The dietary level of C18:2n-6 may explain results seen in human feeding studies by Snook *et al* (131) and Montoya *et al* (132), where healthy subjects had high

serum total- and LDL-cholesterol concentrations after consuming diets high in C16:0 and low in C18:2n-6 (<4%en).

In summary, C16:0 appears to be a conditionally cholesterolemic SFA. Levels of dietary cholesterol (103;121;122), C18:2n-6 (96;104;123;126;129;130;133;134), and contribution of energy from C12:0 and C14:0 fatty acids (81;111;113-115) all influence how C16:0 affects serum lipid and lipoprotein concentrations in both animals and humans (67;81;89;96;103;104;111;115;116;121-123;125;126;129;130;134-137).

6. Effects of Dietary Cholesterol on Serum Lipids and Lipoproteins

In many animal models, dietary cholesterol raises serum cholesterol levels and very high intakes cause atherosclerosis; while in other animals the rise in serum cholesterol is minimal (11;103;123;133;134;138-141). Dietary cholesterol appears to modulate the effects of other fatty acids through changes to the LDL receptor (121;142;143). In a recent study by Idris and Sundram (103), the cholesterolemic response of different fatty acid combinations was determined in the absence and presence of dietary cholesterol. Cynomolgus monkeys were fed either cholesterol-free or cholesterol-rich (0.1%en) diets differing in fatty acid composition. With the addition of dietary cholesterol, serum total-and LDL-cholesterol was almost doubled in all the animals in all diets. This finding in non-human primates suggests that feeding a cholesterol-rich diet increases the serum cholesterol response of other fatty acids.

Research in human infants has shown sensitivity in serum cholesterol concentrations to feeding dietary cholesterol from breast milk compared to formula (144-146). Cruz *et al* (145) showed that infants fed breast milk had higher serum total and LDL-cholesterol concentrations than infants fed low-cholesterol infant formula. Bayley *et al* (147) demonstrated that serum cholesterol concentrations were significantly higher in breast-fed infants compared to milk-based formula-fed infants with and without added cholesterol at 4 months of age. These differences however failed to persist once these infants reached 11- and 12-months of age; infants had similar serum cholesterol concentrations. One must consider that differences between breast milk and formula are greater than just the cholesterol component, as fatty acid composition also differ significantly. The fatty acid composition of the diet appears to have a greater influence on

serum cholesterol concentrations than dietary cholesterol feeding (89;147;148). Additionally, the form of dietary cholesterol may have been responsible for the dissimilarity between supplemented formula and breast milk in infant studies (147). Breast milk contains about two-thirds of its cholesterol in esterified form (149;150), a form that may undergo absorption at a different efficiency compared with free, unesterified cholesterol found in formula.

In controlled metabolic studies conducted in adult humans, dietary cholesterol raises levels of serum total-, HDL- and LDL-cholesterol (75), but the effects are small compared to SFA and TFAs (89;148) and individuals vary widely in their response (47;72). Because individuals do not experience a uniform response to dietary cholesterol, it is difficult to accurately predict its effect on plasma cholesterol and lipoprotein concentrations (151). The existence of a hypo- or hyper-responders to dietary cholesterol has been acknowledged (151-154) and is considered to be determined by genetic factors such as E4 polymorphisms of the apolipoproteinE gene (155-157). Howell *et al* (89) found that for every 100mg/day increase in dietary cholesterol, serum total cholesterol rises an average of 0.57 mmol/L (158). Although serum total cholesterol tends to increase with increasing dietary cholesterol, this effect is seen in both the LDL- and HDL-cholesterol fractions as determined by a meta-analysis of metabolic ward cholesterol feeding studies reported by Clarke *et al.* (148). Thus, it is possible to increase total plasma cholesterol concentrations without a significant change in CHD risk provided that the LDL/HDL ratio remains constant (159). The American Heart Association has recommended the public to consume less than 300mg cholesterol per day to avoid unhealthy elevations in blood cholesterol and reduce CHD risk (160;161). The nutrition recommendations for Canadians are based on the Dietary Reference Intakes Reports, Institute of Medicine of the National Academies of Sciences and advises that intakes of dietary cholesterol be minimized as much as possible while consuming a nutritionally adequate diet (162).

7. Dietary Fat and Serum Triglycerides

The type and amount of fat affects fasting serum TG concentrations, reflecting VLDL concentrations. Saturated fatty acids, MUFA and n-6 PUFA appear to have a

modest effect on serum TG (62) while intakes of TFA rich diets increase serum TG (163). A meta-analysis of all studies conducted on this subject showed that fasting serum TG concentrations were not significantly affected when SFA were replaced by either MUFA or n-6 PUFA (164). When MUFAs replace carbohydrates in the diet, fasting serum TG concentration decreases (77). In some persons, PUFA of the n-6 variety may lower fasting TG, but not in others (77). The difference between a MUFA-rich diet and a PUFA-rich diet is of borderline statistical significance when compared directly. At present, it is not possible to provide a definitive answer to the question of whether n-6 PUFA and MUFA differ in regard to serum triglyceride concentrations; any differences that exist are likely to be of marginal clinical significance (62). Conversely, numerous studies have demonstrated that long-chain n-3 PUFAs (EPA and DHA), have distinct TG lowering properties (94). This TG-lowering potency is not shared by the shorter chain n-3, ALA, in physiological doses (94).

EXOGENOUS LIPID METABOLISM AND POSITIONAL DISTRIBUTION OF FATTY ACIDS

1. Classification of Dietary Fats and Oils

Dietary fats and oils are commonly referred to as being either predominately saturated or unsaturated. This classification is based on the fact that dietary fats and oils are unique mixtures of many different saturated and unsaturated fatty acids. Fats and oils containing a sizable portion of saturated fatty acids (SFAs) are deemed saturated, while unsaturated fats and oils are typically rich in unsaturated fatty acids.

Fatty acids (FAs) within dietary fats and oils are found as triacylglycerol (triglyceride) molecules. Glycerol serves as the backbone and contains three distinct and non-equivalent attachment sites for FAs, which are connected to glycerol by an ester bond. These sites are numbered *sn*-1, *sn*-2 and *sn*-3, where *sn* stands for stereospecific numbering, and each site is termed positional distribution. There are at least twenty common FAs that can be esterified to glycerol. Fatty acids at these sites may differ in chain length, degree of unsaturation, and configuration of double bonds (i.e. deeming them saturated or unsaturated). Specific FAs preferentially occupy the *sn*-1, -2 or -3 sites in dietary fats and oils; hence, the positional distribution of FAs is not random in fats from biological sources (24).

Dietary studies focusing on feeding high levels of different dietary fats and oils containing specific amounts of saturated or unsaturated fatty acids can be difficult to interpret. This difficulty arises because fats and oils having similar amounts and types of saturated and unsaturated fatty acids may have dramatically different positional distributions. The positional distribution of FAs determines their degree and rate of digestion, absorption and perhaps even their metabolic influence in humans. This section will review the metabolism of dietary lipids and examine how positional distribution may affect this process. Specifically, focus will be on palmitic acid (C16:0), the major SFA found in the human diet, as conclusions about its metabolic effects have not been clearly established.

2. Overview of Exogenous Triglyceride Metabolism

A. Triglyceride Digestion

Once dietary triglycerides (TGs) are consumed, they are pre-digested by lingual and gastric acid lipases in the stomach. Lingual and gastric lipases preferentially hydrolyze short- and medium-chain fatty acids in the *sn*-3 position of TGs to produce 1,2-diglycerides and free fatty acids (FFAs). Emulsification also occurs in the stomach; this step is an important pre-requisite for efficient hydrolysis by pancreatic lipase in the intestinal lumen (165).

The partially hydrolyzed lipid emulsion leaves the stomach and enters the duodenum as fine lipid droplets. Bile and pancreatic juice are released from the pancreas to bring about marked changes in the chemical and physical form of the lipid emulsion. Pancreatic lipase primarily acts on FAs in the *sn*-1 position and secondarily on the *sn*-3 position (166). Colipase is a heat-stable protein required for pancreatic lipase activity when bile salt is present; it is synthesized and secreted by the pancreas as procolipase and is activated to colipase in the small intestine by proteolytic cleavage by trypsin. Colipase binds to the lipid droplet triglyceride/aqueous interface and allows binding of pancreatic lipase to this interface. Prolonged digestion of lipid droplet leads to hydrolysis of all FAs in the *sn*-1 and *sn*-3 positions and formation of 2-monoglycerides (2-MG) characteristic of the *sn*-2 position (166). Only a small percentage of TG is totally hydrolyzed to free glycerol because pancreatic lipase does not hydrolyze fatty acids in the *sn*-2 position (the 2-MG). The fatty acid chain is released from the 2-MG only after chemical migration of fatty acids in the *sn*-2 position to the *sn*-1 or *sn*-3 position whereupon glycerol is produced (166).

B. Triglyceride Absorption

The products of the partial digestion of TGs, primarily 2-MG and FFAs, combine with bile salts in the intestine to form negatively charged polymolecular aggregates called micelles (79). Bile salts stabilize micelles and make them sufficiently water-soluble to penetrate what is called the unstirred water layer (UWL), bathing the enterocytes. Micelles interact at the entrocyte brush border membrane (BBM), but are not absorbed as intact structures. Within the UWL, an acidic microclimate aids micellar dissociation

(167). Lipid monomers transverse the intestinal BBM either by passive diffusion or by protein-mediated mechanisms (166). Passage of long-chain fatty acids (LCFAs, longer than 12 carbons) across the UWL is rate-limiting, whereas passage of short- and medium-chain fatty acids may be facilitated by a BBM fatty-acid binding protein in the membrane (166).

C. Intestinal Re-synthesis of Triglycerides

After crossing the enterocyte brush border membrane, FFAs and 2-MGs must be delivered from the cytosol to their site of processing at the endoplasmic reticulum. The primary candidates for this function appear to be the intracellular fatty acid binding proteins (166). In the endoplasmic reticulum, intracellular reformation of TGs takes place. The process of re-esterification is a function of FA. Fatty acids having more than 10 to 12 carbons (LCFA) are first activated, as they are coupled to coenzyme A (CoA) by the enzyme acyl CoA synthetase. The LCFA are then re-esterified into TGs by specific pathways. There is evidence that some LCFA, particularly polyunsaturated fatty acids (PUFAs), may pass directly into the portal blood (167;167). On the other hand, fatty acids containing fewer than 10 to 12 carbons (short chain fatty acids, SCFAs) indefinitely pass from the cell directly into the portal blood. In blood, SCFAs attach to albumin for transport to other tissues for processing. The different fate of the LCFAs and SCFAs is due to specificity of acyl CoA synthetase for LCFAs only (79).

Re-esterification of 2-MGs and LCFAs occurs by two routes: the monoacylglycerol pathway and phosphatidic acid pathway. The monoacylglycerol pathway accounts for the majority of intestinal cell TG synthesized in the fed state (168). This pathway proceeds by direct acylation of the 2-MG with coenzyme A and involves re-esterifying enzymes located on the cytoplasmic surface of the smooth endoplasmic reticulum known as the "Triglyceride Synthetase Complex" (166). In the phosphatidic acid pathway, FAs are esterified to α-glycerol-phosphate as the glycerol donor. This metabolite can be formed either from the phosphorylation of free glycerol or from reduction of dihydroxyacetone phosphate, an intermediate in the pathway of glycolysis (79). Phosphatidic acid is hydrolyzed to form diacylglycerol, which is then converted to

TG as well as glycerophospholipids (165). The phosphatidic acid pathway is utilized predominately during fasting conditions when no 2-MG is absorbed.

Although the two pathways of intestinal TG formation have generally been considered to function separately, evidence for convergence at the level of 2-MG via a deacylation/ reacylation cycle has been presented (168-170). The basis for this finding is the observation that TGs formed by these two pathways appear in the lymph as the same lipid particle rather than separate particles (i.e. chylomicrons). It appears that acylglycerol products of the phosphatidic acid pathway are hydrolyzed to 2-MG prior to reconversion to TGs via the monoacylglycerol pathway (168;170).

D. Integration of Triglyceride into Developing Chylomicrons

TG synthesized in intestine is translocated across the membranes of the smooth endoplasmic reticulum to the Golgi cisternae, where nascent lipoproteins are formed (168). The TG droplet is united with lipoprotein membrane components including phospholipid, cholesterol and apolipoprotein-B to produce immature chylomicrons. Golgi enzymes add terminal sugars to the glycoproteins on the surface of these particles to form mature chylomicron particle. Golgi vesicles migrate to the basolateral plasma membrane and chylomicrons are secreted from enterocytes by a process of reverse pinocytosis into the intercellular space (166). Chylomicrons are secreted into the mesenteric lymph and transported to the thoracic duct, from which they enter the systemic circulation via the subclavian vein.

Chylomicrons range in size from 50 to 500 nm and function to transport dietary TG to peripheral tissues. They are predominantly made of lipid, with protein accounting for only 1% of their mass. About 90% TG and 1% cholesteryl ester make up chylomicron core components, and about 6% to 8% phospholipid and 1% free cholesterol are surface components (171). The FA composition of the chylomicron TG usually reflects the FA composition of the diet (171). Chylomicrons are characterized by virtue of their density as well as their unique apolipoprotein, apoB-48. Therefore, following digestion of dietary fat, there is a significant increase in plasma TG and apoB-48 concentrations (172).

E. Regulation of Chylomicron Secretion

Chylomicrons cannot be formed or secreted by the intestine unless there is apoB-48 in membrane. ApoB-48 is combined with lipoproteins by the action of microsomal transfer protein (1). It appears that apoB-48 is the only apolipoprotein that forms an integral part of the lipoprotein particle and does not dissociate at any time during peripheral metabolism (173). ApoB-48 is one of two forms of apoB found in human plasma; the other is apoB-100. In humans, apoB-48 is only synthesized in the intestine, whereas apoB-100 is predominantly of hepatic origin (1). ApoB-100 is the integral protein of very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and low-density lipoproteins. Both apolipoproteins are essential for normal plasma TG and cholesterol metabolism. The absolute requirement of apoB-48 for chylomicron production is demonstrated by disorders of lipoprotein metabolism caused by genetic defects resulting in impaired apoB-48 synthesis and/or secretion. These disorders include abetalipoproteinemia, severe familial hypobetalipoproteinemia, and chylomicron retention disease (1).

Phospholipid is also important for sustaining normal chylomicron formation and secretion. Defects of lipid transport by enterocytes observed under experimental conditions in animals with essential fatty acid deficiency may be based on abnormal membrane structure and function related to the formation of phospholipids of abnormal composition. Of interest in this regard is the observation that luminal phospholipid (particularly phosphatidylcholine, PC)- in some way causes more efficient processing of absorbed dietary lipids that are re-esterified by the 2-monoacylglycerol pathway, resulting in greater transport of dietary lipid into lymph as chylomicrons. This effect is not due to a delay in the digestion and uptake of luminal lipid, but is rather due to an unexplained action of PC on the intracellular events that occur during chylomicron synthesis and secretion. Luminal PC of either biliary or dietary origin can be readily hydrolyzed to lyso-PC, quickly absorbed and re-esterified back to PC to meet the needs of the enterocyte. PC can also be synthesized from de novo synthesis in the enterocyte utilizing α-glycerol phosphate, FFA, and choline (166).

F. Chylomicron Metabolism

When chylomicrons enter the bloodstream, they rapidly undergo lipolysis via the action of lipoprotein lipase (LPL), an enzyme bound predominately to the vascular endothelium of muscle and adipose tissue (173). LPL hydrolyzes core TGs of the chylomicron, and hydrolytic products enter muscle cells for oxidation and adipocytes for storage (173). LPL, like pancreatic lipase, is specific for FAs at the *sn*-1,3 position of chylomicron TGs (174). FAs retained at the *sn*-2 position may then be preferentially transported to the liver instead of extrahepatic organs (174). During this process, apolipoproteinE, apolipoproteinC-II and cholesteryl ester are acquired from HDL in the circulation via the action of cholesterol ester transfer protein (CETP) (1). As TG hydrolysis progresses, chylomicron surface lipid (free cholesterol and phospholipid) are transferred in return to HDL. The chylomicron particle progressively becomes TG-depleted and relatively cholesteryl ester-enriched to become a chylomicron remnant (173). The liver takes up the chylomicron remnant via a receptor-mediated process that may use apoE as a receptor ligand. ApoB-48 does not transfer from the chylomicron remnant at any time. Dietary cholesterol in chylomicron remnants is effective in the down-regulation of cholesterol synthesis in the liver although synthesis by this tissue is minor compared to extrahepatic tissues (173;175;176).

The liver has a complex chylomicron remnant removal system that is comprised of a combination of different mechanisms, including the low-density lipoprotein receptor and the LDLR-related-protein (177). One major concern has arisen regarding the association of chylomicron remnants and coronary heart disease in man. This concern is that slow removal of chylomicron remnants, as reflected by a prolonged postprandial state, is now commonly observed in patients with CHD and those with abnormal lipid disorders (177).

3. Effect of Positional Distribution on Exogenous Triglyceride Metabolism

A. Digestion and Absorption

The stereospecific positions of FAs are important because they influence how TGs are digested and absorbed. After TG hydrolysis, FAs released from the *sn*-1 and *sn*-3 positions often have different metabolic fates than FAs retained in the *sn*-2 position as 2-

MGs. Dietary fats with LCFAs in the *sn*-1 and *sn*-3 positions may exhibit different absorption patterns from fats with these FAs in the *sn*-2 position. In contrast, most of the *sn*-2 FAs as 2-MG are absorbed, enter the mucosal pool, and are re-acylated to TGs, retaining the original FA in the *sn*-2 position (178).

Unesterified (i.e. free) ^{13}C-labelled LCFAs, C16:0 and C18:0 have been shown in animals and humans to be less well absorbed from the lumen than shorter chain saturated or carbon chain 18 unsaturated fatty acids such as C18:1 and C18:2n-6 (179-183). This may be due in part to the fact that C16:0 and C18:0 have melting points above body temperature (>60°C) and are partly excreted in the feces through formation of insoluble calcium soaps (184;185). However, when ^{13}C-labeled C16:0 and C18:0 are administered within a lipid-casein-glucose-sucrose-emulsion, the absorption of C16:0 is improved over that of C18:0 (182). It has been suggested that absorption of labeled fatty acids increases when administered as part of an emulsion in comparison with the simple addition of the tracer fat within a test meal (179;182;183;186). This agrees with previous studies that show absorption of C16:0 and C18:0 is improved when high-melting point solid-fat particles are avoided, or when fat sources containing these fatty acids as mixed TGs are used (186). These findings suggest that although C18:0 is absorbed less, the absorption comparisons between all SFAs is small and should not impart differences in metabolic utilization.

In dietary fat and oil research, increased absorption of C16:0 found at the *sn*-2 position of TG is supported by studies of fecal fat loss and chylomicron and liver lipid analysis in rats and piglets. Aoyama *et al* (187) and Lien *et al* (188) studied the absorption of C16:0 in rats from dietary TG with C16:0 predominately in the *sn*-1, 3 position or most of the C16:0 in the *sn*-2 position. Both found higher fecal losses of C16:0 when C16:0 was in the *sn*-1, 3 positions versus the *sn*-2 position. Renaud *et al* (178) fed rats diets containing either palm oil (58% C16:0 at *sn*-1,3), lard (65% C16:0 at *sn*-2), or their interesterified counterparts. Fecal excretion of C16:0 was greatest in diets containing high amounts of C16:0 at the *sn*-1 and *sn*-3 positions. Interesterification of lard (i.e. alteration of positional distribution of C16:0) decreased incorporation of C16:0 into plasma lipids and decreased total plasma TGs; these decreases are consistent with reduced absorption of C16:0 (178). Fatty acid absorption studies in piglets have

demonstrated that when C16:0 is fed predominately in the *sn*-2 position of dietary TG, the incorporation of C16:0 into plasma TGs and cholesteryl esters is increased compared with feeding C16:0 in *sn*-1,3 positions (189-191).

These animal studies are similar to findings in human infants. Filer *et al* (192) indicated the importance of the location of C16:0 in the *sn*-2 position of human milk to reduce fecal loss of energy in infants. Infants fed formulas containing randomized lard containing less C16:0 in *sn*-2 position than native lard excreted six times as much fat. Clinical trials in formula-fed infants confirmed better absorption of C16:0 from TGs with preferential esterification of C16:0 in the *sn*-2 position compared to randomly esterified C16:0 (193;194) The specific positioning of C16:0 at the *sn*-2 position of human milk TGs has been suggested as one of the reasons for the high efficiency of absorption of fat from human milk (192;195;196). However, because of the role of the milk enzyme, bile salt-stimulated lipase, which in newborns is responsible for the hydrolysis of 2-MGs to free glycerol and FFAs (195-197), the contribution of *sn*-2 C16:0 to total amount of absorbed milk fat is questionable. The above studies suggest that modifying dietary TG structure to increase the level of C16:0 in the *sn*-2 position leads to increased absorption in animals and human infants.

Dietary TG-fatty acid absorption studies are limited in adult humans. Snook *et al* (131) and Park *et al* (198) suggested that FA absorption is dependant on chain length and saturation. Both found that despite source of dietary fat administered (i.e. palm kernel oil, butter, or lard) to supply C16:0 or C18:0, which differ greatly in positional distribution, absorption of these FAs was decreased relative to C14:0, C18:1 and C18:2n-6 in all cases. Apparent digestibility in these studies was determined by administration of 50mg Brilliant Blue marker and analysis of fecal excretion. Tholstrup *et al* (199) measured postprandial lipemia in adult humans to six test meals high in either C18:0, C16:0, C16:0 + C14:0, C18:1, elaidic acid (*trans* 18:1), or C18:2n-6. The test fats containing C16:0 and C18:0 generally resulted in a lesser increase in plasma total and chylomicron TG after 4 hours than did other test fats. Moreover, the return to post-absorptive values was slower. The authors concluded that saturated LCFAs (C16:0 and C18:0) were absorbed less and at a lower-rate gastrointestinally; conclusions that were also supported by analyses of FA composition of chylomicron TGs (199). Recently, in a novel multi-isotope tracer study

undertaken by Emken *et al* (200), deuterium-labeled C16:0 (^{2}H-C16:0) within a lipid-casein-glucose-sucrose-emulsion was found to be absorbed equally from all three positions of TG. As evidenced by relative absorption and clearance of labeled FA into developing chylomicron TGs, fatty acid positional distribution had no effect on ^{2}H-C16:0 absorption. The multiple isotope tracer used in this study provides a more accurate comparison of FA incorporation and metabolite synthesis than single isotope tracer designs (such as ^{13}C-labelled isotopes) because all deuterated FAs in the mixture fed to subjects are influenced equally by the same metabolic variables (200). Thus, the *sn*-1,3 vs. the *sn*-2 position comparisons are highly accurate. These investigators also noted that there was much unlabeled FA incorporated into chylomicron TGs. The amount of unlabeled FA incorporated was not trivial and undoubtedly compromises results from studies such as that by Tholstrup *et al* (200) that use unlabeled fats to investigate absorption and metabolic effects of TG structure.

Another factor to consider regarding absorption is that stereospecific location of SFAs may be influenced by dietary calcium levels. Free long-chain SFAs form calcium soaps that are 10-20 times less soluble than the calcium salts of C18:1 and C18:2n-6 (185). Brink *et al* (201) showed lower SFA absorption in rats fed a high fat blend of 1-oleoyl-distearate and high dietary calcium compared to low dietary calcium and the same fat blend. In pre-term infants, administration of oral calcium supplements fed as calcium lactate decreased fat absorption from pre-term mother's milk and formula (202). Moreover, Denke *et al* (203) found that calcium fortification (2200 mg/d) of a diet with 34%en fat, from mainly beef tallow, decreased SFA absorption in adult humans by increased fecal excretion. Additionally, serum total- and LDL-cholesterol concentrations significantly decreased and HDL increased with supplemental calcium. The observations seen in animals, infants and adults may explain reduced absorption of C16:0 and C18:0 seen by Bonanome and Grundy (118) when a high fat meal from dairy fat was fed to human subjects.

B. Influence of Positional Distribution on Chylomicron Triglyceride

The 2-monoacylglcerol pathway is believed to account for 70-80% of the TGs synthesized and secreted in chylomicrons following absorption of dietary fat, with the

remaining TG synthesis occurring via the *de novo* phosphatidic acid pathway (190;204). Triglyceride synthesis by means of this pathway, rather than the phosphatidic acid pathway, results in formation of chylomicron TGs with the same FA retained in the 2-position as in the original dietary fat. Evidence for this conservation of FA structure in plasma chylomicron TGs has been observed in many studies. Innis *et al* (189-191) showed higher levels of C16:0 in chylomicron TG *sn*-2 position in piglets fed either sow's milk or a formula high in *sn*-2 C16:0 compared to formula lower in *sn*-2 C16:0. Innis *et al* (205) showed higher proportions of C16:0 esterified to the *sn*-2 position of plasma TGs in breast milk fed infants (containing more dietary *sn*-2 C16:0) versus formula fed infants (containing less dietary *sn*-2 C16:0). Nelson and Innis (204) confirmed these findings, showing that infants fed formula with C16:0 enriched at the *sn*-2 position had higher amounts of C16:0 at the *sn*-2 position of plasma chylomicron TGs than infants fed formula with a similar total amount of energy from C16:0, but with 5% less energy from *sn*-2 C16:0.

In a study of adult men given a diet with 31%en from an interesterified high palm oil blend (more C16:0 in the *sn*-2 position than native palm oil), ~70% of the dietary *sn*-2 C16:0 was recovered in the sn-2 position of chylomicron TG (206). Yli-jokipii *et al* (207) used an efficient tandem mass spectrometric method to analyze the regioisomers of chylomicron TGs after two oral fat loads (palm oil and interesterified palm oil) in humans. They confirmed that chylomicron TGs reflected dietary TGs fed to each subject. Palmitic acid in chylomicron TGs from the palm oil diet were preferentially located in the *sn*-1,3 positions, similar to the dietary distribution of palm oil, whilst C16:0 was randomly distributed in the chylomicron TGs from the interesterified palm oil diet (207). These findings in adult humans are consistent with the recent findings of Emken *et al* (200) that 80-90% of *sn*-2 dietary TGs are retained in chylomicron *sn*-2 position.

C. Effects of Positional Distribution on Postprandial Chylomicron Metabolism

Some studies in rats have provided evidence that metabolism of chylomicrons is influenced by the distribution of the component TG-FAs (204). Chylomicron TGs with C16:0 in the *sn*-2 position may be transported faster into the lymph (208). SFAs in the *sn*-2 position of dietary TG have been shown to slow down the clearance of chylomicrons

(209;210). The *sn*-2 fatty acid may remain on the chylomicron surface, causing changes in surface layer physical properties. Imbalance between chylomicron production and clearance results in remnant accumulation.

In contrast, Yli-Jokipii *et al* (207;211;212) found no clear trends indicating selective clearance of chylomicron TGs regioisomers from palm oil or transesterified palm oil, and lard or transesterified lard in adult humans. Despite differences in the positional distribution of FAs in the test fats, clearance of chylomicron TGs was similar after both treatments, suggesting that positional distribution of FAs may not have a major effect on clearance of TGs from chylomicrons. Similar results were observed by Zamples *et al* (213), who found that feeding healthy male subjects diets consisting of palm oil (*sn*-1,3 C16:0) or a modified fat blend with C16:0 predominately in the *sn*-2 position (Betapol) resulted in the same plasma total and chylomicron TG concentrations. Therefore positional distribution was suggested to not be an important determinant of postprandial lipemia. Jensen *et al* (214) also found that in healthy females, the 8-hour postprandial lipemia response to an acute high fat test meal (65%en from fat) from either palm oil or lard was the same. The postprandial plasma TG and chylomicron TG area-under-the-curve (AUC) clearance was not significantly different between the two high fat test meals with different positional distributions of C16:0 (214).

These unaltered clearance rates may be explained by recent observations that production and clearance are not affected by dietary fatty acid positional distribution because plasma TG- and chylomicron TG-fatty acids migrate to positions that are more normal for human chylomicron TGs. SFAs will be mainly found in the *sn*-1,3 position and PUFAs will be in the *sn*-2 position (200). Using deuterated fatty acids Emken *et al* (200), found that C16:0 in the *sn*-2 position of dietary TG initially was retained in the *sn*-2 position of chylomicron TG during absorption, but then migrated to the *sn*-1 position through a previously unrecognized isomerization mechanism. The stereospecific position of C16:0 therefore did not influence incorporation or turnover of fatty acids in plasma TG and cholesteryl ester. This FA rearrangement process appears to be an initial step in the overall metabolic sequence that is ultimately responsible for human plasma chylomicron TG structures to contain mainly SFAs at the *sn*-1,3 position (200). Since lipoprotein lipase lyses FAs preferentially in the *sn*-1,3 positions of chylomicron TGs, the FAs

retained in the *sn*-2 position would be transported to the liver in chylomicron remnants. The liver is the major site of action of fatty acids on LDL metabolism (215-218). Observations by Spady, Woolett, and Dietschy (215-218), indicate that SFAs in hepatocytes may inhibit cholesterol esterification and elevate the ratio of unesterified cholesterol to cholesteryl esters. Unesterified cholesterol concentrations in the hepatocyte are inversely related to LDL receptor activity and LDL clearance; higher concentrations of free cholesterol reduce LDL receptor activity (215-217;219-221). Therefore, rearrangement of SFAs to the *sn*-1,3 position of chylomicron TGs would reduce the amount of SFAs in the *sn*-2 position reaching the liver and prevent possible increases in LDL cholesterol concentrations because of reduced clearance.

ENDOGENOUS LIPID AND LIPOPROTEIN METABOLISM

1. Introduction

Cholesterol, triglycerides and other lipids are transported through the circulatory system in lipoprotein carriers. The lipoproteins of interest with regards to Coronary Heart Disease (CHD) are very low-density lipoproteins, low-density lipoproteins and high-density lipoproteins. The most abundant of the circulatory lipoproteins are LDL and HDL (77). The serum total cholesterol value measure used to assess CHD risk encompasses the measurements of LDL and HDL cholesterol; the individual components are just as important as the absolute value, and must be considered. All lipoproteins are in dynamic interaction with each other and this promotes exchange of cholesterol, triglycerides and other lipids among different lipoproteins (177). This section will discuss the homeostasis of endogenous lipids and lipoproteins and the proposed mechanisms behind alteration of their homeostasis by dietary lipids.

2. Endogenous Lipid Transport

The liver is the main tissue responsible for the regulation of whole-body lipid homeostasis; it is the key player in lipid transport, as it is the site of synthesis of lipoproteins formed from endogenous lipids. The liver also takes up exogenous lipids (eg. dietary fatty acids and cholesterol) transferred to it through chylomicron remnants. The lipid portion of chylomicron remnants is hydrolyzed in liver cells (hepatocytes) to free fatty acids, 2-monoglycerides, diglycerides, glycerol and cholesterol; but re-synthesis of these compounds promptly occurs once again in a manner analogous to events in the enterocyte (222). Chylomicron remnant cholesterol and cholesteryl ester may be converted to bile salts or remain as neutral sterol and secreted in bile, or be incorporated into VLDL or HDL and released in plasma (222).

Hepatocytes secrete predominately VLDL into the plasma (223). These lipoproteins contain a large structural apolipoprotein, known as apolipoproteinB-100 (apoB-100). Most of the cholesterol contained in this lipoprotein is present in its esterified form and the main function of VLDL is to transport hepatic TG. Therefore, the main determinant of VLDL production is the rate of hepatic TG synthesis (77). Once in

the plasma, VLDL are acted upon by lipoprotein lipase (LPL) which hydrolyzes a proportion of the TG, enabling delivery of free fatty acids (FFAs) to extrahepatic tissues (223). Concurrently, VLDL exchange TG for cholesteryl esters with HDL in a process promoted by cholesterol ester transfer protein (CETP). They also acquire apolipoproteinE (apoE) from HDL. As a consequence, the catabolic products of VLDL, known as intermediate density lipoproteins are enriched in cholesteryl esters and apoE and are depleted of TG relative to the parent VLDL (224).

Intermediate density lipoproteins are normally present in plasma at very low concentrations, since they are either rapidly taken up by the liver or are further catabolized into LDL (224). Hepatic lipase hydrolyzes TG in IDL and apoE is returned to HDL, resulting in the formation of cholesterol-rich LDL. Low density lipoproteins are catabolized slowly in human plasma and therefore are present at relatively high concentrations. The rate of LDL production is largely determined by the rate at which VLDL are metabolized to LDL (225). The concentration of cholesterol carried in LDL is predominantly dictated by metabolic events occurring in liver (218).

Low density lipoproteins are removed from plasma following binding to cell surface LDL-receptors. This receptor binds the major protein component of LDL, apoB-100, after which the LDL particle is internalized and degraded, delivering its load of cholesterol to the cell (226). Most of the LDL receptor activity is found in the liver, although a proportion of the uptake of plasma LDL is via LDL receptors in extrahepatic tissues.

The LDL receptor is the most important component in the tightly controlled maintenance of cholesterol homeostasis in the body (227). Clinically, the most important effect of LDL receptor deficiency is hypercholesterolemia with accelerated development of atherosclerosis and its complications. The LDL receptor, as an active interface between extra- and intracellular cholesterol pools, is subject to regulation (227). Activity of the LDL receptor is determined in part by the intracellular cholesterol concentration: if the level of cell cholesterol is high, the receptor is downregulated; if low, the receptor is upregulated (224). In this fashion, membrane cholesterol is maintained at a constant level. Since the liver is the main site of LDL clearance, when the amount of cholesterol input increases from exogenous sources (via chylomicron remnants), there is expansion

of sterol pools within hepatocytes and down-regulation of receptors responsible for clearing LDL from the bloodstream. As a consequence, concentration of LDL in plasma increases (218).

It should be noted that uptake of LDL cholesterol by extrahepatic tissues is not essential, since cells in every tissue of the body have ability to synthesize all the cholesterol they need for membrane construction (224). When LDL is available, however, cells primarily use the LDL receptor to import LDL cholesterol and keep their own synthetic activity suppressed (227). Liver is the only organ that has ability to catabolize cholesterol. Thus, as cells and cell membranes undergo normal metabolic turnover, there must exist a mechanism for elimination of cholesterol, which would otherwise accumulate in the cell (224). This is achieved by delivery of cholesterol from cell membranes to plasma HDL in the first step of the reverse cholesterol transport pathway (226). Thus, a constant level of cholesterol is maintained within the cell, while the external supply in the form of lipoproteins can undergo large fluctuations (227). The homeostatic pathways of cholesterol metabolism operate through an efficient regulatory system of membrane-bound transcription factors that are sensitive to membrane and intracellular cholesterol levels and affect activity of genes encoding cholesterol synthesizing enzymes and the LDL receptor of each cell (227).

3. Reverse Cholesterol Transport

Reverse cholesterol transport (RCT) is a pathway transporting cholesterol from extrahepatic cells and tissues to HDL for subsequent catabolism in the liver. By reducing the accumulation of cholesterol in the walls of arteries, RCT may prevent atherosclerosis. The RCT consists of five steps: (i) uptake of cholesterol from cells by specific acceptors (cholesterol efflux); (ii) esterification of cholesterol within HDL by lecithin: cholesterol acyltransferase (LCAT); (iii) transfer of cholesteryl esters to the apoB-containing lipoproteins (cholesterol transfer) by CETP; (iv) remodeling of HDL; and (v) uptake of HDL cholesterol by the liver and possibly also by kidney and small intestine through lipoprotein receptors (cholesterol uptake) (228). HDL is involved in all RCT steps; however, the plasma concentration of HDL is not necessarily the key determinant of the rate of RCT.

High density lipoproteins are secreted from liver or gut as very small particles, or are formed in plasma during lipolysis of TG in VLDL and chylomicrons (77). HDL consists primarily of phospholipids, cholesteryl esters and the lipoproteins apoA-I and/or apoA-II. Several studies have indicated that there are important metabolic differences between apoA-I and apoA-II (132;229;230); HDL particles that contain apoA-I are considered anti-atherogenic (231). It has been reported that the apoA-I fraction of HDL is the preferred plasma acceptor of cell cholesterol (224).

In plasma, HDL is reshaped due to the action of LCAT and the transfer of lipids between HDL and other lipoproteins by CETP. LCAT converts unesterified cholesterol received by HDL to esterified cholesteryl ester via an enzyme system that uses apoA-I as a co-factor. This action of LCAT allows HDL to pack more cholesterol into its core as cholesteryl ester, which is much more hydrophobic than free cholesterol, thereby increasing the capacity of HDL to receive additional cholesterol. CETP, a hydrophobic glycoprotein, circulates in plasma bound mainly to HDL and promotes the redistribution of cholesteryl esters, TG, and to a lesser extent, phospholipids between HDL and other plasma lipoproteins (224). The overall effect of CETP is a net mass transfer of cholesteryl esters from HDL to TG-rich lipoproteins (VLDL and chylomicrons) and LDL, and transfer of TG from TG-rich lipoproteins to LDL and HDL. Consequences of these CETP-mediated transfers of cholesteryl esters from HDL are reductions in the cholesterol content, the apoA-I content, and the size of HDL particles (224). Therefore, CETP could be considered pro-atherogenic through the redistribution of cholesteryl esters from the non-atherogenic HDL to the potentially atherogenic VLDL and LDL, and the decrease in overall concentration and remodeling of HDL. A recent study in humans using torcetrapib, a potent drug inhibitor of CETP, showed that administration of this drug causes HDL levels to significantly increase and LDL levels to decrease (232), suggesting the importance of reduced CETP activity to decrease CHD risk. On the other hand, CETP is considered anti-atherogenic by virtue of its ability to increase the rate of RCT (228).

High density lipoproteins are most likely removed from circulation by the liver, which has receptors or binding sites for HDL. It is uncertain whether the entire HDL

particle is taken up by receptor-mediated endocytosis, or if only HDL cholesterol is taken up by the liver (77).

4. Mechanisms of Lipid and Lipoprotein Alterations by Dietary Lipid

A. Alterations in Low Density Lipoprotein Metabolism

One hypothesis on the effect of dietary fats on serum lipoprotein concentrations relates to alterations in LDL receptor activity. The steady state concentration of LDL is determined by the rate at which this lipoprotein is formed within the plasma, (i.e. the LDL production rate from VLDL, and level of LDL receptor activity in the liver) (215). It is reported that SFAs increase serum LDL concentrations by decreasing the number and activity of LDL receptors in animal and tissue cultures (218-220;225;233). A decrease in the number and activity of LDL receptors decreases the rate of LDL cholesterol catabolism and therefore increases circulating LDL concentrations. VLDL remnants also attach to LDL receptors. As a result, if the number of LDL receptors decreases, more VLDL remnants will remain in the circulation and be converted to IDL and LDL (234).

Hepatic LDL receptor activity varies inversely with the steady state concentration of unesterified cholesterol in hepatocytes (215;216). Hepatic cholesterol and cholesteryl ester concentrations have been shown *in vitro* to be altered in response to exogenous fatty acids (219;221). Using a novel mouse model, Xie *et al* (215) found *in vivo* that C14:0 significantly reduced hepatic esterified cholesterol concentrations, increased unesterified cholesterol concentrations, and led to decreased LDL receptor activity compared to C18:2n-6. These findings are in agreement with studies reported in perfused liver of the African green monkey (235); feeding dietary TGs rich in SFAs resulted in significantly higher plasma LDL concentration, and suppressed LDL receptor activity compared to feeding MUFA-rich TGs (235). Gill *et al* (236) found that decreasing the SFA content of the diet by replacement with MUFAs in mildly hypercholesterolemic male subjects, resulted in decreased LDL concentrations but similar VLDL and IDL levels. This suggests that decreased LDL production was not the main mechanism behind reduced LDL concentration; rather the lower concentrations likely occurred as a result of increased LDL receptor activity. Moreover, Mazier and Jones (237) found that feeding

high-SFA diets (high in C8:0, C10:0, C14:0 and C18:0) to normocholesterolemic men resulted in significantly lower cholesterol fractional esterification rates (FER) and lower serum cholesteryl ester concentrations than feeding diets rich in C18:2n-6. A lower rate of cholesterol esterification in response to SFA intake may result in an expanded hepatic pool of free (unesterified) cholesterol, which is thought to contribute to the down-regulation of LDL receptors and an increase in circulatory LDL concentrations (176). Indeed, serum total- and LDL-cholesterol concentrations were higher in response to the high-SFA diets.

PUFAs (mainly C18:2n-6) have been reported to decrease both LDL and HDL cholesterol by several mechanisms. In monkeys, C18:2n-6 decreases LDL apoB production rates, increases HDL apoA-1 catabolism and increases LDL apoB catabolism (238;239). It has also been established that high dietary levels of C18:2n-6 up-regulate LDL-receptors (240) by increasing the concentration of esterified cholesterol in the liver (215). In contrast, up-regulation of LDL receptors was not found in a recent study (241): C18:2n-6 was shown to down-regulate the LDL receptor of human HepG2 cells (241) with or without cholesterol in culture media, which is consistent with other *in vitro* animal work (242;243). If n-6 PUFA has the same effect *in vivo* as *in vitro*, then decreased LDL receptor activity may not be the reason for reductions in serum LDL cholesterol. One hypothesis for the reduction of LDL with C18:2n-6 is decreased hepatic VLDL synthesis and secretion (244;245) which in turn lowers the level of LDL cholesterol even when LDL receptor activity is reduced. In contrast, research by Xie *et al* (215) showed *in vitro* that C18:2n-6 increased hepatic VLDL secretion more than C14:0, although LDL receptor activity was not decreased. These contradictory findings regarding activity of the LDL receptor and VLDL secretion could be due to the different *in vivo* and *in vitro* models investigated.

Dietary cholesterol is shown to decrease LDL receptor activity *in vivo* (176;217;246;247). However, increases in serum total- and LDL-cholesterol are usually found when cholesterol-rich diets are fed in conjunction with high dietary SFAs (148;248-250), indicating that an interaction effect between cholesterol and fatty acids may exist. Otherwise, in humans, serum concentrations of cholesterol are affected

minimally by feeding dietary cholesterol alone, or when consuming less than 400mg/day (251).

B. Fecal Sterol Excretion and Cholesterol Absorption

Alterations in serum cholesterol and lipoprotein concentrations seen with dietary fatty acids could be a result of fat-induced shifts in cholesterol metabolism including fecal sterol excretion affecting cholesterol absorption. In fecal sterol excretion studies, investigators have shown that feeding dietary fats rich in PUFAs increase cholesterol excretion rates in humans. Moore *et al* (252) measured fecal sterol excretion in men after 16 days of consuming diets containing 40%en as safflower oil (high PUFA) or butter (source of SFA). Fecal sterol excretion increased with safflower oil, and serum total cholesterol concentrations decreased. Similarly, Conner *et al* (253) fed six normocholesterolemic men formula diets containing 40%en as either cocoa butter (high SFA) or corn oil (high PUFA) for three weeks. Fecal sterol excretion was greater with corn oil, indicating that PUFA caused loss of cholesterol from tissues. Grundy (254) observed that feeding safflower oil (high PUFA) compared with lard (high SFA) increased fecal elimination of endogenous sterols in hypertriglyceridemic individuals. Nestel *et al* (255;256) found that feeding diets high in PUFAs to humans resulted in similar increases in total sterol excretion with lower serum total cholesterol concentrations compared to feeding diets high in dairy fats (high SFAs). These observations were made when diets were both high and low in cholesterol (255).

Opposing these observations are those that show variable changes in fecal sterol excretion with different dietary lipids. Avigan and Steinberg (257) fed hypercholesterolemic subjects liquid-formula diets containing corn oil (PUFA) or coconut oil (SFA) for 2-wk periods, then collected feces over 2 to 4 days. Total sterol excretion was unaffected by corn oil consumption (high PUFA) compared with coconut oil (high SFA) although subjects had lower serum total cholesterol when fed the high PUFA diet. Shepard *et al* (258) noted no consistent changes in fecal sterol excretion in subjects fed diets containing either dairy fat (high SFA) or safflower oil (high PUFA). The high PUFA safflower oil diet did however significantly lower serum total cholesterol concentrations. Most of the serum cholesterol reduction was LDL cholesterol (67%), but

VLDL and HDL also fell by 27% and 20% of their respective control value. This observation of serum cholesterol concentration reductions is in agreement with much research in humans fed high PUFA diets (78;87;88). McNamara *et al* (259) fed normocholesterolemic subjects diets containing fats with low to high P:S ratio for 12 weeks, with variable dietary cholesterol levels. Quality of dietary fat had no effect on fecal sterol excretion. Individuals were heterogeneous in their responses i.e. no clear trend was observed (259).

These findings suggest that consumption of PUFA-rich fats enhances cholesterol excretion in most humans although the response is not homogenous. Individuals respond differently to dietary fats, and rates of cholesterol absorption may change with age and vary between sexes (260). Changes in cholesterol excretion may initially change with an alteration in diet, but when studies are extended, a new steady state of fecal excretion may result.

Moreover, accuracy of fecal sterol excretion studies is dependant on complete stool collection in addition to sensitivity of gas-chromatography equipment to ensure that plant sterols are not counted as dietary or fecal cholesterol. In recent years, the role of phytosterols (plant sterols and stanols) to reduce cholesterol absorption has been established (261-274). As a result of reduced cholesterol absorption, serum concentrations of total- and LDL-cholesterol decrease in many human subjects (261;262;270;271). Plant oils are the major dietary source of phytosterols occurring in the typical North American diet. Recent data has shown that phytosterols found in plant oils, such as corn oil, constituting less than 1% by weight of the oil, significantly reduce cholesterol absorption and serum cholesterol concentrations compared to phytosterol-free corn oil (262). This observation and others (275) suggests that phytosterols found in PUFA-rich plant oils may be one of the mechanisms behind reductions in cholesterol absorption and total- and LDL-cholesterol concentrations observed rather than effects of PUFAs themselves.

C. Endogenous Cholesterol Synthesis

The ability of fatty acids to influence endogenous cholesterol synthesis is another mechanism proposed to explain alterations seen in serum cholesterol and lipoprotein

concentrations. Cholesterol enters the body pool from only two sources: absorption from the diet (approximately 250-500mg/day) and synthesis within various tissues in the body (i.e. *de novo* synthesis: 700-1000mg/day) (223). *De novo* synthesis of cholesterol typically contributes two-thirds of the total-body input in humans (276;277). This substantial contribution of endogenous cholesterol synthesis to whole-body input has led to an interest of understanding control factors (175;276;277) because elevated circulatory pools of cholesterol (serum cholesterol) are associated with greater risk of CHD.

All tissues can synthesize cholesterol, and extrahepatic tissues contribute most to whole body cholesterol concentrations. It is proposed that extrahepatic tissues synthesize 80% of total body cholesterol, while liver and intestine each synthesize approximately 10% (175;176). The capacity to synthesize cholesterol is important, as cholesterol is an essential component of cell membranes and the precursor to steroid hormones and vitamin D (222). Cholesterol synthesis is essential during growth and when dietary intake is limited (278;279).

Cholesterol biosynthesis is complex; at least 26 steps are known to be involved in formation of cholesterol from acetyl CoA (222). The pathway can be thought of as occurring in three stages. First, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) is formed from acetyl CoA in a cytoplasmic sequence. Second, HMG-CoA is converted to squalene; this conversion includes the reduction to mevalonic acid by HMG-CoA reductase, which is an important physiological regulatory step in the pathway. Finally, cholesterol is formed from squalene. HMG-CoA reductase can be inhibited by a variety of physiological factors, the most important of which is the intracellular level of cholesterol (222). The enzyme is also the site of action for statin drugs known as HMG-CoA reductase inhibitors (280;281).

In order to fully understand how alterations in endogenous cholesterol synthesis by dietary fatty acids may affect serum lipids and lipoprotein concentrations, a detailed discussion of cholesterol synthesis must be addressed.

5. Endogenous Cholesterol Synthesis

A. Measurement of Endogenous Cholesterol Synthesis

Available methods for measuring cholesterol synthesis either measure substrate flux through the biosynthesis pathway or determine concentrations of one or more pathway intermediates (175;276;277). Prior to the use of stable isotopes, endogenous cholesterol synthesis could be measured by sterol balance, HMG-CoA reductase activity, cholesterol turnover with ^{14}C-cholesterol, and quantitation of plasma cholesterol precursor levels (282;283). These methods each possess various limitations, including the requirement for extended measurement periods and/or indirect or overly invasive approaches (282;283). In contrast, stable isotope tracer methods including deuterium incorporation and mass isotopomer distribution analysis (MIDA) are more immediate, direct, and not overly invasive. The fundamental outcome of both methods is the cholesterol fractional synthesis rate (FSR), which is defined as the fraction of the rapidly turning over cholesterol pool that is newly synthesized from a precursor over a 24-hour period (175;276;277;284).

Deuterium incorporation methodology is based on the rate of incorporation of deuterium-labeled water tracer into *de novo* synthesized cholesterol. Deuterated water is a non-radioactive tracer that can be safely ingested orally and the enrichment of the precursor of cholesterol, plasma water, can be measured (285). Deuterium enrichment of cholesterol is also analyzed and measured by isotope ratio mass spectrometry (IRMS) after isolation and combustion, and reduction to hydrogen gas. The use of deuterium incorporation as an *in vivo* tracer measure of lipogenesis dates back almost 70 years and refinement of this method was dependant on improved precision measurements by IRMS (175)

In the deuterium incorporation technique, deuterated tracer water equilibrates among a precursor pool of known enrichment (the intracellular site of synthesis) and extracellular fluids such as plasma and urine. Plasma is a homogenous representative pool that is accessible for sampling. Extracellular fluids can therefore be used to measure the degree of labeling.

To accurately measure endogenous cholesterol synthesis using deuterium incorporation, the ratio of incorporation of deuterium versus hydrogen into cholesterol

must be examined. The cholesterol molecule contains 46 hydrogen atoms originating from precursors, and some are replaced by deuterium. Because not all precursors along the cholesterol biosynthetic pathway incorporate label at a rate proportional to that of body water, the fraction of each of the 46 protons incorporated into each of the newly synthesized cholesterol molecules must be known (282). Protons (deuterium and/or hydrogen) can be incorporated into cholesterol from only three sources: 7 directly from water, 15 from NADPH, and 24 from cytosolic acetyl-CoA (283). Therefore, the major theoretical concern using deuterium incorporation lies in establishing D_{max}, which represents the maximum number of deuterium atoms that can be incorporated into newly synthesized cholesterol (286). Over periods as long as 48 hours, protons that derive from water and NADPH are in equilibration with total body water, whereas those originating from acetyl-CoA are unlabeled, thereby limiting D_{max} to 22 of the total 46 hydrogens in cholesterol (282;283;286). Over longer periods ranging up to 8 weeks, D_{max} has been shown to increase to 30 from the contribution of the acetyl-CoA pool that may become labeled due to label recycling (282;283). Changes in source of protons used to synthesize NADPH may also influence D_{max} (282;283). NADPH protons originate from two potential sources, the pentose phosphate pathway and the malic enzyme system; there are questions as to whether labeling of NADPH is consistent across different metabolic states (286). Therefore, although the effects of changing metabolic conditions remain to be defined fully, D_{max} is considered to be 22 (0.478 x deuterium enrichment of body water)(283) for studies of cholesterol synthesis less than 48 hours. Periods shorter than 6 hours will not be as accurate, since deuterium may not have equilibrated across body water compartments.

B. Theoretical Assumptions of Deuterium Incorporation

Three fundamental assumptions underlie deuterium incorporation. Firstly, it is assumed that a constant fraction of deuterium atoms in *de novo* synthesized free cholesterol originates from plasma water (282;287). A second assumption is that cholesterol rapidly exchanges between intracellular sites of synthesis and the rest of the central pool (i.e. plasma, liver, intestine) (282). Free plasma cholesterol is considered to be a component of the rapidly turning over central pool of unesterified cholesterol

associated with the liver and small intestine. Within this central pool, cholesterol transmigrates rapidly between cellular plasma membranes and lipoproteins (282). Therefore, plasma water deuterium level following equilibrium of an oral bolus provides a measure of precursor pool enrichment, whereas the deuterium enrichment of plasma free cholesterol corresponds to that of the cholesterol central pool. The third assumption is that deuterium uptake into plasma free cholesterol represents synthesis in the central pool only and does not represent influx of newly formed cholesterol from other pools (282). Since the rate of inter-pool cholesterol exchange is slow, this assumption is justified (286).

C. Diurnal Rhythm in Cholesterol Synthesis

A greater incorporation of deuterium into cholesterol at night as compared to daytime was first identified using indirect markers (288). Since this time, a diurnal pattern of sterol synthesis has been confirmed (279;289). In one study, 5 healthy subjects consumed three self-selected meals/day over a 48-hour period (289). Following a priming dose of deuterium, 4-hour blood samples were collected and deuterium uptake into cholesterol identified. The results revealed distinct differences in deuterium uptake over each 24-hour period, with peak synthesis occurring shortly after 0600 and the lowest levels occurring in the late afternoon and evening. This data suggests that whole body cholesterol synthesis is not constant throughout the day, but varies in a predictable manner.

6. Modulation of Endogenous Cholesterol Synthesis by Dietary Factors

Studies in animals have defined many dietary modulators of cholesterol synthesis, including food restriction (278;290;291), food frequency (292), types of dietary fat (176;246;292-294) and cholesterol intake (176;246;295). Results provide evidence that synthesis can be altered through dietary practices thereby altering the whole-body cholesterol pool size and plasma concentrations. Despite data obtained in animals, it is understood that hepatic contributions of cholesterol to total cholesterol synthesis in humans is much lower (176). These differences emphasize the need to understand dietary influences on cholesterol synthesis in humans.

A. Dietary Lipid Composition, Cholesterol Synthesis and Serum Lipids and Lipoproteins

Animal data have provided inconsistent data regarding the magnitude of effects of dietary fat composition on cholesterol synthesis (176;246;292-294). The variable nature of data in animals is likely due to factors such as the animal model used, duration of feeding, cholesterol content of the diet, and precise fatty acid composition. There are many recognized differences between cholesterol homeostasis regulation between animals and humans (176;277) suggesting limitations in interpreting data from many animal models.

Effects of dietary fat type on cholesterol synthesis have been studied by deuterium incorporation technique in humans. Jones *et al* (296;297) studied cholesterolgenesis in mildly hypercholesterolemic patients consuming diets containing 30%en fat from corn oil, olive, canola, or rice bran oils or beef tallow. Higher cholesterol synthesis rates were observed when subjects consumed high-PUFA corn oil diets compared with other diets. The observations of higher synthetic rates with PUFA consumption have also been seen by Mazier *et al* (298) in normocholesterolemic male subjects when feeding high PUFA safflower oil diets compared to feeding high MUFA olive oil diets. French *et al* (104) showed using the deuterium incorporation method that diets with 5%en from *trans* fatty acids (5%en as TFAs) consumed with a high PUFA oil (6.5%en as C18:2n-6) resulted in a significantly higher FSR of free cholesterol and increased endogenous synthesis of cholesterol versus a high SFA diet containing predominately C16:0 and a lower PUFA intake (3.5%en as C18:2n-6). Matthen *et al* (299) found that as the amount of TFA in the diet increased with decreasing C18:2n-6 content, the FSR of free cholesterol decreased. Clandinin *et al* (129;130) demonstrated that C16:0 at high and low levels with high and low levels of C18:2n-6 in the diets of normocholesterolemic subjects had no significant effect on endogenous cholesterol synthesis suggesting no relationship between C16:0 and cholesterolgenesis. However, the diets in this study that were higher in C18:2n-6 resulted in a slightly greater FSR of free cholesterol even though the FSR for free cholesterol was not statistically significant (129;130). Overall, these observations suggest that dietary PUFA increases cholesterol synthesis (296-298); C16:0 appears to have a neutral effect

(129;130); and effects of TFAs are less clear (104;299) but may be contingent on dietary content of C18:2n-6.

The results of the above studies (129;130;296-298) showed increased cholesterol synthesis rates using the deuterium incorporation method in humans when consuming high dietary PUFA despite finding decreased serum cholesterol concentrations. This suggests that an inverse association may exist between these two measures. The deuterium incorporation method measures contribution of both hepatic and extrahepatic tissues to total cholesterol synthesis (298). It is proposed that extrahepatic tissues synthesize 80% of total body cholesterol, while liver synthesizes approximately 10% (175;176); therefore the majority of changes in cholesterol synthesis would represent extra-hepatic alterations. The inverse relationship between synthesis and serum cholesterol concentrations may be explained by the homeostatic mechanisms responsible for maintaining extrahepatic cellular cholesterol concentrations at certain levels. When less serum cholesterol in the form of lipoproteins is available to each cell, cells must then up-regulate their own internal synthesis to maintain homeostasis (224;227) The type of dietary fat may also influence cholesterol synthesis through regulation of the hepatic free cholesterol pool. Dietary PUFA may increase cholesterol loss from tissues and enhance hepatic synthesis because of increased esterification and lower intrahepatic pool size (175;176). The observation that serum cholesterol concentrations are inversely correlated with cholesterol synthesis (300) is consistent with the notion that hepatic synthesis increases as circulating cholesterol levels decrease. Because the regulatory pool of free cholesterol in liver is small, synthesis in this organ remains elevated with PUFA feeding. Steady state may be maintained through greater biliary cholesterol excretion to offset the higher total body synthesis as seen in studies described showing enhanced fecal excretion of cholesterol with dietary PUFA (175;176;252-255).

B. Dietary Cholesterol, Cholesterol Synthesis and Serum Lipids and Lipoproteins

The ability of dietary cholesterol to modulate cholesterol synthesis has been addressed in animals and humans. In animals, addition of dietary cholesterol results in a marked and immediate suppression of synthesis (246;295;301) accompanied by a noticeable increase in serum cholesterol concentrations, thus also supporting an inverse

relationship between these two measures. Much of the reduction occurs in the liver, where in many species, a majority of body cholesterol in synthesized. In adult humans, dietary cholesterol at various concentrations has been shown to decrease (248;259;302-304) or have no effects (296;298;305-307) on cholesterol synthesis while affecting plasma concentrations variably.

It can be concluded that any effect of dietary cholesterol concentration on cholesterol synthesis is modest in humans, in agreement with the limited influence on serum cholesterol concentrations (250). The recognition of responders and non-responders to dietary cholesterol may explain these observations (151-154;250). Given that the liver contributes 10-20% to cholesterol synthesis (176), it is not unexpected that total production responds only slightly to increases in dietary cholesterol if the majority of the body pool is produced by extrahepatic tissue, which does not take up cholesterol-rich chylomicron remnants. The liver, although responsible for a smaller fraction of whole-body cholesterol synthesis, is relatively sensitive to dietary cholesterol from chylomicron remnants and from endogenous routes via LDL (173). The modest suppression of synthesis that may occur with dietary cholesterol feeding reflects suppression of hepatic synthesis while synthesis from extrahepatic tissues remain relatively unaffected (175).

ENDOGENOUS LIPIDS AND LIPOPROTEINS AND POSITIONAL DISTRIBUTION OF FATTY ACIDS

1. Effect of Fatty Acid Positional Distribution on Lipids and Lipoproteins

A. Animal Research

The stereospecific position of FAs may influence production and elimination of lipoproteins from circulation thereby altering serum lipid and lipoprotein concentrations. An impetus for looking at positional distribution in relation to lipids, lipoproteins and CHD comes from series of animal studies that investigated influence of different fats on atherogenesis. Kritchevsky *et al* (308-311) compared the cholesterolemic effects of native fats and randomized fats and found that those with SFAs in the *sn*-2 position resulted in a greater degree of atherosclerosis than the same fat with SFAs in the *sn*-1,3 positions. This increased atherogenic effect was seen despite similar serum concentrations of lipids and lipoproteins. Hence the differences in atherogenicity could not have been predicted by serum lipid and lipoprotein concentrations of the animals. Even though these studies can not be applied directly to humans, they are of interest because most human studies can not readily assess the atherogenic potential of a dietary component (196).

Innis and coworkers conducted several experiments with growing piglets to evaluate the effect of dietary TG on plasma lipoprotein concentrations (189-191). Piglets fed sow's milk containing 55% C16:0 in *sn*-2 position had higher serum total-cholesterol, HDL cholesterol and serum TGs than piglets fed synthetic TGs (32% C16:0 in *sn*-2) and palmolein (4% C16:0 in *sn*-2). Renaud *et al* (178) showed in rats that feeding interesterified lard (less C16:0 in *sn*-2) resulted in lower plasma TG concentrations than feeding native lard (more C16:0 in *sn*-2) but no significant differences were seen in total- or HDL-cholesterol concentrations. It appears from animal research that altering the positional distribution of C16:0 in dietary TG has little impact on lipids and lipoproteins concentrations except in growing animals such as piglets.

B. Human Infant Research

One human infant study showed that consumption of triglycerides with C16:0 in predominately in the *sn*-2 position resulted in a greater cholesterolemic response than consumption of triglycerides with C16:0 in *sn*-1,3 positions. Nelson and Innis (204) found significantly lower plasma apoA-1 and HDL cholesterol concentrations and higher apoB in infants fed formula made with synthesized TG (39% C16:0 in *sn*-2) than infants fed a standard formula (6% C16:0 in *sn*-2). Total cholesterol and serum TG concentrations were not different between the groups of formula-fed infants. Both formula-fed groups had significantly lower plasma total cholesterol concentrations than infants fed breast milk containing 56% C16:0 in *sn*-2. Breast milk naturally contains a modest content of dietary cholesterol (100 to 120 mg/L) (147) whereas the formulas were cholesterol-free. In addition to the positional distribution of C16:0, differences in dietary cholesterol content, and type of cholesterol (esterified versus unesterified) between breast milk and formula may have influenced the findings in lipid and lipoprotein concentrations.

C. Adult Human Studies

Zock *et al* (170) fed 60 normocholesterolemic male and female subjects two diets of equal fatty acid composition for three weeks in a cross-over design. Each diet supplied 40%en as total fat, 16%en as SFA, and 11%en from C16:0 (with 65% or 18% C16:0 in the *sn*-2-position). Both diets provided 4.5%en from C18:2n-6 and 360mg cholesterol. The high *sn*-2 C16:0 diet was administered as a margarine and oil blend from interesterified palm oil, while the low *sn*-2 C16:0 diet was from natural palm oil. No significant differences were found in serum total cholesterol or lipoprotein cholesterol concentrations in fasting plasma of males and females. The only significant effects were observed in the men who showed small increases in total- and LDL-cholesterol (0.10 mmol/L and 0.08mmol/L respectively) on the diet with C16:0 in the sn-2 position (170). The HDL/LDL ratio and serum TG concentrations were unchanged. In consideration of the more modest differences that can be achieved in everyday diets, the influence on serum cholesterol and lipoprotein concentrations in free-living subjects should be much smaller than the increase observed in men (170). Results suggest that fat with C16:0

mainly in the *sn*-1,3 position does not confer any significant advantage in terms of cholesterol and lipoproteins over fats with the same fatty acid composition but different positional distribution (170).

Nestel *et al* (206) fed 27 moderately overweight hypercholesterolemic men diets containing a special margarine made from either a palm oil blend (C16:0 primarily in *sn*-1,3 position) or an interesterified palm oil blend (C16:0 distributed evenly among all positional distributions). Total fat intake for the palm diets was 32%en, SFA intake was 15%en, C16:0 intake was 7%en and C18:2n-6 was 5%en. After three weeks on each diet, fasting blood samples were taken for three days and averaged. Total cholesterol, HDL and LDL concentrations were not different between each of the palm oil dietary treatments, suggesting that altering the positional distribution of C16:0 does not influence the plasma lipid and lipoprotein response in hypercholesterolemic male subjects (206).

Meijer and Weststrate (312) fed 60 healthy male and female subjects a diet consisting of a control blend of commonly consumed vegetable fats or a interesterified blend from coconut oil, palm oil, palmstearin, a low *trans* partially-hydrogenated rapeseed oil, and soybean oil. The control and interesterified blends had the same fatty acid composition, but the control blend contained 7% *sn*-2 C16:0 and the interesterified blend contained 18% *sn*-2 C16:0. Diets provided 34%en as total fat, 15%en as SFA, 5%en as PUFA, and 300mg cholesterol. Each fat blend was supplied at two energy levels (4%en or 8%en) included in normal foods for half of the subjects in a parallel design. In both energy level groups, the two fat blends were given to all subjects according to a cross-over design, with each fat blend included in the diet for 3 weeks. Neither the type of fat blend nor the energy level at which the fat blends were consumed resulted in significant differences in blood lipids and lipoproteins, blood enzymes and hemeostasis parameters between the groups; all lipoprotein concentrations were within the normal recommended range (312). Total cholesterol and HDL cholesterol concentrations were higher in females after all diet treatments (312). This study used a blend of commonly consumed vegetable oils at a realistic level of intake in normocholesterolemic adults. No effect was noted on blood lipid and lipoprotein concentrations despite differences in enrichment of C16:0 at the *sn*-2 position of dietary TG.

Thus far, research comparing feeding dietary treatments differing in positional distribution of C16:0 from interesterified oils in adult humans suggests that positional distribution does not alter the response of serum lipids and lipoproteins.

2. Positional Distribution of Fatty Acids in Palm Oil and Lard

Palm oil and lard are two biological dietary lipids that have a high content of C16:0, but differ greatly in positional distribution of this SFA. In palm oil, C16:0 is mostly esterified in the *sn*-1 and *sn*-3 position. Conversely, in lard, C16:0 is found primarily in the *sn*-2 position. The principal TG species found in palm oil are POP, POO, POL and PLP (P = palmitic C16:0, O = Oleic C18:1n-9, L= linoleic C18:2n-6). In lard, the principle TG species are SPO, OPL, and OPO (S= stearic C18:0) (196). Palm oil must be distinguished from palm kernel oil, as the FA composition of these two oils is quite different. Palm kernel oil has less C16:0 and is a lauric acid-rich oil (45% C12:0), containing 83%en as SFAs (158). Palm oil is mainly used for food, while palm kernel oil is mainly used for the oleochemical industry (313). Dry fractioning of palm oil produces palmolein, a liquid fraction, and palmstearin, a solid fraction. Both fractions contain the same positional distribution of the major fatty acids, but palmolein contains more C18:1 and C18:2n-6 and less C16:0 than palmstearin (314).

Palm oil has been compared to lard as a preferable dietary lipid source mainly due to the stereospecific configuration of fatty acids (most of the SFAs occur at the *sn*-1,3 positions). Moreover, because palm oil is a vegetable oil, it is cholesterol-free, whereas lard is not (313;315). Other beneficial aspects of palm oil include an anti-clotting effect in blood, and vascular relaxation in animals and humans (313;316-318) and lower atherosclerotic buildup in rabbits (319-321).

A number of human feeding studies have reported that palm oil (fed mostly as palmolein) in the diet does not have negative effects on concentrations of serum lipids and lipoproteins (117;127;135;136;322-324). In one study, feeding palm oil (as refined bleached palm oil) was compared to lard in diets of normocholesterolemic Chinese subjects (135). Palm oil feeding was found to result in lower serum concentrations of total- and LDL-cholesterol and higher TC/HDL ratio than lard feeding (135). Total cholesterol concentrations were 3.36 ± 0.53 and 3.99 ± 1.24 mmol/L; LDL-cholesterol

was 2.13 ± 0.53 and 2.85 ± 1.23 mmol/L; and TC/HDL ratio was 3.5 ± 0.9 and 4.3 ± 1.5, for palm oil and lard diets respectively (means ± SD). These results showed that palm oil diets were less cholesterolemic, but both diets resulted in serum cholesterol concentrations that were within the recommended range. The diet in this study provided 30%en as total fat and was administered for six weeks. The palm oil diet had 12%en as SFA, 11%en as C16:0, 11%en as C18:1, 5%en as C18:2n-6 and 220mg cholesterol, while the lard diet had 10%en as SFA, 6.5%en as C16:0, 14%en as C18:1, 4.5%en as C18:2n-6 and 320mg cholesterol. Observed lipid and lipoprotein differences seen here may be attributed to differences in dietary cholesterol and C16:0 and C18:2n-6 levels between palm oil and lard diets. Hence, if contribution from these components was more similar between the diets, the lipid and lipoprotein concentrations may have not been significant.

Research with lard/pork products in normocholesterolemic humans showed that when pork fat contains higher amounts of n-6 PUFA (C18:2n-6), fasting lipid and lipoprotein concentrations were decreased (325). Stewart *et al* (325) fed 24 normocholesterolemic females diets for 4 weeks consisting of modified pork fat which was high in n-6 PUFA, or a unmodified pork fat lower in n-6 PUFA. Both diets provided 42%en as total fat, and 300mg of cholesterol. The standard pork diet had 16%en as SFA and 6%en as C18:2n-6, while the modified pork diet had 10%en as SFA and 16%en as C18:2n-6. The modified pork products were produced by altering the feed given to the swine, thereby resulting in higher amounts of n-6 PUFA and lower amounts of SFA and MUFA than standard pork. Concentrations of serum total- and LDL cholesterol were lower with feeding diets of modified pork fat compared to the unmodified pork fat, however, the TC/HDL ratio was not significantly different (325). Additionally, serum lipid and lipoprotein concentrations after feeding each diet were within the recommended range. Total cholesterol concentrations were 3.39 ± 0.09 mmol/L and 4.01 ± 0.15; and LDL cholesterol concentrations were 1.79 ± 0.17 and 2.34 ± 0.14 mmol/L for modified and unmodified pork fat respectively (means ± SEM). There were also significant changes from SFAs and MUFAs to n-6 PUFAs in plasma and erythrocytes (325). These changes confirmed compliance to each dietary treatment, since dietary fatty acid intake is reflected at the cellular level (326-328). These results support findings that effects of

dietary C16:0 are modulated by levels of C18:2n-6, rendering them less cholesterolemic (104;123;126;129;130;134).

Overall, there have been few human feeding studies that compared palm oil and lards effects on lipids and lipoproteins (135), and results must be carefully interpreted. Levels of dietary C16:0, C18:2n-6 and cholesterol must all be evaluated before these two natural fat sources of C16:0 can be compared to assess their cholesterolemic effects.

RESEARCH OBJECTIVE

The purpose of the current research study is to compare the cholesterolemic response of feeding C16:0 in the *sn*-1,3 position from palm oil with C16:0 in the *sn*-2 position from lard in normocholesterolemic male subjects. It is hypothesized that feeding a diet rich in C16:0 in the *sn*-1,3 position will be less hypercholesterolemic than feeding a diet rich in C16:0 in the *sn*-2 position.

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CHAPTER 2-RESEARCH STUDY

1. Introduction

The association between dietary fat and coronary heart disease (CHD) risk has been established by numerous epidemiological and clinical investigations (1;2). Dietary fat containing a high proportion of saturated fatty acids (SFAs) has generally been shown to increase the risk of CHD by elevating fasting concentrations of serum total- and LDL-cholesterol; polyunsaturated fatty acids (PUFAs) reduce these concentrations and lessen risk; and monounsaturated fatty acids lower these concentrations or have minimal effect (3). Within the class of SFAs, individual fatty acids do not influence cholesterol concentrations to the same extent. Lauric (C12:0), myristic (C14:0), and possibly palmitic (C16:0) acid, are suggested to be the most hypercholesterolemic, while stearic acid (C18:0) is suggested to be neutral (4;5). As an initial step in the prevention of hypercholesterolemia, most health agencies recommend a diet that restricts total SFAs and/or dietary cholesterol (6;7). In this restriction, SFAs should contribute less than 10% total energy (%en) (6).

The limitation of consumption of dietary fats rich in C16:0 has been debated on the basis of several investigations that suggest C16:0 is not as hypercholesterolemic as once implicated (8;9). Animal (10-12) and human (9;13-16) research has demonstrated that C16:0 may be considered neutral or 'conditionally' hypercholesterolemic. This neutrality is contingent on the dietary content of linoleic acid (C18:2n-6) and cholesterol (9;13-17). When C18:2n-6 intake is greater than 5%en and cholesterol is less than 400mg/day, C16:0 does not negatively effect serum total- or LDL-cholesterol in healthy humans (12;15;16;18-20).

Positional distribution of C16:0 in dietary triglyceride may also be a factor determining its cholesterolemic properties. During normal intestinal digestion, fatty acids are hydrolyzed from the *sn*-1,3 positions resulting in production of free fatty acids and 2-monoglycerides (21). Saturated fatty acids at the *sn*-1,3 position may be absorbed less efficiently as they form insoluble soaps with calcium and magnesium and are excreted in feces (22;23). Studies in animals (24;25) infants (26;27) and humans (28;29) indicate that

75-80% of fatty acids in the *sn*-2 position of triglycerides are retained during digestion and absorption and incorporated into chylomicrons. Palmitic acid in the *sn*-2 position of dietary triglyceride is more atherosclerotic than C16:0 in the *sn*-1,3 position when fed to rabbits (30;31). In both rats (25) and human infants (32), feeding C16:0 in the *sn*-2 position suggests that fasting serum cholesterol concentrations are higher than feeding C16:0 predominately in the *sn*-1,3 position. These observations imply that C16:0 in the *sn*-2 position of dietary triglyceride may impart hypercholesterolemia.

Investigations of normocholesterolemic and hypercholesterolemic adult human have failed to show that feeding interesterified oils containing more C16:0 in the *sn*-2 position, compared to oils containing less C16:0 in the *sn*-2 position, result in a significant increase in serum total- or LDL-cholesterol (14;33-35). Altering the natural triglyceride structure of dietary fats through interesterification to provide less C16:0 in the *sn*-2 position does not appear to alter fasting serum cholesterol concentrations in adults. Few studies however have compared feeding unaltered biological sources of dietary fat, such as palm oil or lard which contain C16:0 in different positional distributions, on serum cholesterol and lipoprotein concentrations in humans (36). In palm oil, C16:0 is mostly esterified in the *sn*-1 and *sn*-3 position (37). In lard, C16:0 is found primarily in the *sn*-2 position (38). Human research has investigated the dietary influences of palm oil and shown that it is not hypercholesterolemic (13;36;39-42); this may be due to predominant esterification of C16:0 in the *sn*-1,3 position.

The purpose of this study is to compare the cholesterolemic response of feeding C16:0 in the *sn*-1,3 position from palm oil with C16:0 in the *sn*-2 position from lard in normocholesterolemic male subjects. It is hypothesized that feeding a diet rich in C16:0 in the *sn*-1,3 position will be less hypercholesterolemic than feeding a diet rich in C16:0 in the *sn*-2 position at both low and high levels of C18:2n-6. This hypothesis will be tested by assessing fasting serum cholesterol concentrations in response to feeding each dietary treatment for two weeks. Endogenous fractional cholesterol synthesis rates will be assessed using the deuterium incorporation method. C-reactive protein, a marker of acute inflammation and independent risk factor for CHD and atherosclerosis (43) will also be determined in subjects after feeding each dietary treatment.

2. Experimental Methods

Subjects

The protocol was approved by the Human Research Ethics Review Committee at the University of Alberta. All subjects gave written consent prior to the investigation. Ten male subjects with no history of medical problems and no family history of heart disease or hyperlipidemia were recruited through advertisements posted at the University of Alberta. Eight subjects completed all aspects of the study. Each subject was interviewed to inform them of purpose of the study, to explain expected responsibility and time commitment, and outline dietary treatments. An in-depth questionnaire was completed by each participant to characterize activity level, food intake, and sleeping patterns, and provide information about food allergies and food dislikes. It was important that subjects were willing to consume lard and pork products for a portion of the study; that they would only eat food given to them; and that they would refrain from alcohol and caffeine during the study. Subjects were not taking any medications or supplements that would influence blood lipids or cardiovascular function. Blood samples were taken from each subject to give baseline values of serum cholesterol and triglyceride concentrations.

Caloric requirements for each subject were determined using 3-day food records and the Mayo Clinic Nomogram, incorporating the subject's height, weight, age, sex and activity factor (44). Subjects were weighed daily before breakfast to ensure body weight remained stable. Adjustments were made to energy intake if sustained weight changes were observed or if total energy expenditure was altered.

Diets

The study consisted of four dietary treatments of 14 days each with a 14-day washout period between. Each treatment consisted of a 3-day rotational menu portioned into three isocaloric meals (refer to Appendix A for example menus) with a total of approximately 30% energy from fat, 15% energy from protein, and 55% energy from carbohydrate. Diets were based on normal foods and designed using Food Processor II nutrient analysis computer software program (ESHA Research, Salem, Oregon, USA). Each dietary treatment was balanced for omega-3 fatty acids, cholesterol, and fiber content. Fatty acids were calculated from nutrient composition tables and laboratory

analysis to provide high levels of C16:0 (~8%en) with low and high levels of C18:2n-6 (3%en and 7-9%en respectively). Lard and Canadian pork products such as ham, bacon, sausage, and pork chops were fed to provide a dietary triglyceride source of C16:0 in the *sn*-2 position of triglyceride. Palmstearin and a small amount of low-fat dairy products were fed to provide C16:0 in the *sn*-1,3 position. The four dietary treatments were: 1) *sn*-2 C16:0 Low C18:2n-6; 2) *sn*-2 C16:0 High C18:2n-6; 3) *sn*-1 C16:0 Low C18:2n-6; and 4) *sn*-1 C16:0 High C18:2n-6. Laboratory analysis of the fatty acid composition of lard and palmstearin is shown (Table 2-1).

Table 2-1. Laboratory Analysis of Fatty Acid Content of *sn*-1 and *sn*-2 Dietary Fat[1]

C16:0 Source	SFA	C16:0	C18:0	MUFA	C18:2n-6	Cholesterol
	g	g	g	g	g	mg
Palmstearin	70.1	63.4	4.92	24.3	5.29	0.0
Lard	39.6	26.5	14.7	42.5	8.43	95.0

[1]Data expressed per 100grams

A high polyunsaturated safflower oil and/or sunflower oil was fed to provide a dietary source of C18:2n-6. The majority of the fat was provided by lard and pork products in the *sn*-2 C16:0 Low C18:2n-6 diet. In the *sn*-1 C16:0 Low C18:2n-6 diet, the majority of fat was provided by palmstearin and monounsaturated fat from olive oil, mixed nuts, and peanut butter (non-hydrogenated). The remainder of the fat sources in diets were: low-fat dairy, egg yolks, beef, chicken, bagels, low-fat dinner buns, English muffins, and waffles. Pre-prepared entrées obtained from Bassilis Best (Fabko Foods, Ltd, Edmonton, Alberta, Canada) were used for the majority of the lunch and dinner meals. These entrees contained low amounts of total fat and were analyzed before use to confirm fat and fatty acid composition and to calculate fatty acid intake (Table 2-2).

Table 2-2. Laboratory Analysis of Fat Composition of Bassilis Best Entrée's[1]

Entrée	Total Fat	SFA	MUFA	PUFA	Cholesterol
	g	g	g	g	mg
Teriyaki Chicken	4.92	0.94	1.84	1.79	31.8
Thai Chicken	4.66	1.65	1.53	1.39	30.8
Country Beef and Potato	9.13	2.91	4.26	1.91	35.2

[1] Based on 300gram serving size

In order to make all diets palatable and enjoyable, the lard and palmstearin were incorporated into common food items such as hashbrowns at breakfast, pitas and sandwiches at lunch, and the pre-prepared Bassilis Best entrées at dinner. The palmstearin presented an interesting challenge as it was completely solid at room temperature, so it had to be incorporated into hot food items for consumption. If the palmstearin was not in a hot food, it had a consistency that was comparable to wax, and this was not enjoyable for the subjects. The lard was a little easier to work with since its consistency was similar to soft margarine, and could be used as a sandwich spread. The lard also would not solidify at room temperature like the palmstearin.

Meals for each dietary treatment were prepared daily in the Human Nutrition Research Center kitchen for consumption in the dining room (breakfast and lunch) or packaged for take-out (supper). Meals were consumed at regular intervals; 0730-0900h, 1130-1300h, and 1730-1900h for breakfast, lunch and supper respectively, depending on the individual participants' schedule. Additional foods allowed were some fruits and/or vegetables devoid of any fat. Beverages allowed were clear herbal tea, decaffeinated coffee, some sugar-free fruit juices, and permitted beverages devoid of caffeine. To assess compliance, dishware and containers were inspected to ensure that all visible fat was eaten.

Protocol

Subjects consumed the same dietary treatment during the same 14 day period and were fed diets in the following order:

Diet 1: *sn*-2 C16:0 (8% of kcal), Low C18:2n-6 (3% of kcal)
Diet 2: *sn*-2 C16:0 (8% of kcal), High C18:2n-6 (7-9% of kcal)
Diet 3: *sn*-1 C16:0 (8% of kcal), Low C18:2n-6 (3% of kcal)
Diet 4: *sn*-1 C16:0 (8% of kcal), High C18:2n-6 (7-9% of kcal)

A 30ml fasting blood sample was obtained by venipuncture for serum cholesterol determinations and cholesterol synthesis measurements was taken between 0730h and 0900h on day 13 (background day) for each diet phase. Prior to this day, subjects were instructed to perform the same amount of physical activity and refrain from eating after 2000h. Subjects consumed a priming dose of deuterium oxide (D_2O) at 0.5g D_2O/kg

estimated body water (estimated as 60% of body weight) before breakfast was eaten. A maintenance dose of 1.0g D_2O/kg estimated body water was provided in a 2L bottle of water for consumption over the next 24 hours to maintain plasma deuterium enrichment at plateau and to compensate for unlabeled water obtained in the diet. Twenty-four hours after the priming D_2O dose (day 14), a second fasting blood sample was collected. Plasma was obtained by centrifugation (3000rpm for 10 min) and frozen at –20C. Day 13 background samples were analyzed for total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, and C-reactive protein determination. The background sample of plasma was used to determine baseline deuterium concentration in plasma water, plasma cholesterol and cholesteryl ester, and the enriched sample (day 14) was used to determine deuterium enrichment over the 24 hour period.

Fat Analysis of Diets

Diets were analyzed for total fat and fatty acid composition. Duplicate samples for each meals in the 3-day menu of each dietary treatment were prepared. Meals were homogenized in a Sybron/Brinkman polytrin (Model PT/10/35) with the addition of water to a smooth paste. An aliquot of the blended food paste containing approximately 0.3 to 0.5g of fat was weighed into a beaker and extracted with 40 ml of chloroform-methanol (2:1) (vol:vol) mixture with a magnetic stirrer for 10 minutes (45). The mixture was then transferred into a separatory funnel and the beaker rinsed repeatedly with the extracting solvent mixture of chloroform-methanol up to a total volume of 100 ml. A known amount of internal standard (tripentadecanion, Sigma Chemical T4257) was added. This was followed by the addition of an aqueous solution of 0.0125% $CaCl_2$, equivalent to 20% of the volume of extracting mixture. The funnel was shaken vigorously and left to stand overnight in the cold room for separation of the two phases.

For quantitative determination of fat, the bottom layer containing the dietary lipid was collected into a pre-weighed 50 ml culture tube. The solvent was then evaporated under nitrogen until the total weight of the tube plus fat was relatively constant. For qualitative determination of the fatty acid composition of the dietary fat, duplicate samples of 50 µl of the solvent collected were transferred into screwed cap culture tubes (13x100mm) and evaporated to dryness under nitrogen. Dietary fat was saponified with 1

ml of 0.5N KOH in methanol for one hour on a heating block at 110°C. After cooling, 1 ml of BF_3 in methanol (14% BF_3) and 2 ml of hexane was added to the tube and the sample was methylated for 1 more hour at the same temperature. At completion of methylation, 1 ml of water was added to the methylation tube and the tube vortexed. The hexane upper layer was transferred to a GC vial and the volume of hexane further reduced to 0.5 ml for GC analysis.

Fatty acid methyl esters were analyzed by gas-liquid chromatography equipped with a hydrogen flame ionization detector and an auto-sampler (Vista 3400 CX GLC and Vista 8200 data system; Varian Instruments, Georgetown, Ontario). Fatty acid methyl ester separation and identification was performed on a capillary column (DB20 25m x 0.22mm ID; SGE Inc, Austin, Texas, USA). Pre-purified helium gas was used as the carrier gas at the flow rate of 1.25 ml per minute. The temperature programming of the column running condition was as follow: initial column temperature of 90°C maintained at 4 min, followed by an increment of 25°C/min to 170°C and held for 13.8 min, then increased at 5°C/min to 190°C, held for 20 minutes, and finally increased at 10°C /min to 230°C, and held for 16 minutes. One µl of hexane was injected into the GC and the retention times of each of the fatty acid methyl ester was compared with those of a standard fatty acid methyl ester (NU-CHEK PREP, Inc) containing 32 known fatty acid methyl esters.

Determination of Deuterium Enrichment

Deuterium enrichment was measured in plasma cholesterol, plasma cholesteryl ester and plasma water. To obtain free and esterified cholesterol, a 0.2ml aliquot of plasma and 1 ml of diethyl ether was added to a solution of 2 units of phospholipase C in 2 ml of 17.5 mM Tris buffer (pH 7.3) and 8 mg (0.8 ml) of 1% $CaCl_2$ (46;47). The mixture was incubated with stirring for 2 hours in tightly closed screw cap vials at 37°C. After 2 hours, the reaction was extracted with 10 ml chloroform-methanol (2:1) (vol/vol) and 100µg of internal standard, tridecanoylglycerol was added. The solvent phases were separated by centrifugation for 10 min at 200 *g* after extraction. The upper layer was removed with a vacuum pipette and the clear lower chloroform phase was passed through a Pasteur pipette containing 2 g anhydrous Na_2SO_4. The solvent was evaporated to

dryness under a stream of nitrogen. The lipids were then reacted for 30 min at room temperature with 100 µl SYLON BFT (Sigma Chemical) plus 5 drops dry pyridine. This procedure converts the free fatty acids into silyl esters and the free sterols, diacylglycerols, and ceramides into silyl ethers leaving the cholesteryl esters and triacylglycerols unmodified (46;48). The reaction mixture was evaporated to dryness, diluted with 100 µl hexane and used for direct GLC analysis (49). The analyses were performed on an Agilent Model 6890 ThermoFinnigan Delta*Plus*XL Gas Chromatography/ Pyrolysis/ Isotope Ratio/ Mass Spectrometer (GCIRMS, Bremen, Germany). The injector temperature was 325°C to ensure that the entire sample was volatilized. The split ratio was 10:1. Analyses were performed using an HP-5 capillary column (30m x 0.32 mm ID and 0.25µm film thickness). For analysis in the DeltaPlusXL, the sample is injected on-column at 150°C, held for 1 min and then the oven temperature was increased 30°C/min to 200°C, then 20°C/min to 325°C and held for 5 min (46). The analytical precision of the instrument was calculated from multiple analyses (n=96) of the hydrogen produced from the pyrolysis of the internal standard, tridecanoylglycerol. The coefficient of variability of the instrument was 4%. All samples were analyzed in duplicate.

Samples of day 14 plasma (enriched plasma), intended for plasma water enrichment measurement were diluted twenty-fold with 5% bovine serum albumin solution to lower the deuterium enrichment to within the analytical range of the IRMS instrument. Baseline day 13 samples were not diluted. Plasma water samples (10 µL) were vacuum distilled into Pyrex tubes containing zinc. The water samples from plasma were reduced by zinc to hydrogen gas by placing the reaction tubes in a heating block at 500°C for 30 minutes. The reaction tubes could be attached directly to the mass spectrometer without further purification. The deuterium enrichment in plasma water was measured by use of a Finnigan MAT 251 Isotope Ratio Mass Spectrometer (IRMS, Bremen, Germany) against hydrogen prepared from a water standard. The mass three abundance was corrected for H_3^+ contribution and was determined daily. Multiple analyses of hydrogen produced from the reduction of laboratory water standard demonstrated the analytical precision (coefficient of variation) of this instrument at <1%. All samples were analyzed in duplicate.

Cholesterol fractional synthesis rates (FSR) were determined from the initial incorporation rate of deuterium-labeled cholesterol into the rapid exchangeable cholesterol pool, relative to the initial precursor enrichment determined using body water deuterium level (50). Maximum attainable enrichment was calculated as body water pool enrichment corrected for the fraction of protons in *de novo* synthesized cholesterol that derive from water, relative to non-water sources (50;51) using the equation:

$$FSR_{FC} = \frac{\delta_{FC}}{(\delta_{PW} X0.478)}$$

where δ_{FC} is the change in enrichment in the free cholesterol fraction and δ_{PW} is the change in enrichment in the plasma water over 24 h. The enrichment in each fraction of free cholesterol (FC) and cholesteryl ester (CE) are determined separately and the total FSR calculated.

Isotopic enrichments are expressed in per mil using the δ notation defined as:

δ = [(R sample/R standard) -1] x 1000

where R is the ratio of the heavy to light isotope. Because not all the various precursors along the cholesterol biosynthetic pathway incorporate label at a level proportional to body water, the constant of 0.478 must be used (50). This constant represents the number of hydrogen atoms per cholesterol molecule that are predicted to be replaced by deuterium (50).

3. Statistical Analysis

A two-way analysis of variance (ANOVA) with repeated measures was used to determine the significance of dietary treatments on plasma lipids, lipoproteins and fractional synthetic rates (SAS vs. 8.2 Inc., Cary NC, USA). Significant differences were determined among dietary treatments by a Duncan's multiple range test. Statistical significance was set at $p<0.05$.

4. Results

Subjects

The study began with 10 male subjects, but two subjects dropped out. One subject left because he developed digestive problems from eating more fat than he was accustomed. The other subject discontinued participation due to other time commitments that restricted him from being able to eat at the Human Nutrition Research Center each day. Eight subjects successfully completed each dietary treatment. Every effort was made to accommodate each subject, and due to individual time commitments, some subjects had to complete a diet treatment at a different time than the main group. In diet treatment 1, all eight subjects completed the diet at the same time. In diet treatment 2, one subject completed the diet 4 weeks after the seven other subjects. In diet treatment 3, one subject completed the diet 2 months after the seven other subjects. In diet treatment 4, three subjects completed the diet two weeks after the four other subjects.

Descriptive data of subjects are shown (Table 2-3). The subjects ranged in age from 21-28 years old.

Table 2-3. Descriptive Data of Subjects

Age yr	Mean Wt kg	Height cm	BMI
28	89.3	170	28.0
26	75.3	175	24.5
27	64.1	173	21.7
28	86.3	180	26.5
26	108	187	28.3
24	88.5	195	24.5
21	73.8	190	20.8
27	69.3	182	20.8
25.9 ± 2.4	**81.8 ± 14.1**	**182 ± 8.7**	**24.4 ± 3.1**

Data expressed as mean ± SD.

Habitual dietary intake of subjects is shown (Table 2-4) and was determined using self-reported 3-day dietary intake records. Dietary records were analyzed using Food Processor II. The average caloric intake was 3019 ± 614 kilocalories and total fat intake ranged from 22-46% of total energy. Habitual saturated fat intake was similar to the amount of saturated fat fed in each dietary treatment.

Table 2-4. Habitual Dietary Intake of Subjects

Subject	Calories kcal	Protein %kcal	CHO %kcal	Total Fat %kcal	SFA %kcal	Cholesterol mg
1	2210	18	37	46	10	442
2	3504	21	43	38	10	689
3	2922	14	55	31	9	191
4	3061	12	60	28	9	301
5	3848	11	50	31	9	168
6	3635	17	48	26	8	364
7	2716	24	55	22	7	326
8	2255	18	45	32	12	223
Mean ± SD	**3019 ± 614**	**17 ± 4**	**49 ± 8**	**32 ± 7**	**9 ± 2**	**338 ± 169**

CHO, carbohydrate; SFA, saturated fat

Dietary compliance by each subject to all diet treatments was high as evident by positive feedback and lack of returning evening and weekend meals unfinished. Each subject adhered to requests to only eat foods and drinks given to them or allowed during the study, and refrained from all alcohol and caffeine.

All subjects maintained their baseline weight on the calorie level assigned to them at the beginning of the study. Body weight fluctuations over the 14-day feeding periods were negligible (Table 2-5).

Table 2-5. Body Weight Changes of Subjects During Each Dietary Treatment

Subject	*sn*-2 C16:0 Low C18:2	*sn*-2 C16:0 High C18:2	*sn*-1 C16:0 Low C18:2	*sn*-1 C16:0 High C18:2	Overall Change
1	0.1	-0.3	-0.4	-0.2	0.2
2	-0.4	0.5	0	-0.1	-0.1
3	-0.1	0.2	-0.1	-0.2	-0.1
4	1	0.7	0.3	0	1.8
5	0	0	-1	-1	-1
6	-0.2	1	0.2	0.5	0.1
7	0	-1	0	0	-0.5
8	0.5	-0.5	0	-0.2	0
Mean ± SD	**0.1 ± 0.4**	**0.1 ± 0.7**	**-0.1 ± 0.4**	**0.0 ± 0.5**	**0.0 ± 0.8**

Data expressed in kilograms and are differences between weight measured on Day 1 and Day 14 of each treatment phase.

Diets

Diets were consumed from the end of January 2003 to the end of September 2003. Formulated nutrient content of diets and laboratory analysis of diets, indicated in brackets, are shown (Table 2-6). The contribution of total energy from fat in each diet was 30-36%. The dietary treatment with highest quantity of fat was *sn*-2 C16:0 High C18:2n-6 diet (36%en) due to the fact that it was difficult to keep total fat lower and achieve at least 6%en C16:0 in combination with at least 6%en C18:2n-6. The difference in total fat and fatty acids between laboratory analysis of diets and diets formulated by Food Processor was negligible except for the *sn*-2 C16:0 High C18:2n-6 diet that had 5% more energy from monounsaturated fat than originally formulated. The content of cholesterol between each diet treatment ranged from 259 to 347mg. Diets that provided *sn*-1 C16:0 from palmstearin contained less cholesterol than diets that provided *sn*-2 C16:0 from pork fat because palmstearin does not contain any cholesterol, whereas pork fat does contain cholesterol. There were no differences between dietary treatments in regards to omega-3 fatty acids (0.24 ± 0.11%en) calcium (1052 ± 62mg), or types and amounts of dietary fiber (23.2 ± 8.3g). The polyunsaturated to saturated fat ratio (P: S ratio) was approximately 0.32 in the Low C18:2n-6 diets and 0.82 in the High C18:2n-6 diets. Energy contribution of total C16:0 in the *sn*-2 position was estimated based on data from Sheppard *et al* (52) with dairy lipid containing 43% C16:0 in *sn*-2; lard (pork lipid) containing 65% C16:0 in *sn*-2; and palm oil containing 23% C16:0 in *sn*-2 (52). Total contribution of energy as C16:0 from dairy lipid, lard, and palm oil are also presented.

Table 2-6. Nutrient Content of *sn*-1 C16:0 and *sn*-2 C16:0 Dietary Treatments

Energy Source	*sn*-2 C16:0 Low C18:2	*sn*-1 C16:0 Low C18:2	*sn*-2 C16:0 High C18:2	*sn*-1 C16:0 High C18:2
Energy: kcal	3000	3000	3000	3000
Total Fat	33.1 (31.5)	29.6 (30.2)	35.7 (36.8)	31.0 (30.1)
C16:0	8.33 (7.91)	9.94 (11.0)	7.49 (6.69)	9.06 (9.58)
C18:0	4.05 (4.00)	1.42 (1.66)	3.81 (4.47)	1.44 (1.47)
MUFA	12.8 (12.1)	11.3 (12.0)	11.5 (16.4)	7.73 (10.1)
C18:2n-6	3.06 (3.11)	3.02 (2.69)	8.21 (6.77)	8.96 (8.68)
C18: 3n-3	0.31 (0.26)	0.21 (0.14)	0.25 (0.39)	0.15 (0.17)
Cholesterol mg	347	259	332	296
Dietary Fiber g	19.2	25.9	23.7	24.1
Calcium mg	1000	1140	1050	1020
Dairy C16:0	0.35	1.80	0.00	1.67
Lard/Palm C16:0	7.23	7.62	6.55	6.84
Total *sn*-2 C16:0	5.74	1.98	6.39	1.80
P: S Ratio	0.35	0.30	0.84	0.81

All values derived using Food Processor II nutrient analysis software Version 7.71 (Esha Research, Salem, OR, USA) except for C16:0 and C18:2 n-6 which are derived from laboratory analysis. Values in brackets indicate those obtained by laboratory analysis. Meals were analyzed in duplicate. Values are expressed as % kilocalories of total energy. Kcal=kilocalorie, mg=milligram, MUFA=monounsaturated fatty acid.

Plasma Lipid and Lipoprotein Concentration

Mean plasma lipid and lipoprotein concentrations in subjects fed each dietary treatment are shown (Table 2-7).

Total Cholesterol. The normal concentration of serum total cholesterol for individuals within the age range of subjects studied is 3.20-5.20 mmol/L. Mean concentrations of total cholesterol after consumption of all test diets was within the normal range and there was no difference between total cholesterol observed after each of the diets (Table 2-7). One subject's total cholesterol concentration was higher than the normal range after consumption of diets containing *sn*-1 C16:0 with Low or High C18:2n-6 (5.42mmol/L and 5.32 mmol/L respectively). Individual serum total cholesterol levels are shown (Figure 2-1). Feeding *sn*-2 C16:0 resulted in significantly lower levels of mean total cholesterol compared to feeding *sn*-1 C16:0 (Table 2-7) ($p=0.02$). The effect of Low vs. High C18:2n-6 on total cholesterol was not significant.

Figure 2-1. Individual Responses of Total Cholesterol in Subjects Consuming Each of the Dietary Treatments

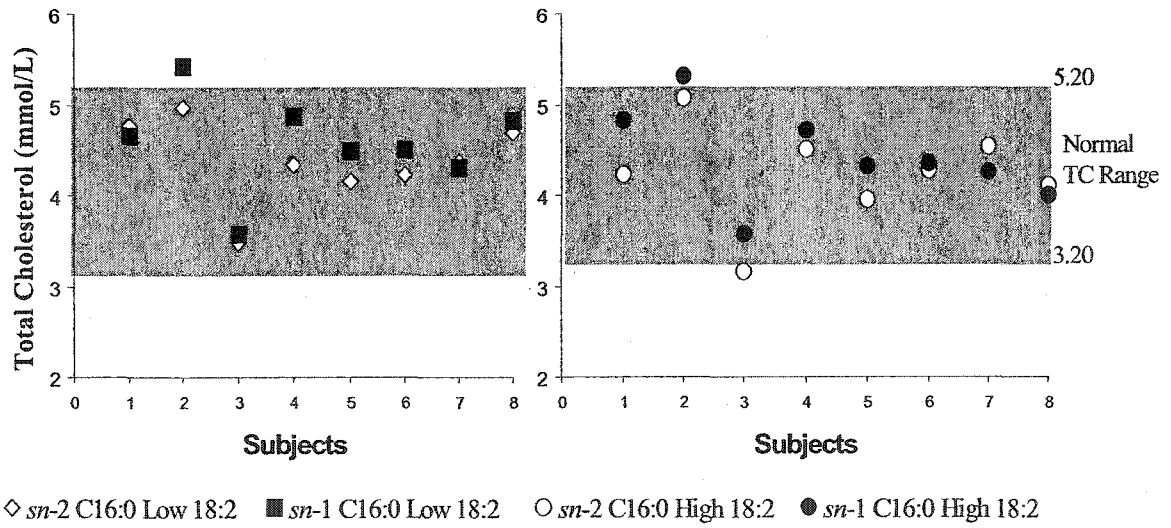


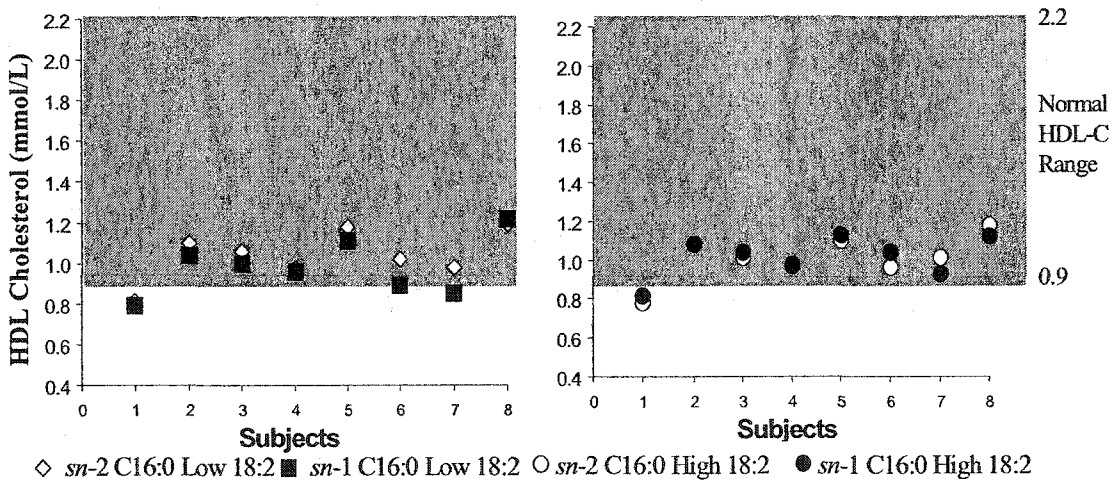
Table 2-7. Plasma Lipid and Lipoprotein Concentration in Subjects Consuming Each Dietary Treatment[1]

	Diet Treatment Mean Values				Main Effects	
Plasma concentration (mmol/L)	***sn*-2 C16:0 Low C18:2n-6**	***sn*-1 C16:0 Low C18:2n-6**	***sn*-2 C16:0 High C18:2n-6**	***sn*-1 C16:0 High C18:2n-6**	***sn*-2 vs. *sn*-1 C16:0**	**Low vs. High C18:2n-6**
Total Cholesterol	4.37 ± 0.16	4.58 ± 0.19	4.23 ± 0.20	4.42 ± 0.19	p = 0.02	NS
HDL Cholesterol	104 ± 0.04	0.98 ± 0.05	1.01 ± 0.04	1.02 ± 0.04	NS	NS
LDL Cholesterol	2.78 ± 0.14	2.88 ± 0.17	2.67 ± 0.17	2.72 ± 0.18	NS	NS
Triglyceride	1.22 ± 0.23	1.59 ± 0.33	1.20 ± 0.22	1.53 ± 0.45	NS	NS
Total Chol/HDL Ratio	4.3 ± 0.3	4.8 ± 0.3	4.2 ± 0.3	4.4 ± 0.3	p = 0.0001	p = 0.003

[1] values are means ± SEM. No significant interactions were found.

HDL Cholesterol. The normal range of HDL cholesterol is 0.90-2.20mmol/L. Mean concentration of HDL cholesterol was within the normal range after each of the dietary treatments (Table 2-7). One subject's HDL level was <0.9mmol/L after all of the dietary treatments and two other subjects had HDL levels <0.9mmol/L after consuming the *sn*-1 C16:0 Low C18:2n-6 diet treatment. Individual HDL cholesterol levels are shown (Figure 2-2). No significant effects of diet were observed on HDL cholesterol, and no significant interaction effects were seen (Table 2-7).

Figure 2-2. Individual Responses of HDL Cholesterol in Subjects Consuming Each of the Dietary Treatments



LDL Cholesterol. The normal LDL cholesterol range is 1.70-3.40 mmol/L. Throughout the study period, mean LDL cholesterol concentrations were within the low range of normal (Table 2-7). Only one subject had LDL cholesterol higher than normal, and this occurred after consuming the *sn*-1 C16:0 High C18:2n-6 diet treatment. No significant effects of diet were observed on LDL cholesterol, and no significant interaction effects were seen (Table 2-7).

Triglycerides. Desirable fasting concentrations of TG are <2.30mmol/L. Mean TG concentrations after each dietary treatment were below this recommended level (Table 2-7). One subject had TG concentrations > 2.30mmol/L after all diet treatments and another subject had TG concentrations >2.30mmol/L after consuming the *sn*-1 C16:0 Low C18:2n-6 diet (Table 2-8). There was no difference among TG concentrations for any of the diet treatments. The effects of diet were not statistically significant. The effect of feeding Low vs. High C18:2n-6 was not significant and no interaction effects were found.

Table 2-8. Individual Responses of Fasting Triglycerides in Subjects Consuming Each Dietary Treatment

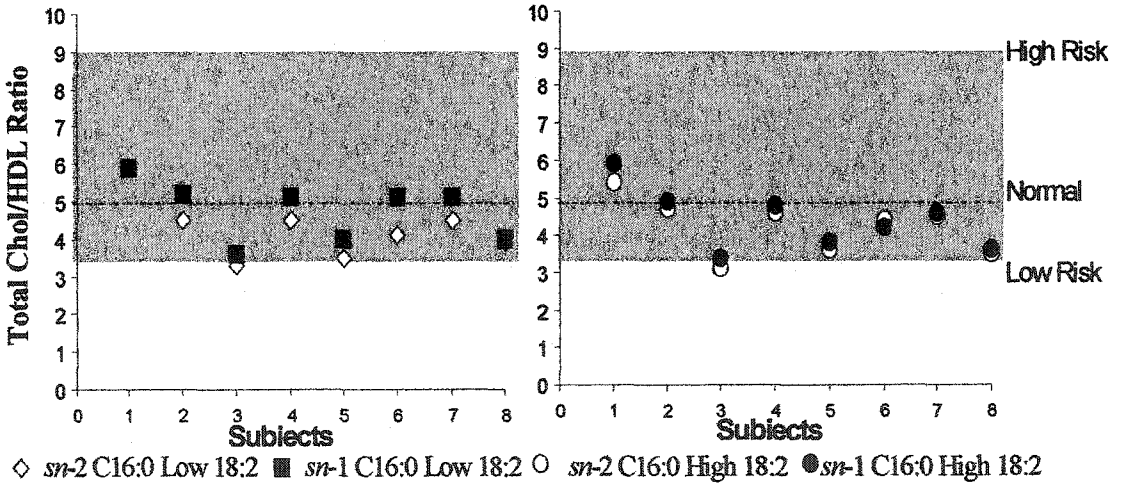
TG (mmol/L)

Subject	***sn*-2 C16:0 Low C18:2**	***sn*-1 C16:0 Low C18:2**	***sn*-2 C16:0 High C18:2**	***sn*-1 C16:0 High C18:2**
1	2.55*	3.35*	2.43*	4.55*
2	1.80	2.61*	1.80	1.78
3	1.16	1.10	0.89	0.99
4	1.18	1.51	1.25	1.59
5	0.79	1.60	1.06	0.95
6	0.64	0.66	0.74	0.78
7	0.81	0.99	0.86	0.80
8	0.79	0.86	0.55	0.74

* Higher than desirable.

Total Cholesterol/ HDL Ratio. The normal Total cholesterol/ HDL ratio (TC/HDL ratio) for males of the age range studied is 5. High risk for development of CHD in males is identified when the ratio exceeds 9, and low risk is identified when the ratio is less than 3.5. The mean TC/HDL ratio after consumption of all diets was less than normal for males (Table 2-7). No subject had a ratio value that indicated high risk. Individual HDL cholesterol concentrations are shown (Figure 2-3). Consuming diets that contained *sn*-2 C16:0 with Low or High C18:2n-6, resulted in a lower TC/ HDL ratio than those containing *sn*-1 C16:0 with Low or High C18:2n-6 ($p=0.0001$) (Table 2-7). Consuming High C18:2n-6 diets resulted in a statistically lower TC/ HDL ratio than consuming Low C18:2n-6 diets ($p=0.003$).

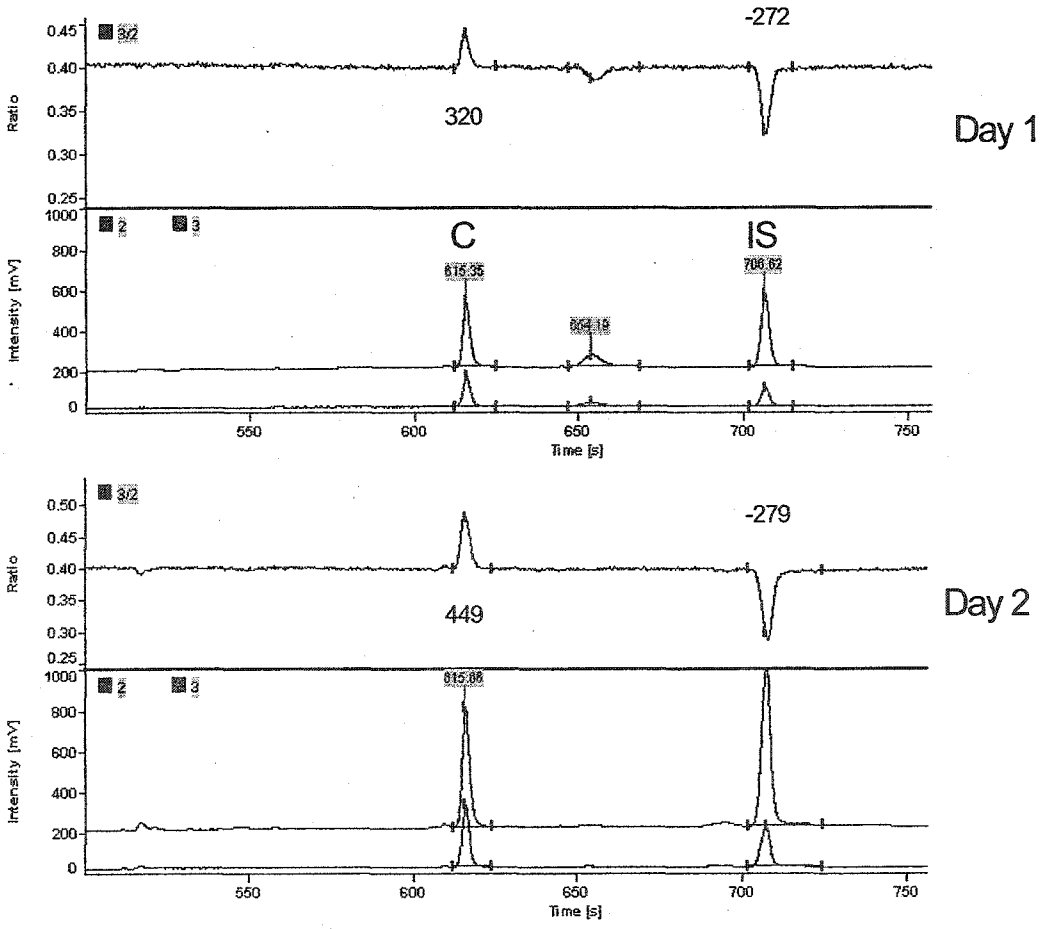
Figure 2-3. Individual Responses of Total Cholesterol/HDL Cholesterol Ratio in Subjects Consuming Each of the Dietary Treatments



Cholesterol Fractional Synthesis Rate

Deuterium Enrichment in Plasma Cholesterol: Figure 2-4 shows an example of the GCIRMS chromatogram obtained on day 1 (day 13, background) and day 2 (day 14, enriched sample), where C is the cholesterol silyl ether, and IS is the internal standard: tridecanoylglycerol. Day 2 chromatogram shows that the ratio of mass 3/2 (expressed as δ vs. standard mean ocean water: SMOW) is higher than day 1 for cholesterol, demonstrating enrichment of cholesterol with deuterium, whereas enrichment of IS does not change.

Figure 2-4. GCIRMS Chromatogram of Day 1 and Day 2 Plasma Cholesterol Deuterium Enrichment Analysis



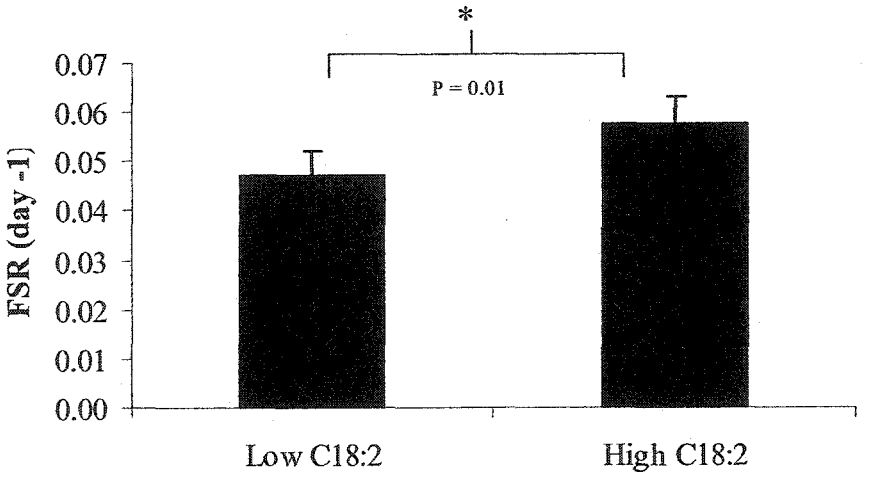
Cholesterol Fractional Synthetic Rate: The fractional synthesis rate (FSR) for free cholesterol was significantly lower in subjects consuming the *sn*-1 C16:0 Low C18:2n-6 diet compared to consuming all other diets ($p = 0.01$) (Table 2-9). The effect of *sn*-1 vs. *sn*-2 C16:0 was not significant. The effect of Low vs. High C18:2n-6 was significant ($p = 0.01$) (Figure 2-5).

Table 2-9. Fractional Synthesis Rates for Free Cholesterol in Subjects Consuming Each Dietary Treatment

Diet Treatment Mean Values	Fractional Synthesis Rate (pools/day^{-1})[1]
sn-2 C16:0 Low C18:2	0.054 ± 0.005^{a}
sn-1 C16:0 Low C18:2	0.040 ± 0.004^{b}
sn-2 C16:0 High C18:2	0.053 ± 0.004^{a}
sn-1 C16:0 High C18:2	0.062 ± 0.003^{a}

[1]FSR Values are means ± pooled SEM. Means not sharing common superscripts are significantly different from each other ($P<0.05$). A significant interaction effect was found ($p=0.0001$).

Figure 2-5. Low vs. High C18:2n-6 on Cholesterol Fractional Synthesis Rate



Fasting C-Reactive Protein

C-Reactive Protein: Lowest indication of acute inflammation and CHD risk are CRP levels less than 1.0mg/L; levels between 1.0 and 3.0mg/L indicate average risk, and levels above 3.0mg/L are suggestive of high risk (53). The interquartile range of CRP for men is 0.80-3.20mg/L (53). A mean CRP level was not calculated as many subjects had levels less than 1.0mg/L (Table 2-10).

Table 2-10. Fasting C-Reactive Protein Measurements in Subjects Consuming Each Dietary Treatment[1]

CRP (mg/L)				
Subject	***sn*-2 C16:0 Low C18:2**	***sn*-1 C16:0 Low C18:2**	***sn*-2 C16:0 High C18:2**	***sn*-1 C16:0 High C18:2**
1	1.32	<1.0	2.00	<1.0
2	<1.0	<1.0	1.10	1.13
3	<1.0	<1.0	<1.0	<1.0
4	<1.0	<1.0	1.30	1.19
5	1.76	1.40	2.20	3.48
6	<1.0	<1.0	<1.0	<1.0
7	1.11	<1.0	<1.0	<1.0
8	<1.0	1.20	<1.0	1.77

[1]Values obtained on Day14 of each diet treatment.
A denotation of <1.0 mg/L indicates a value of less than 1.0 mg/L

5. Discussion

The results of the present study (Table 2-7) find that feeding diets with C16:0 in the *sn*-2 position of dietary triglyceride to normocholesterolemic male subjects results in significantly lower fasting serum total-cholesterol concentrations and a significantly lower fasting TC/HDL ratio than feeding C16:0 in the *sn*-1,3 position. This suggests that *sn*-2 C16:0 may be less cholesterolemic than *sn*-1 C16:0, which is opposite to the hypothesis. It noteworthy that although these measures were significantly lower after feeding dietary treatments providing C16:0 in the *sn*-2 position, all dietary treatments resulted in mean fasting serum lipoprotein concentrations that were within the recommended range for subjects of the age group studied. The TC/HDL ratio is considered more sensitive and specific than total- or lipoprotein-cholesterol concentrations in estimating risk of CHD (54). The TC/HDL ratio emphasizes that dietary-induced reductions in HDL will increase CHD risk due to the protective nature of HDL concentrations. Use of the TC/HDL ratio includes triglyceride-rich VLDL along with levels of LDL cholesterol, which is important to consider since high fasting concentrations of serum triglycerides positively correlate with CHD risk (55). Therefore, despite the finding that feeding diets with C16:0 predominately in the *sn*-2 position was more cholesterolemic, consuming dietary lipid sources with C16:0 predominately *sn*-1 position of triglyceride may not increase the risk of CHD in healthy young men.

The effect of dietary C18:2n-6 level on fasting lipid and lipoprotein concentrations was significant for the TC/HDL ratio. Low levels of C18:2n-6 in diets resulted in a higher TC/HDL ratio than high levels of C18:2n-6 (Table 2-7). Although it was not significant, diets containing high levels of C18:2n-6, resulted in lower concentrations of total- and LDL cholesterol than diets containing low levels of C18:2n-6 (Table 2-7). The significant finding of decreased TC/HDL ratio after feeding high dietary levels of C18:2n-6 with high levels of C16:0 agree with previous human research showing a cholesterol-lowering effect of C18:2n-6 at levels above 5%en (15;16;18;56).

Cholesterol fractional synthesis rate (FSR) was determined by the deuterium incorporation method to investigate whether positional distribution of C16:0 and/or content of C18:2n-6 has an effect on FSR and if fasting lipid and lipoprotein concentrations in each diet could be explained by 24 hour cholesterol FSR. In the present study, feeding High C18:2n-6 diets resulted in a significantly higher FSR for free cholesterol than feeding Low C18:2n-6 diets (Table 2-9). The *sn*-1 C16:0 Low C18:2n-6 diet treatment had the lowest FSR for free cholesterol compared to all other diets. This diet treatment also resulted in the highest fasting mean serum total cholesterol concentration relative to other dietary treatments (Table 2-7). These results suggest that measurement of 24-hour FSR for free cholesterol in humans may not explain changes seen in fasting serum total cholesterol concentrations in response to feeding dietary C16:0 and C18:2n-6.

Measurement of fasting CRP levels in the present study indicate that there was no effect of dietary treatment on CRP in healthy male subjects (Table 2-10). This agrees with other investigations in human populations that show a minimal relationship between saturated fat intake and CRP levels (57;58).

The finding of lower fasting total cholesterol concentrations in response to feeding C16:0 in the *sn*-2 position compared to the *sn*-1,3 position to normocholesterolemic males found here is not similar to research by Zock *et al* (33) and Meijer and Weststrate (35) who saw no differences in lipid and lipoprotein concentrations. In the study by Zock *et al* (33), men and women subjects were fed diets containing 40%en as total fat, and positional distribution of C16:0 was evaluated using palm oil and interesterified palm oil. When results for men and women were considered separately, significant effects were observed in men who experienced a small increase in fasting total cholesterol after consuming the interesterified palm oil diet with more C16:0 in the *sn*-2 position; this finding is opposite to the present finding (Table 2-7). Meijer and Weststrate (35) also studied both men and women and fed interesterified fat blends to determine the cholesterolemic effects of positional distribution of C16:0. Results showed no differences in lipids and lipoproteins between the fat blends.

Another study that disagrees with increased fasting total cholesterol concentration and increased TC/HDL ratio by feeding C16:0 in the *sn*-1,3 compared to *sn*-2 position

found in the present study, is research by Zhang *et al* (36). Feeding diets rich in palm oil (refined bleached palm oil) were compared with feeding diets rich in lard to 120 normocholesterolemic Chinese men. Results showed that palm oil diets resulted in significantly lower serum total- and LDL-cholesterol concentrations and a lower TC/HDL ratio than lard. However, in agreement with the present study, all lipid and lipoprotein concentrations were within the recommended range. Diets provided 30%en as total fat and were administered for six weeks using normal Chinese foods (36). The diet composition was similar to the current study (Table 2-6). Differences in lipid and lipoprotein concentrations compared to the current study could be attributed to ethnicity of the subjects studied, the number of subjects studied, the types of foods eaten, the type of palm oil administered (refined bleached palm oil vs. palmstearin), and the time frame of investigation.

Several studies evaluating the cholesterolemic effect of palm oil fed as palmolein, a dry fraction of palm oil containing more C18:1 and C18:2n-6 and less C16:0 than palmstearin (59), have shown that palmolein has beneficial effects on serum cholesterol concentrations. Palmolein has been found to behave like the monounsaturated-rich olive oil, resulting in similar desirable concentrations of serum total- and LDL-cholesterol in humans (13;36;39;40;42). Similarly, research in humans fed dietary treatments containing mostly lard and pork products demonstrate that fasting serum cholesterol concentrations were within the recommended range (29;36;60). The present finding that diets rich in palmstearin and lard resulted in recommended concentrations of lipids and lipoproteins is supported by these observations.

In summary, results of the present study suggest that in normocholesterolemic male subjects, consumption of C16:0 in the *sn*-2 position of dietary TG may result in significantly lower fasting serum total cholesterol concentrations and TC/HDL ratio than consumption of C16:0 in the *sn*-1,3 position when fed through natural dietary sources. Feeding diets with C18:2n-6 levels greater than 5%en result in a significantly lower fasting TC/HDL ratio than feeding diets with less than 5%en C18:2n-6. The 24 hour FSR for free cholesterol does not appear to explain changes observed in fasting serum total cholesterol and TC/HDL ratio in humans in response to feeding high levels of C16:0 and C18:2n-6.

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CHAPTER 3-SUMMARY AND FUTURE DIRECTION

Discussion

Palmitic acid (C16:0) is the most abundant saturated fatty acid in the North American diet. Canadian and American health authorities recommend restricting saturated fatty acid consumption to help prevent elevations in serum cholesterol and lipoprotein concentrations and decrease the risk of developing coronary heart disease (1;2). This recommendation is complicated by findings in humans that diets rich in C16:0 do not appear to increase serum cholesterol concentrations when diets also contain recommended levels of linoleic acid (C18:2n-6) and moderate dietary cholesterol (3-5).

The stereospecific position of C16:0 on dietary triglyceride has been suggested to dictate its metabolic and physiological properties. Animal research has shown that feeding fats with C16:0 mostly in the *sn*-2 position of dietary triglyceride results in increased arterial lesion formation than feeding fats with C16:0 mostly in the *sn*-1,3 position (6). When dietary fats containing more C16:0 in the *sn*-2 position are fed to rats (7) and growing infants (8), serum cholesterol concentrations are higher than feeding dietary fats containing less C16:0 in the *sn*-2 position. Results from human feeding studies show that when native fats are modified by interesterification to provide more C16:0 in the *sn*-2 position, as in the case with lard, or less C16:0 in the *sn*-2 position, as in the case with palm oil, serum lipid and lipoprotein concentrations are not different (9-11).

Findings of the present study suggest that when unmodified biological sources of C16:0 are fed to young, normocholesterolemic male humans for two weeks, C16:0 primarily in the *sn*-2 position from lard may result in significantly lower fasting total cholesterol concentrations and a significantly lower fasting TC/HDL ratio than feeding C16:0 primarily in the *sn*-1,3 position from palmstearin. The TC/HDL ratio is also decreased in these subjects when diets provided greater than 5%en from C18:2n-6.

Conclusion

Restricted intake of dietary fats containing high quantities of C16:0 to decrease risk of CHD may not be warranted based on observations that C16:0 appears to not negatively influence fasting serum cholesterol concentrations especially when the diet contains recommended levels of C18:2n-6 and cholesterol. Dietary fats containing C16:0 mostly in the *sn*-2 position may be less hypercholesterolemic than dietary fats containing C16:0 in the *sn*-1 position based on the current findings in healthy human men.

Future Research

The current research study may be limited by the following factors. First, this study was only able to investigate the cholesterolemic effects of C16:0 in the *sn*-2 position with that of C16:0 in the *sn*-1,3 position from natural sources of dietary fat in eight human subjects. Future research should be conducted to determine if these effects are repeated in a larger group of subjects. Second, this study limited its subjects to healthy men. Subsequent research should investigate the effects of positional distribution of C16:0 in biological sources of fat in hypercholesterolemic men, and in normo- and hyper-cholesterolemic women. Previous research has shown that men and women differ in lipoprotein response to dietary fat and cholesterol feeding (12) so investigating sex-related responses to positional distribution of C16:0 is warranted. Third, palmstearin was fed to investigate the cholesterolemic effects of C16:0 in the *sn*-1 position. Palmstearin is a dry fraction of palm oil containing less C18:1 and C18:2n-6 and more C16:0 than palmolein (13), another form of palm oil often fed to humans to study its cholesterolemic effects. Further research should investigate the cholesterolemic response of feeding C16:0 in the *sn*-1,3 position from palmolein to healthy North American subjects compared to feeding C16:0 in the *sn*-2 position from lard with high and low levels C18:2n-6. Fourth, cholesterol fractional synthesis rate (FSR) is a measure of cholesterol synthesis over a 24 hour period where measurements of serum cholesterol concentrations in this study were performed at a single time period and in the fasting state. Since humans the majority of their lives in the postprandial period rather than in a fasting state (14),

measurements of cholesterol concentrations in the fasting state may not provide a complete picture of cholesterolgenesis in response to dietary fat intake. Future research should investigate postprandial lipemia in human subjects in response to feeding diets with C16:0 in the *sn*-1 position of triglyceride compared to C16:0 in the *sn*-2 position and determine if any differences in postprandial lipemia are explained by changes in FSR for cholesterol. Finally, animal research has suggested that C16:0 in the *sn*-2 position of dietary triglyceride is more atherogenic than C16:0 in the *sn*-1,3 position (15). Although it is not possible to measure atherogenesis in the same way in humans, vascular health can be assessed by a noninvasive technique that evaluates endothelial-dependent flow-mediated vasodilation (FMD) of an artery (16). Future research may evaluate the effect of positional distribution of C16:0 on this measure of vascular health in humans.

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APPENDIX A- EXAMPLE OF DIETARY TREATMENT MENUS

Day 1: *sn*-1,3 C16:0, High C18:2n-6
3000 Calorie Level

BREAKFAST

DRY FOOD:

1 % Low Fat Yogurt (Yoplait)...100 grams
Grape Juice...250 ml
2% Milk...250 ml
English Muffin...60 g
1 Package of Jam
Banana...1 Medium

COOKED/PREPARED FOOD:

Hash browns...175 g frozen... *cooked in* Palmstearin...11 g and Safflower Oil...12 g
Medium Egg (fried, boiled, poached)....1 each

LUNCH

DRY FOOD:

Grapes...80 g
Soda Pop of choice...355 ml
J-ello Gelatin Snacks...1 each
Granola Bar...27 g (1 each)

COOKED/PREPARED FOOD:

White Pita...60 g *with*
Tuna...100 g and Safflower Oil...14 g and Olive Oil...4 g
Fat Free Mayo...1 tbsp
Tomato and Romaine Lettuce
RTS Fat Free Vegetable Soup...200 g *with* Palmstearin...14 g
Fat Free Saltine Crackers...2 each

SUPPER

DRY FOOD:

1 % Milk...240 ml
Fruit Cocktail... 1 can
Jello Pudding Snack...1 each
Apple Juice...200 ml
Dinner Bun...40 g

COOKED FOOD:

Teriyaki Chicken Entrée...300 g *with* Frozen Vegetables...65 g
mixed with Palmstearin...14 g and Safflower Oil...9 g and Olive Oil...4 g

Day 1: *sn*-1,3 C16:0, Low C18:2n-6
3000 Calorie Level:

BREAKFAST
DRY FOOD:
Low Fat Yogurt…175 grams
Grape Juice…250 ml
2% Milk…250 ml
Whole Wheat Bread…29 g
1 Package of Jam
Banana…1 Medium
Singles Cheese Slice…1 (21 g)

COOKED/PREPARED FOOD:
Hash browns…175 g frozen… *cooked in* Palmstearin…12 g and Olive Oil…12 g

LUNCH
DRY FOOD:
Rice Krispie Square…1 (37 g)
Soda Pop of choice…355 ml
Chocolate Covered Almonds…27 g

COOKED/PREPARED FOOD:
Instant Rice…200 g *with* Palmstearin…15 g
Chicken Breast…100 grams *with* BBQ Sc…1tsp
Diced Tomatoes…1/4 C

SUPPER
DRY FOOD:
1 % Milk…240 ml
Medium Apple…1 each
Jello Pudding Snack…1 each
Apple/Passionfruit Juice…200 ml
Dinner Bun…40 g

COOKED FOOD:
Teriyaki Chicken Entrée…307 g
mixed with Palmstearin…14 g and Olive Oil...9 g

Day 1: *sn*-2 C16:0, High C18:2n-6
3000 Calorie Level:

BREAKFAST
DRY FOOD:
FF Yogurt…125 grams
Juice…200 ml
Skim Milk…250 ml
½ Cinnamon Bagel…71 g
1 Package of Jam

COOKED/PREPARED FOOD:
Hash browns…170 g frozen… *cooked in* Lard…15 g and Safflower Oil…8 g
Bacon…17 grams (cooked weight)
Medium Egg…1 each

LUNCH
DRY FOOD:
Medium Orange…131 g
Soda Pop of choice…355 ml
Kraft Single Cheese Slice…1 each (21 g)
Gummy Bears…25 g
Angel Food Cake…48 g *with* Strawberries…46 g
Safflower Oil…8 g

COOKED/PREPARED FOOD:
Whole Wheat Bread…2 slices (60 g) *with*
Bacon…15 grams
*Lard…20 g *and and* FF Mayo… 2 tsp
Lettuce… 2 pieces *and* Tomato Slices…2 pieces

SUPPER
DRY FOOD:
Skim Milk…250 ml
Very Cherry Fruit Mix…1 each (142 ml)
Gelatin Snacks…1 each
Tossed Salad…1.5 cups *with*
Safflower Oil...6.5 g and FF Dressing…1 tbsp

COOKED FOOD:
Thai Chicken Entrée…270 g
mixed with Lard…28 g
Fresh/ Frozen Mixed Vegetables...155 g

Day 1: *sn*-2 C16:0, Low C18:2n-6
3000 Calorie Level:

BREAKFAST
DRY FOOD:
Medium Banana…118 grams
Apple Juice…284 ml
1% Milk…120 ml
English Muffin…57 grams
2 Packages of Jam

COOKED/PREPARED FOOD:
Hash browns…165 grams frozen… *cooked in* Lard…25 grams
Sliced Ham…70 grams (raw weight)

LUNCH
DRY FOOD:
Canned Mandarin Oranges in juice…117 grams (1/2 cup)
Soda Pop of choice…355 ml
Jello Gelatin Snack…1 each
Quaker Chewy Granola Bar…1 each (28grams)

COOKED/PREPARED FOOD:
Flour Tortilla…1 each (57 grams) *with*
Pork Bologna…100 grams
*Lard…12 grams *and* FF Mayo… 2 tsp *and* Mustard…1 tbsp *with*
Dill Pickles…2 *slices and* Chopped Lettuce…42 grams *and*
Tomato Slices…2 pieces…60 grams

SUPPER
DRY FOOD:
Skim Milk…250 ml
Jello Pudding Snack…1 each
Angel Food Cake…36 grams *with* Strawberries…65 grams

COOKED FOOD:
Thai Chicken Entrée…305 grams *mixed with* Lard…18 grams
Pork Sausage…40 grams (cooked weight)