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IMPACT OF HYDROGENATED FAT CONSUMPTION ON IN VIVO LIPID METABOLISM IN MODERATELY HYPERCHOLESTEROLEMIC WOMEN

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

ABSTRACT

The negative health effects of *trans* fatty acids from hydrogenated fats on plasma lipid profile have been well documented. However, the mechanisms responsible for these changes remain to be elucidated. Hence the overall objective of the thesis was to examine the effect of consuming different forms of hydrogenated fats on cholesterol and triglyceride metabolism, specifically fractional and absolute synthesis rates of free (FSR-FC and ASR-FC) and esterified (FSR-CE, AER and ER) cholesterol, and the functioning of the ASP pathway. In addition, validation of the newer deuterium incorporation (DI) method for measurement of endogenous cholesterol biosynthesis against the cholesterol precursor assessment approach was also performed. Fourteen moderately hypercholesterolemic (LDL-C $\geq$ 130 $mg.dl^{-1}$) postmenopausal women (65-71yrs) participated in this study. Subjects consumed, in random order, each of 6 diets for 5 week periods, separated by washout periods ranging from 2 to 4 weeks in duration. The experimental diets included a baseline (BL) diet (39% kcal fat) and 5 reduced fat diets (30% kcal) where $2/3^{rd}$ of the fat was either soybean oil (SO), low *trans* squeeze (SQM), medium *trans* tub (TM), high *trans* stick (SM) margarines, or butter (BT). Results obtained from the series of analyses performed demonstrate that (i) the DI method and levels of some cholesterol precursors correspond as methods for the study of *in vivo* cholesterol biosynthesis in humans (ii) elevations in endogenous cholesterol synthesis (FSR-FC and ASR-FC) are not responsible for the increase in circulating cholesterol levels seen after consumption of the high *trans* SM, and high SFA rich BT and BL diets (iii) suppression of cholesterol esterification rates on the SM diet may account for the decreased HDL-C levels observed on this diet and finally (iv) dysfunction of the ASP

pathway, with lower ASP and higher FFA levels could be responsible for the higher secretion of hepatic B 100 particles. In conclusion, our results provide evidence that alterations in TG clearance and cholesterol biosynthesis are responsible for the adverse lipid profile seen after consumption of high *trans* containing diets.

RESUME

Les effets négatifs sur la santé des acides gras *trans*, qui proviennent des gras hydrogénés, sur le profile lipidique dans le plasma ont été largement étudié. Cependant, les mécanismes responsables de ces changements restent encore non élucider. D'où, l'objet de notre travail qui consiste à examiner l'effet de la consommation des différentes formes de gras hydrogénés sur le métabolisme du cholestérol et des triglycérides, plus spécialement les taux de synthèse absolus et fractionnels du cholestérol libre (FSR-FC et ASR-FC) et estérifie (FSR-CE, AER et ER) ainsi que le fonctionnement de la voie ASP. En outre, la validité de la méthode d'incorporation du deutérium (DI) pour mesurer la biosynthèse endogène du cholestérol contre l'approche d'évaluation du précurseur a été également déterminée. Quarante femmes post-ménopausées (67-71 ans), modremment hypercholestérolémique (LDL-C ≥ 130 mg.dl^{-1}) ont participé à cette étude. Les sujets ont consommé chacun, et dans un ordre arbitraire, 6 diètes pendant une période de 5 semaines séparée par une période de " Wash out " allant de 2 à 4 semaines. Les diètes expérimentales comportent les diètes de base (BL) (39 % kcal en gras) et 5 diètes à faible teneur en gras (30 % kcal) dans lesquelles le 2/3 de gras est composé de l'huile végétale de soja (SO), de la margarine avec des teneurs en *trans* soient faibles (SQM), moyennes (TM) ou élevées (SM), ou du beurre (BT). Les résultats obtenus à partir des séries d'analyses utilisées démontrent que (i) la méthode DI et les taux de certains précurseurs de cholestérol correspondent comme pour les méthodes d'étude de la biosynthèse de cholestérol *in vivo* chez l'homme (ii) l'augmentation de la synthèse du cholestérol endogène (FSR-FC et ASR-FC) n'est pas responsables de l'augmentation des taux de cholestérol circulants observé après consommation des diètes SM a teneur élevée en *trans*

et des diètes de base et du beurre riche en SFA (iii) la suppression du taux d'estérification de cholestérol sur les diètes SM pourrait compter pour une baisse du niveau de HDL-C observée dans cette diète et finalement (iv) le disfonctionnement de la voie ASP, avec un faible niveau d'ASP et un niveau élevé de FFA pourrait être responsable de la sécrétion élevée des particules hépatiques B100. En conclusion, nos résultats fournissent l'évidence que les altérations dans la clairance des TG et la biosynthèse des cholestérols sont responsable du profile lipidique défavorable observé après consommation des diètes contenants des teneurs élevées en *trans*.

PREFACE

In this thesis, the effects of consuming different forms of hydrogenated fats on fractional and absolute synthesis rates of free and esterified cholesterol, and the acylation stimulating protein (ASP) pathway were examined in moderately hypercholesterolemic postmenopausal women. In addition, a validation of the newer deuterium incorporation methodology against the more commonly used plasma cholesterol precursor assessment method for determination of endogenous cholesterol synthesis was performed.

The results are presented in manuscript format, with pertinent literature reviews on each chapter of the thesis. Chapter 1 provides an introduction, with a rationale for the project, including objectives and hypotheses stated in the null form. Chapter 2 presents an extended literature review for topics discussed in the body of the thesis. Chapter 3 compares the deuterium incorporation method for measurement of endogenous cholesterol synthesis against plasma cholesterol precursor levels. Chapter 4 addresses the effect of consumption of different forms of hydrogenated fat on fractional and absolute synthesis rates of free cholesterol. Chapter 5 explores the impact of degree of hydrogenation on cholesterol esterification rates. Chapter 6 investigates the effects of differing *trans* and fatty acid profile on components of the ASP pathway, specifically plasma ASP, free fatty acid, glucose and insulin levels.

The thesis ends with a summary and general conclusions drawn from all aspects of the research. Limitations of the thesis and potential directions for continued future research are also discussed. This thesis is based on five manuscripts, one published, one accepted, two submitted to peer-reviewed journals and one in preparation.

STATEMENT FROM THESIS OFFICE

According to the regulations of the Faculty of Graduate Studies and Research of McGill University, the following statement from the Guidelines for Thesis Preparation (McGill University, October, 1999) is included:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature (in addition to that covered in the introduction to each paper), and a final conclusion and summary.

As manuscripts for publication are frequently very concise documents, where appropriate additional material must be provided where appropriate (e.g. in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's best interest to clearly specify the responsibilities of all the authors of the co-authored papers.

ADVANCE OF SCHOLARLY KNOWLEDGE

1. Original contributions to knowledge

This thesis examines three potential mechanisms that could explain the adverse plasma lipid and lipoprotein profile seen after consumption of different forms of hydrogenated fats. In addition, comparison of two techniques namely D uptake and cholesterol precursor levels, used to measure endogenous cholesterol synthesis was also performed. The results from the thesis work have contributed to knowledge in the field of cholesterol and lipid research, specifically the area of *trans* fatty acid metabolism by:

i) demonstrating that the D uptake method is one of the most practical and direct techniques currently available to study *in vivo* cholesterol biosynthesis in humans.

ii) showing that endogenous cholesterol synthesis (FSR-FC and ASR-FC) was reduced after consumption of the SM, BT and BL diets, and thus could not be responsible for the higher plasma TC and LDL-C levels seen on the above diets.

iii) illustrating that cholesterol esterification rates are markedly lowered with consumption of diets high in *trans* fatty acids and consequently providing an explanation for the lowering of HDL-C levels with hydrogenated fat consumption.

iv) suggesting that *trans* fatty acid feeding disrupts the normal functioning of the ASP pathway resulting in lower plasma ASP and higher FFA levels.

v) demonstrating for the first time that alterations in both cholesterol and triglyceride metabolism are responsible for the adverse plasma lipid and lipoprotein profile seen with high *trans* SM feeding.

vi) documenting these results in moderately hypercholesterolemic postmenopausal women, a population at risk of developing CVD.

2. Research publications in refereed scientific journals

i) Matthan NR, Jones PJH. Differential effects of individual *trans* fatty acid isomers on lipoprotein assembly and metabolism. Nutr Rev 1999; 57: 282-284.

ii) Matthan NR, Ausman LM, Lichtenstein AH, Jones PJH. Hydrogenated fat consumption affects cholesterol synthesis in moderately hyper-cholesterolemic women. J Lipid Res 2000 (in press)

3. Research manuscripts submitted to refereed scientific journals or in preparation

i) Matthan NR, Cianflone K, Lichtenstein AH, Ausman LM, Jones PJH. Hydrogenated fat consumption affects plasma acylation stimulating protein levels and consequently triacylglycerol and cholesterol metabolism in hypercholesterolemic women (submitted).

ii) Matthan NR, Raeini-Sarjaz M, Lichtenstein AH, Ausman LM, Jones PJH. Deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women (submitted).

iii) Matthan NR, Lichtenstein AH, Ausman LM, Jones PJH. Degree of hydrogenation affects cholesterol esterification rates in moderately hypercholesterolemic women (in preparation).

CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS

This project was a collaborative venture with Drs. Lichtenstein and Ausman at the Jean Mayer US Department of Agriculture, Human Nutrition Research Center (HNRC) on Aging at Tufts University in Boston. The principal investigator at Tufts University was Dr. Lichtenstein, who was responsible for development of the study protocol and execution of the study at the metabolic unit of HNRC. The study was designed to evaluate the impact of consuming different forms of hydrogenated fats relative to butter on plasma lipid and lipoprotein profile. The concept of exploring mechanisms that could explain the adverse lipid profile observed after consumption of these diets was developed by the candidate along with Dr. Jones, after obtaining consent from the collaborators at Tufts University. In addition, the candidate spent a week during the trial familiarizing herself with all on site clinical procedures at the metabolic unit of HNRC.

The candidate was responsible for assisting the supervisor in the development of the validation paper, which compared the DI technique against plasma levels of some cholesterol precursors. The candidate performed all analyses and was responsible for writing the paper entitled " Deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women". Dr. M. Raeini-Sarjaz helped train the candidate to perform the GC analysis and also helped with the statistical analyses for the above manuscript, where he is co-author.

For the samples generated for measuring free and esterified cholesterol biosynthesis, the candidate was responsible for all stages of sample analysis, from lipid extraction to mass spectrometric analyses. The candidate performed all calculations and statistics on the collected data, and was responsible for writing the manuscripts presented in chapters 4 and 5. Enzyme activity levels, LCAT and CETP, were measured in collaboration with Drs. Frohlich and Jauhiainen respectively.

The concept of determining whether alterations in the ASP pathway following dietary fat modification affect plasma lipid response, was developed by the candidate along with Dr. Cianflone and Dr. Jones. Dr. Cianflone provided the facilities to measure plasma ASP and FFA levels. Steve Phelis prepared the monoclonal antibody for the ASP assay, however all analyses were performed by the candidate. Dr. Cianflone also provided valuable comments on the manuscript for which she is co-author.

Dr. Wykes and Dr. Van de Voort, the candidate's committee members, edited the final draft of the thesis. Dr. Lichtenstein and Dr. Ausman edited all four manuscripts. Dr. Jones, the candidate's supervisor, edited all four manuscripts including the brief critical review (Appendix II). In addition, Dr. Jones conducted weekly meetings with the candidate to monitor progress of the work completed and set milestones for future accomplishments.

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First, I would like to acknowledge and thank my supervisor Dr. Peter Jones, for giving me the opportunity to conduct this research project, and for his encouragement and guidance throughout the course of this undertaking. He made me realize that good old fashioned hard work does indeed pay off ! I would also like to express my gratitude for the advice and support provided by my thesis committee members, Dr. Linda Wykes and Dr. Fred Van de Voort.

I would like to acknowledge my international collaborators at Tufts University- Dr. Alice Lichtenstein and Dr. Lynne Ausman, without whom this study would not be possible. I am grateful to Dr. Cianflone for providing me with the opportunity to delve into the world of ASP research. The professionalism, commitment and integrity shown by my collaborators have been a model for my own burgeoning capacity to work. I would also like to thank the other Faculty members of the School who made my graduate experience one to remember. Special thanks are due to Anne, Leslie, Francine, Nicole and Lise, for their kindness and friendship.

I salute my fellow colleagues of the Peter Jones lab group-past and present, Tim, Tanya, Fady, Marco, Andrea, Jayne, Diane, Ming, Crystal, Jian Ying, Mahmoud, Catherine, Geoff and Jode. I would like to thank Tony, Mark, Lindi, Erika, Susan, Nancy, Kathleen, Chris, April and Xu-Jing for their good humor and support. To Shaila, Suman, Uma, the Sahai's and Sircar's- I cannot find words to express my gratitude.

Finally, I would like to thank my family, especially Ammachy for her prayers and Nandini and Dipak who saw me through it all. I dedicate this thesis to my parents whose sacrifices made my dreams come true and my achievements seem worthwhile. Appa and Amma- thank you for believing in me and my abilities.

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LIST OF ABBREVIATIONS

ACAT	Acetyl coenzyme A: acyl transferase
AER	Absolute esterification rate
ASP	Acylation stimulating protein
ASR	Absolute synthesis rate
BT	Butter
BL	Baseline
CE	Cholesteryl ester
CETP	Cholesterol ester transfer protein
D	Deuterium
DI	Deuterium incorporation
D_2O	Deuterium oxide
DGAT	Diacylglycerol acyl transferase
ER	Esterification ratio
FFA	Free fatty acid
FSR	Fractional synthesis rate
HMG-CoA	3-hydroxyl-3-methylglutaryl coenzyme A
LCAT	Lecithin cholesterol acyl transferase
LPL	Lipoprotein lipase
NCEP	National Cholesterol Education Program
MIDA	Mass isotopomer distribution analysis
PC	Phosphatidylcholine
SM	Stick margarine
SMOW	Standard Mean Ocean Water
SO	Soybean oil
SQM	Squeeze margarine
TM	Tub margarine
TTR	Transthyretinin

CHAPTER 1

INTRODUCTION

1.1 Rationale and Statement of Purpose

Cardiovascular disease (CVD) is the single leading cause of death in most industrialized countries (1). Numerous epidemiological, clinical and experimental studies have resulted in the identification of a number of primary and secondary risk factors for CVD (2-5). Among these factors, the role of dietary fat and cholesterol in the etiology and prevention of CVD has been extensively reviewed (6).

Results from the above studies have been instrumental in the formulation of several guidelines issued by various National Heath Agencies that include the most "prudent" dietary approach towards reducing plasma cholesterol levels and the associated risk from CVD. The historical consensus has been to reduce saturated fat (SFA) in favor of monounsaturated (MUFA) and polyunsaturated (PUFA) fat in the diet (6). To minimize the sensory impact of these recommendations, the food industry, fueled by customer demand developed the "hydrogenation" process to produce foods such as margarines and shortenings that look and taste like the original animal fat but are made from vegetable oils. However, during the hydrogenation process a change in fatty acid profile occurs, with saturation of a portion of the existing double bonds in the fatty acid chain, thereby decreasing the PUFA and increasing the SFA and MUFA content of the resultant fat. In addition some of the naturally occurring *cis* double bonds are isomerized to the *trans* configuration resulting in decreased bond length angle and an acyl chain resembling a saturated fatty acid (7).

In order to better understand the metabolic and health implications of this artificial element introduced into the food supply, a number of feeding trials have been conducted that have either directly or indirectly evaluated the effect of hydrogenated fat or *trans* fatty acid intake on plasma lipoproteins and risk of CVD. Results indicate that *trans* fatty acids from hydrogenated fats increase total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C) and triglycerides (TG), as well as decrease high density lipoprotein cholesterol (HDL-C) levels (8-16). However, the larger feeding trails have included either young normocholesterolemic subjects with a broad age range (25-65 years) but relatively normal plasma cholesterol levels (mean 205 $mg.dl^{-1}$) (11-14). Except for the study by Lichtenstein et al (8) and Sundram et al (16) the level of *trans* fatty acids provided to the study subjects in the other trials were at the high range of the estimated intake levels in North America or many fold higher. Thus, missing from the literature are data derived from controlled metabolic studies using diets that conform to current dietary guidelines (30% energy as fat) and focussing on the parameters in subjects with plasma TC or LDL-C levels in the borderline or high risk range. Subjects fitting this criteria are over represented by older individuals, especially postmenopausal women. In addition, the usual focus of attention in most trials has been on plasma lipid and lipoprotein levels. Unclear to date are the actual mechanism(s) responsible for the changes in lipid levels. Possibilities include alterations in the rate of endogenous synthesis of free and esterified cholesterol, and in the functioning of the acylation stimulating protein (ASP) pathway (Figure 1-1).

In humans, biosynthesis of cholesterol accounts for 60-80% of whole body cholesterol input (17,18) and has been shown to be influenced by dietary fatty acid composition. Saturated fats tend to suppress, while polyunsaturated fats increase cholesterol synthesis rates (19,20). Cuchel and colleagues (21) have shown that compared to a corn oil diet, feeding subjects corn oil margarine in stick form increased plasma lipid and lipoprotein levels in spite of a trend toward decreased *de novo* cholesterol synthesis. However, this declining trend in fractional synthetic rate of cholesterol (FSR-FC) did not reach statistical significance, possibly due to sample size limitations and high variability in synthesis among the male and female subjects. With the exception of the above study, the effect of hydrogenation, specifically degree of hydrogenation on cholesterogenesis remains unknown.

Trans fatty acids behave in a manner similar to SFA with regard to their ability to increase TC, LDL-C, VLDL-C and TG levels. However, the lowering of plasma HDL-C concentrations is a distinguishing feature between consumption of *trans* fatty acids from hydrogenated fats and SFA. Since the majority of esterification in plasma occurs in HDL-C and is proportional to the activity of the enzyme lecithin: cholesterol acyltransferase (LCAT), which in turn is closely related with plasma TG and HDL-C levels, it is of interest to determine whether cholesterol esterification rates are altered with consumption of different forms of hydrogenated fats.

Another potential mechanism that could explain the changes observed in plasma lipoprotein levels after consumption of hydrogenated fats is abnormal TG metabolism.

Recent studies (22-26) have shown that the ASP pathway is a major determinant of the rate of adipocyte TG synthesis and fatty acid re-esterification. In addition, Cianflone *et al* (27) have documented alterations in plasma ASP levels in a number of dyslipoproteinemic states that suggest a relationship between the ASP pathway and other major determinants of lipoprotein metabolism. One such determinant is the rate of synthesis of TG rich lipoproteins, which in turn depends on the amount and type of fatty acid consumed. To date no study has evaluated whether *trans* fatty acids from hydrogenated fats alter the functioning of the ASP pathway.

In order to measure *de novo* cholesterogenesis, one requires an accurate and precise tool. Several techniques are available to measure endogenous synthesis. Among them the deuterium incorporation (DI) and plasma cholesterol precursor assessment methods offer potential advantages over existing methods, as they are safe and less invasive. Both methods have been validated against the classic sterol balance method (28,29). In addition, the DI method has been validated against the MIDA (30) technique, while plasma cholesterol precursor levels have been shown to correlate well with levels of HMG-CoA reductase (31). However a direct cross comparison between the D uptake and plasma levels of squalene, desmosterol, lanosterol and lathosterol, which are commonly used as relative indices of cholesterol biosynthesis has not been carried out.

1.2 Project Overall Objectives

The overall objective of the thesis was to explore potential mechanisms responsible for the altered lipid and lipoprotein profile seen with consumption of different

forms of hydrogenated fats. In addition, the objective was to validate the diagnostic accuracy of the DI methodology for measurement of *de novo* cholesterogenesis. Our study subjects were moderately hypercholesterolemic, postmenopausal women, a group whose metabolic responses to different types of hydrogenated fats, have been understudied. Furthermore, with the exception of the baseline diet, all other diets were formulated to conform to current dietary guidelines for CVD risk reduction.

1.2.1 Specific Objectives

a) To determine if the deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterogenesis in moderately hypercholesterolemic postmenopausal women fed different forms of hydrogenated fats.

b) To evaluate the impact of consumption of commonly available sources of dietary fats subjected to different degrees of hydrogenation (within the context of a reduced fat and cholesterol diet) on endogenous cholesterol synthesis rates in moderately hypercholesterolemic postmenopausal women.

c) To evaluate the impact of consumption of commonly available sources of dietary fats subjected to different degrees of hydrogenation (within the context of a reduced fat and cholesterol diet) on cholesterol esterification rates in moderately hypercholesterolemic postmenopausal women.

d) To evaluate the impact of consumption of commonly available sources of dietary fats subjected to different degrees of hydrogenation (within the context of a reduced fat and cholesterol diet) on components of the acylation stimulating protein (ASP) pathway, specifically plasma ASP, free fatty acid, glucose and insulin levels in moderately hypercholesterolemic postmenopausal women.

1.2.2 Null Hypotheses

a) There is no difference between the deuterium incorporation and plasma cholesterol precursor assessment methods for measurement of endogenous cholesterogenesis in moderately hypercholesterolemic postmenopausal women fed different forms of hydrogenated fats.

b) There is no difference in endogenous synthesis rates of free cholesterol when moderately hypercholesterolemic postmenopausal women are fed different forms of hydrogenated fats.

c) There is no difference in cholesterol esterification rates when moderately hypercholesterolemic postmenopausal women are fed different forms of hydrogenated fats.

d) There is no difference in plasma ASP, free fatty acid, glucose and insulin levels when moderately hypercholesterolemic postmenopausal women are fed different forms of hydrogenated fats.

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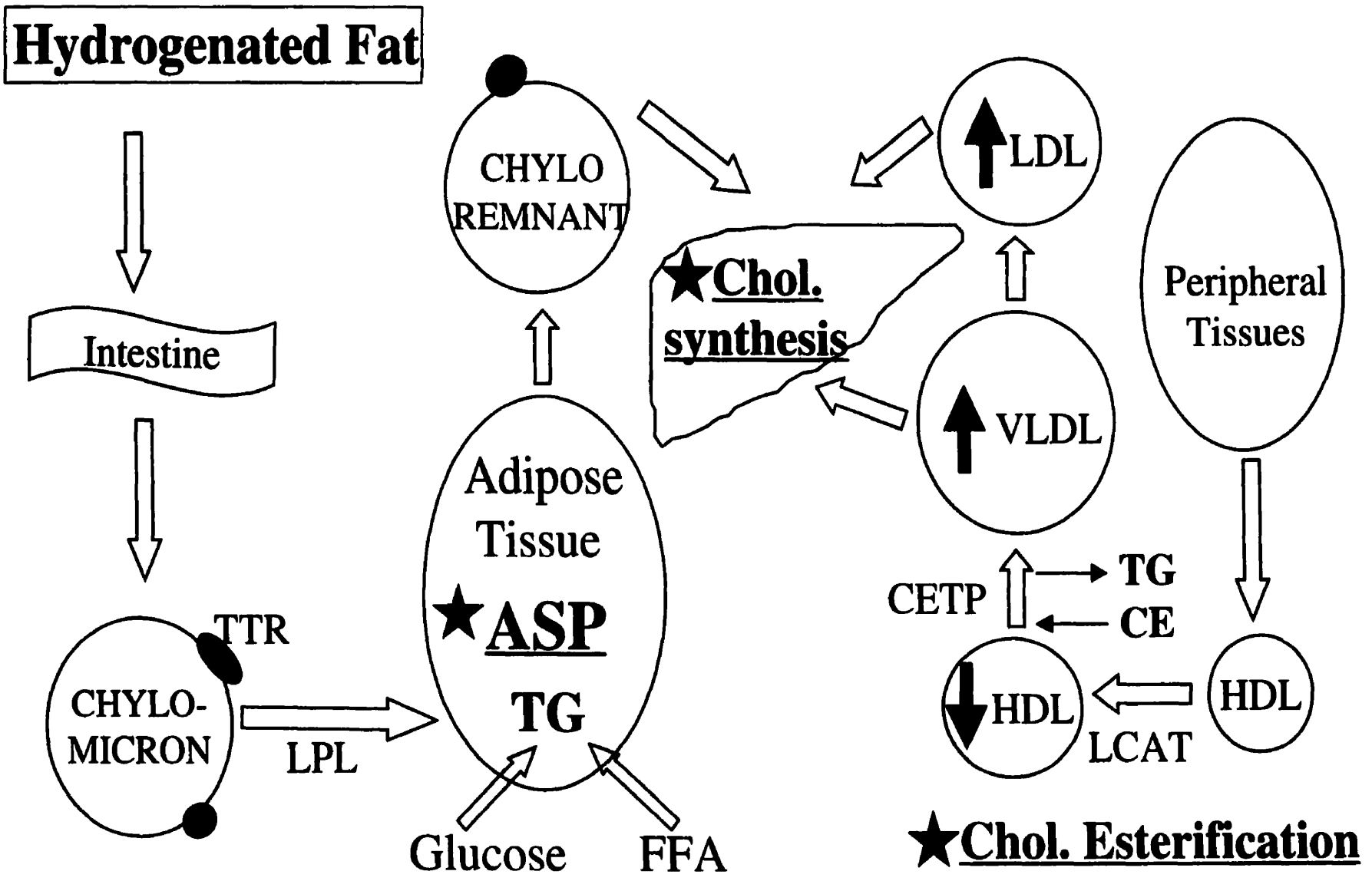
1.4 Figure Legend

Figure 1-1: Proposed mechanisms of action responsible for the adverse plasma lipid and lipoprotein profile seen after consumption of hydrogenated fats. The ★ denotes potential areas in triglyceride and cholesterol metabolism that could be affected by *trans* and SFA feeding. Abbreviations are as follows: TTR: transthyretinin, LPL: lipoprotein lipase, FFA: free fatty acids, ASP: acylation stimulating protein, CETP: cholesteryl ester transfer protein, LCAT: lecithin cholesterol acyl transferase, and CE: cholesteryl ester.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Intestine
CHYLO-
MICRON
TTR
LPL
CHYLO
REMNANT
Adipose
Tissue
★ASP
TG
Glucose
FFA
★Chol.
synthesis
LDL
VLDL
CETP
TG
CE
HDL
HDL
LCAT
Peripheral
Tissues
★Chol. Esterification

CHAPTER 2

LITERATURE REVIEW

2.1 The Hydrogenation Process for Dietary Fats

Toward the end of the 18th century, a process was discovered that uses heat in the presence of hydrogen and certain metal catalysts to convert natural liquid vegetable oils into solid fats (1). This process referred to as "hydrogenation" has resulted in the production of foods that resemble and taste like animal fats that can be used in cooking and baking but are made with vegetable oils (2,3). Common examples are margarine designed to replace butter and shortening intended to substitute for lard or beef tallow.

The success of hydrogenated products has also been due to the economic appeal of their longer shelf life and decreased expense compared to other fats. However, the hydrogenation process results in multiple changes in the composition of the fat. The proportion of saturated (SFA) and monounsaturated (MUFA) fatty acids increase, with a concomitant decrease in the proportion of polyunsaturated (PUFA) fatty acids (4). Additionally, some of naturally occurring *cis* double bonds are isomerized to the *trans* configuration, and the position of some double bonds may also be shifted. These factors contribute to a change in physical characteristics, resulting in acyl chains that are more tightly packed together.

2.2 Structure and Nomenclature of *Trans* Fatty Acids

Figure 2-1 depicts the structure of a *cis* versus *trans* monounsaturated fatty acid. In a *cis* double bond in an unsaturated fatty acid the 2 hydrogen atoms bound to the carbon atoms

that form the double bond are located on the same side of the carbon chain, resulting in a "kink" in the acyl chain and a more flexible molecule. In contrast to the more typical *cis* isomeric configuration the double bond angle of the *trans* fatty acid is smaller as the hydrogen atoms are on opposite sides of the carbon chain. This results in an acyl chain that is linear, and forming a more rigid molecule.

In the official International Union of Pure and Applied Chemistry (IUPAC) nomenclature, the position of double bonds in fatty acids is counted from the carboxyl end of the molecule. For example, an 18 carbon MUFA with a *trans* double bond of unspecified position is denoted *t*-C18:1. When the position of the *trans* bond is known, it is specified with a Δ. Thus *t*-C18:1Δ9 refers to the 18 carbon elaidic acid with 1 double bond located between the 9^{th} and 10^{th} carbons from the carboxyl end. Another nomenclature method used is the "n-minus" system which counts from the methyl end of the molecule, so that the numbers remain the same when carbon atoms are added to or removed from the carboxyl end during metabolism. Examples include *t*-C18:1n-9 and *t*-C18:1n-7 which refer to elaidic and vaccenic acids respectively. In this thesis, the "n-minus" nomenclature system will be used to describe the position of the *trans* double bond with the fatty acid molecule.

2.3 Dietary Sources of *Trans* Fatty Acids

In Western diets, the major source of *trans* fatty acids come from commercially produced hydrogenated products and baked goods made with margarine or shortening. The *trans* fatty acid content of these products range between 10-30% of total fatty acids by weight,

although the range between products is tremendous. *Trans* fatty acids also occur naturally in both the plant and animal kingdom. However, in human diets, *trans* isomers originating from plant sources are an insignificant part of the total dietary *trans* intake. Small amounts of *trans* fatty acids are produced in the body fat of ruminant animals as intermediates in the hydrogenation of unsaturated fatty acids by hydrogen produced during bacterial fermentation. As a result, animal sources such as milk, cheese, dairy and beef fat contribute a variable (2-8% of total fatty acids by weight) but modest amount of *trans* fatty acids in the diet.

2.4 Estimated *Trans* Fatty Acid Intake Levels

The *trans* fatty acid content of diets has been estimated (5-22) using a variety of methods including disappearance or availability data, food frequency questionnaire (FFQ), diet record or recall data, self-selected diet analysis and adipose tissue data. Results from some of these studies estimating *trans* fatty acid intake levels in North America have been summarized in Table 2-1. Estimates vary considerably ranging from as low as 2.2 $g.d^{-1}$ to as high as 13.3 $g.d^{-1}$.

Intake levels from different parts of the world have also shown considerable variation. In the seven countries study (23) which evaluated *trans* fatty acid intake (*t* -C18:1) in 16 different cohorts worldwide, estimates ranged from 0.1 to 5.5 $g.d^{-1}$. In the Scottish Heart study (24), *trans* fatty acid intakes measured using a FFQ were 7.1 $g.d^{-1}$ for men and 6.4 $g.d^{-1}$ for women. In Britain, Robert et al (25) using adipose tissue data, which is

considered to reflect long term *trans* fatty acid intake, reported a mean total *trans* fatty acid content of 2.8%. This level is lower than the 6.3% reported for Canadians (26)

However, given the problems inherent in each of the above methods used to estimate *trans* fatty acid intake, it is difficult to quantify *trans* fatty acid intake levels with precision and accuracy especially for individuals. For example, the disappearance and availability data provide incomplete information on hydrogenated fat consumption, because much of the fat is discarded after use. More accurate estimates based on chemical analysis are narrow in scope as they do not reflect the variation across population groups. Estimates of intake from FFQ reflect the inaccuracies associated with recall and estimation of portion size. The database for *trans* fatty acids in foods is inaccurate and of questionable accuracy (27). An additional problem arises when an average value is used to describe the *trans* fatty acid content of a food product category in which there is a wide variation in the *trans* fatty acid content of individual foods within that category (28). The differences in adipose tissue *trans* fatty acid content could be due to variations in analytical techniques. Thus, the methods that have been used to assess *trans* fatty acid intake collectively provide an approximation of the consumption of *trans* fatty acids.

2.5 Consumption Trends of Hydrogenated Fats

While the estimates of per capita intake of *trans* fatty acids remains highly controversial, the overall consumption trends of *trans* fatty acids appear to have remained relatively constant since the 1970's. This is despite an appreciable increase in the intake of

vegetable fat in salad and cooking oils and shortenings along with the concomitant decrease in intake of animal fat as depicted in Figure 2-2. In recent years, advances in food technology have introduced products on the market that have lower *trans* fatty acid contents. Thus, the amount of *trans* fatty acids in the diet have remained relatively constant over the past few decades in part because the increase in vegetable fat consumption has been counterbalanced by a decrease in the *trans* fatty acid content of many products made with vegetable fat (27).

2.6 Hydrogenated Fat Intake and its Relation to Cardiovascular Disease

2.6.1 Epidemiological Studies

Epidemiological studies have been at the forefront of the *trans* fatty acid debate, and results of these studies are provocative (28). A review of some of the epidemiological studies evaluating the link between *trans* fatty acid intake and coronary vascular disease (CVD) has been summarized in Table 2-2. It can be observed that results are inconsistent among the various studies. Data derived from FFQ (14-16,29) and diet records (23) seem to support a relationship between *trans* fatty acid intake and CVD risk. In addition, a recent prospective study (36) found that women in the highest quintile of *trans* fatty acid intake were at greater risk of CVD when compared to women in the lowest quintile of intake. Data obtained using adipose tissue levels as indices of *trans* fatty acid intake are inconsistent, with some (22,25,32) but not all studies (31,33,34) showing an association with CVD development. Siguel and Lerman (35) suggest an association between plasma cholesterol levels and *t*-C16:1 derived mainly from animal sources but not *t*-C18:1 present predominantly in commercially produced hydrogenated products. These data are

contradictory to those obtained by Ascherio et al (16) who reported that *trans* fatty acids from hydrogenated vegetable oils but not from animal sources contribute to increased CVD risk.

Thus, drawing conclusions about the effect of *trans* fatty acids from hydrogenated fat on cardiovascular health, based on epidemiological evidence is difficult given the conflicting results obtained. Although attempts are made to control for confounding variables, given the limitations of each method of assessment, even the best attempts makes it difficult to establish causation.

2.6.2 Metabolic Studies

The physiological effects of hydrogenated dietary fats on plasma lipids were initially investigated in the 1960s. Some studies (37,38) but not all (39,40) indicate that serum cholesterol levels are increased with consumption of partially hydrogenated vegetable oils. However, results from the above studies (37-40) are difficult to interpret given the insufficient information about dietary intake, lack of sufficient cholesterol to elicit a response, small number of subjects and short feeding durations (41). The following is a review of well controlled clinical trials examining the effects of dietary *trans* fatty acids from hydrogenated fats compared to either unsaturated or saturated native oils, saturated fats or the corresponding *cis* isomers.

2.6.2.1 Hydrogenated fat versus Unsaturated or Saturated Native Oils and/or Saturated Fats

Anderson et al (42) fed 27 men either safflower oil or hydrogenated safflower oil substituted for carbohydrate in a high fat diet. The latter diet, which provided 3% of energy as *trans* fatty acids, was found to raise TC concentrations by 5% compared to the oil diet. Additional experiments feeding 10% of energy as *trans* fatty acids raised TC levels by 16%, suggesting a dose response relation.

Similar quantitative results were seen in the study of Lichtenstein and coworkers (43). Feeding 14 moderately hypercholesterolemic men and women corn oil margarine in stick form providing 4.2 % energy from *t*-C18:1, increased TC by 6%, LDL-C by 8% and apo B concentrations by 13% in comparison to the corn oil diet which contained 0.4% energy as *t* -C18:1. Plasma TG, Lp(a) and apoA-I concentrations were unaffected by stick margarine consumption. Overall, the margarine diet increased the TC: HDL-C ratio by 21%, thereby producing the least desirable plasma lipid profile.

Several studies have simultaneously evaluated how hydrogenated fats compare with their native oil as well as with a more saturated fat. Laine et al (44) evaluated the effect of palm oil versus corn oil or lightly hydrogenated soy oil containing 5.4 % energy as *trans* fatty acids in 12 subjects. Compared to the palm oil rich diet, consumption of the corn oil and hydrogenated fat diet decreased plasma TC levels by 13% and 9%, while LDL-C levels were decreased by 23% and 10% respectively. When the hydrogenated fat diet was

compared to the corn oil diet, TC, and LDL-C were increased by 5% and 15% respectively, while HDL-C and TG concentrations remained unchanged.

Nestel and colleagues performed 2 studies (45,46) evaluating the effect of *trans* fatty acids on plasma lipid profile. In the first study (45) 26 moderately hypercholesterolemic men were fed an average Australian diet containing 35-40% fat calories and 2 hydrogenated fat blends contributing 4% energy as *t*-C18:1. Both test blends reduced TC and LDL-C levels, but HDL-C levels did not change. In the second study (46) elaidic acid providing 5.7% energy was directly exchanged for palmitic or oleic acid. The elaidic acid diet significantly increased TC and LDL-C by 7% and 9% relative to the oleic acid diet, but gave similar results as the palmitic acid diet. However, HDL-C was decreased by 10% with the *trans* compared to the SFA containing diet. In addition, Lp(a) levels were higher on the *trans* compared to the palmitic acid but not the oleic acid rich diets. Triglyceride concentrations were somewhat higher with the *trans* diet compared to the other 2 diets, but changes were not statistically significant.

Judd et al (47) appraised the effects of consuming *trans* fatty acids in amounts currently consumed in the US (7% and 4% energy) versus an oleic acid or SFA diet. Plasma TC levels increased by 4%, 5% and 7%, LDL-C by 6%, 8% and 9%, and TG by 8%, 13% and 4% on the high *trans*, moderate *trans* and SFA containing diets relative to the oleic acid rich diet. In addition, a significant decrease of 2.8% in HDL-C concentrations with the higher *trans* fatty acid containing diet was shown when compared to the oleic acid enriched diet.

Two additional studies have compared *trans* versus SFA. The Norwegian study (48) used 2 types of *trans* preparations-partially hydrogenated soybean oil containing 8.5% energy as *trans* (PHSBO) and partially hydrogenated fish oil with 8% *trans* (PHFO). The SFA diet appeared worse than PHSBO but better than PHFO with regard to lipid response. In a Malaysian study (49), PHSBO providing 7% energy as *trans* was compared to either a palmitic acid, lauric + myristic acid, or oleic acid containing diet. PHSBO was more cholesterolemic than either palmitic or oleic acid containing diets, but equal to the lauric + myristic acid containing diet. The lowering of HDL-C was observed only with the *trans* diet.

2.6.2.2 Trans versus Cis Fatty Acids

An alternative approach to evaluating the effect of hydrogenated fat on serum lipids is the use of fat preparations formulated to provide constant ratios of SFA, MUFA and PUFA. Using liquid based diets, Vergroesen and Gottenbos (50) were one of the first investigators who reported that in the presence of dietary cholesterol, *t*-C18:1 (18% energy) was hypercholesterolemic when substituted for *cis* fatty acids.

Mensink and Katan (51) compared the effect of consuming a *trans* (11% energy), oleic or SFA diet in 59 normocholesterolemic subjects. Compared to the oleic acid rich diet, feeding the *trans* diet resulted in significant increases in TC (5%), LDL-C (11%) and TG (13%) levels, as well as a significant decrease in HDL-C (13%). A subsequent analysis of frozen sera from this study (52) revealed that the *trans* diet also caused a significant elevation in Lp(a), when compared with the oleic acid diet. In a follow up study using

lower amounts of *trans* fatty acids (7.7% energy), Zock and Katan (53) compared the effects of dietary elaidic acid, linoleic acid or stearic acid rich diets in 56 normocholesterolemic subjects. Both the elaidic and stearic acid rich diets produced identical lipoprotein profiles, with significantly higher TC, LDL-C and TG, and significantly lower HDL-C concentrations than the linoleic acid diet.

2.6.2.3 Different Forms of Hydrogenated Fats

Over the past few years there has been a proliferation of new products in the market that vary in their degree of hydrogenation. These include semi-liquid, soft and hard margarines. Wood et al (54) have demonstrated that replacing 60% of a habitual diet with either a soft or hard margarine, resulted in different lipoprotein profiles. The soft margarine resulted in significantly lower TC, LDL-C and TG levels, than the hard margarine. Recently, Lichtenstein and co-workers (55) published the results of a trial in which different forms of commercially available margarines and a vegetable shortening with a wide range of *trans* fatty acid levels were substituted for butter. Moderately hypercholesterolemic subjects (n=18 men and 18 women) were fed each diet for a period of 35 days in a randomized cross-over design. Consumption of diets enriched in soybean oil, semi-liquid margarine, soft margarine, shortening and stick margarine resulted in reductions in LDL-C concentrations of 12%, 11%, 9%, 7% and 5% respectively, when compared to the butter diet. The HDL-C levels were reduced by 3%, 4%, 4%, 4% and 6%, respectively, for the same ordering of diets. Ratios of TC: HDL-C were lowest after the consumption of the soybean oil and semi-liquid margarine diets, and highest after stick margarine feeding. Based on these results the authors concluded that consumption

of products low in *trans* fatty acids and saturated fat have beneficial effects on serum lipoprotein cholesterol levels.

Collectively, all recent metabolic studies indicate that *trans* fatty acid or hydrogenated fat consumption results in higher plasma cholesterol levels than native oils (43,44), and lower plasma cholesterol levels than more saturated fats (45-49). Effects on TG concentrations are variable, with significantly higher levels observed in some (47,48,51,54,55) but not all studies (43,44,46,53). These differences are probably due to the differences in study design, and source and level of *trans* fatty acid in the diets. However, the majority of studies report significant decreases in HDL-C levels with *trans* fatty acids relative to SFA diets. In addition, some studies (46,52,55) have reported that *trans* fatty acids elevate Lp(a) concentration. Consequently, hydrogenated fat consumption appears to have multiple unfavorable effects on lipoprotein profile and subsequent risk of developing heart disease.

2.7 Mechanism of Action of *Trans* Fatty Acids from Hydrogenated Fats

In spite of the important body of evidence showing that dietary *trans* fatty acids from hydrogenated fats have a negative impact on plasma lipids, the mechanisms responsible for this effect are as yet unknown. Different hypotheses have emerged in an attempt to explain the hypercholesterolemic effect of *trans* fatty acids. These include changes in endogenous cholesterol biosynthesis, cholesterol esterification and transfer rates, lipoprotein composition and catabolism, and hepatic LDL receptor number. Another

potential mechanism involves the acylation stimulating protein (ASP) pathway, which has been shown to be altered in several dyslipoproteinemic states.

2.7.1 Alterations in Cholesterol Metabolism

2.7.1.1 Cholesterol Biosynthesis

Data on synthesis and uptake of cholesterol suggest that endogenous synthesis in man plays a major role in the adjustment of the body pool of cholesterol. Hence its regulation is considered a significant factor in influencing circulating cholesterol levels. The 3 compartment or 3 pool model (Figure 2-3) is generally used to represent long-term turnover of plasma cholesterol in humans (56,57). The first compartment (M_1) consists of cholesterol that equilibrates rapidly with plasma cholesterol, and includes that in plasma, red blood cells, liver, intestine, and much of the cholesterol in other visceral tissues, such as the lung, pancreas, spleen and kidney. The second compartment (M_2) consists of cholesterol that equilibrates at an intermediate rate with plasma cholesterol, and includes that in some visceral and peripheral tissues. The third compartment (M_3) consists of cholesterol that equilibrates slowly, and includes cholesterol in the skeletal muscle and arterial walls. Cholesterol turnover studies (58) have estimated that the central or M_1 pool synthesis varies from 61 to 89% of the total cholesterol rate. Consequently, changes in plasma cholesterol levels following dietary fat modification are considered to reflect changes in cholesterol synthesis in M_1 pool organs, given that these organs most rapidly equilibrate with plasma cholesterol.

2.7.1.1.1 Regulation of Endogenous Cholesterol Synthesis by Dietary Fatty Acids

Studies in both animals and humans have documented alterations in endogenous cholesterol biosynthesis with consumption of different fatty acids, but results are conflicting. Fernandez et al (59) fed guinea-pigs diets containing either corn oil, olive oil or lard for 5 weeks and reported that whole body cholesterol synthesis rates were lower in the corn oil group than those in the olive oil and lard groups. A similar reduction in liver cholesterogenesis was observed by Triscari et al (60), when rats were fed corn oil versus a hydrogenated fat. These findings suggest that PUFA consumption may decrease *de novo* cholesterol biosynthesis. However, a study conducted by Spady and Dietschy (61) demonstrated contradictory results. Hamsters fed diets containing PUFA rich safflower oil had higher hepatic cholesterol synthesis as compared with diets containing olive or coconut oils. Jones et al (62) measured cholesterogenesis in mildly hypercholesterolemic individuals consuming diets containing 30% energy as fat in the form of corn, olive, canola, or rice bran oils, or beef tallow. Higher synthesis rates were observed for the PUFA-containing corn oil phase compared with any of the other phases. This observation of higher synthesis rates with PUFA and lower synthesis with SFA diets has also been demonstrated in normolipidemic subjects (63-65).

With regard to the effect of *trans* fatty acids on endogenous cholesterol synthesis, Cuchel and colleagues (66) have shown that feeding subjects corn oil margarine in stick form as compared to a corn oil diet, increased plasma lipid and lipoprotein levels in spite of a trend toward decreased *de-novo* cholesterol synthesis rates. However, the issue remained unresolved since the decline in fractional synthetic rate of cholesterol did not reach

statistical significance, possibly due to sample size limitations and high variability in synthesis among the male and female subjects. With the exception of the above study, the effect of hydrogenation, specifically degree of hydrogenation on cholesterogenesis has not been studied.

2.7.1.2 Cholesterol Esterification and Transfer Rates

It has also been suggested that alterations in cholesterol esterification rates may be another important factor responsible for the different effects of dietary saturated and unsaturated fatty acids on plasma lipid levels. Spady and Dietschy (67), using a hamster model, have demonstrated that dietary saturated fatty acids (SFA) affect LDL cholesterol levels through changes in the activity of the hepatic LDL receptor. The liver enzyme-acyl-CoA: cholesterol acyltransferase (ACAT) that converts free cholesterol to its esterified form has a lower affinity for saturated than unsaturated fatty acids. Woollett and colleagues (68) have demonstrated in animals that the selectivity of ACAT for unsaturated fatty acids is lost when the double bond is in the *trans* configuration, specifically *t*-C18:1n-9. The resulting accumulation of hepatic free cholesterol could lead to down regulation of the LDL receptor, causing accumulation of LDL particles in plasma and increased formation of LDL from its precursor-VLDL.

However, in plasma, the majority of esterification occurs in HDL, and is proportional to the activity of the enzyme lecithin: cholesterol acyltransferase (LCAT). Recently, Subbaiah et al (69) using an *in vitro* model have demonstrated that *trans* fatty acids, are not only poor substrates for ACAT but also LCAT. Additionally, in plasma, increased

transfer of cholesteryl esters from HDL-C to VLDL-C and LDL-C, mediated by the cholesterol ester transfer protein (CETP) have also been reported (70-72). Abbey and Nestel (70) have demonstrated significantly higher cholesterol ester transfer protein (CETP) activity in the plasma of subjects fed *trans* fatty acid rich diets as opposed to subjects fed oleic acid rich diets. Similar results of higher CETP activity with *trans* fatty acids relative to linolenic and stearic (71) or *cis* fatty acids (72) have also been reported. Thus, it is possible that the discrimination against *trans* fatty acids by esterification enzymes, accompanied by increased CETP activity could alter cholesterol esterification rates, which in turn could explain the altered lipoprotein profile observed after consumption of hydrogenated fats.

2.7.1.3 Lipoprotein Composition and Catabolism

The elevations in plasma cholesterol concentrations caused by consumption of hydrogenated fats may also be a result of alterations in plasma lipoprotein composition. Morrisett et al (73) showed that lipoproteins from patients fed PUFA diets had thermotropic transitions at lower temperatures than those from patients fed SFA diets, resulting in greater lipoprotein fluidity. Jackson and Gotto (74) suggest that the alterations in membrane fluidity could be responsible for greater LDL receptor uptake. Given the structural similarities between *trans* and SFAs, enrichment of cell membrane phospholipids with either of these fatty acids could reduce binding or internalization of circulating LDL (75,76), thus decreasing the fractional catabolic rate of cholesterol rich lipoproteins and increasing circulating cholesterol levels.

2.7.2 Alterations in Triglyceride Metabolism

In attempting to explain the mechanisms by which hydrogenated fat intake affects lipoprotein metabolism, attention has been primarily focussed on alterations in cholesterol metabolism. Recent evidence suggests that alterations in TG metabolism could also be involved.

2.7.2.1 Role of the ASP Pathway in Triglyceride Metabolism

In vivo (77) and *in vitro* (78,79) studies have demonstrated that the ASP pathway plays an important role in regulating the rate of TG synthesis in adipocytes. Human adipocytes synthesize and secrete 3 proteins-C3, factor B and adipsin, which interact to produce ASP, the bioactive product of this pathway (80). ASP acts on the adipocyte and stimulates TG synthesis by increasing activity of diacylglycerol acyltransferase (DGAT), the last enzyme involved in formation of the TG molecule. In addition ASP increases specific membrane transport of glucose via translocation of glucose transporters from intracellular storage sites to the plasma membrane. The effect on glucose uptake has been shown to be independent and additive to that of insulin (81,82).

2.7.2.2 Regulation of the ASP Pathway by Dietary Fatty Acids

Studies in humans (77) have shown that the production of ASP is markedly accelerated in the second half of the postprandial period. Furthermore, the increase in ASP production during this period correlates with maximal TG clearance and fatty acid uptake by adipocytes. Since normal function of the ASP pathway appears integral to the normal deposition of dietary fatty acids into adipocytes during the postprandial period, it can be speculated that dysfunction of the ASP pathway may be associated with lipoprotein

abnormalities that are linked to the pathogenesis of CVD. If fatty acid trapping and storage by adipocytes are reduced in the postprandial period, an excessive proportion of dietary fatty acids will be diverted to the liver and hepatic secretion of apoB100 lipoproteins, VLDL-C and LDL-C, will increase (83). In fact, Murray et al (79) have demonstrated in mice lacking ASP, increased VLDL-C, LDL-C and triglyceride concentrations following an oral fat load. However, to date, no study has examined the effects of dietary hydrogenated fat consumption on ASP levels, over the long-term.

Despite this knowledge on the effects of dietary fat intake on cholesterol and TG metabolism, the actual mechanisms by which *trans* fatty acids from hydrogenated fats increase plasma cholesterol concentrations are still not well characterized. Further investigations are warranted to determine these mechanisms, and to test the effect of consumption of different forms of hydrogenated fats on plasma lipid and lipoprotein profile in humans.

2.8 Measuring Cholesterol Synthesis

While it has been established that dietary fatty acid composition alters cholesterol biosynthesis, until recently, no simple procedures were available for monitoring short-term changes in humans. Previously, measurement of endogenous cholesterol synthesis relied on indirect methods or methods that required extended measurement periods. The classic sterol balance method measures cholesterol balance i.e., fecal excretion of cholesterol plus its acidic and neutral metabolites, minus cholesterol intake. Although this method is well established, it has the disadvantage of being laborious and is not suited to

detect changes in cholesterol synthesis within the time frame of less than a few days. Moreover, the method requires that the subjects be in steady state with regard to cholesterol metabolism (84).

Determination of the activity of the hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the synthetic pathway of cholesterol, provides an *in vitro* measurement of cholesterogenesis, but is restricted by the need for a liver biopsy (85). Alternately, assessing levels of cholesterol precursors, offers a faster and less cumbersome approach. Such methods include determination of mevalonic acid (MVA) (86-88), squalene (89,90), desmosterol (89,91), lanosterol (89,92) and lathosterol (84,89,93) concentrations. Despite the well documented correlation of plasma cholesterol precursor levels with hepatic HMG-CoA reductase activity (94) and with cholesterol synthesis as assessed by sterol balance (86), potential sources of error do exist. Plasma MVA is water soluble and reflects not only changes in cholesterol synthesis itself but possibly also changes in renal function (95). In contrast, plasma squalene, desmosterol, lanosterol and lathosterol are transported with VLDL and LDL particles, and are thus influenced by factors that alter VLDL and LDL cholesterol concentrations in plasma (93). However, it is possible to correct for these influences by expressing the levels of the above precursors relative to the concentration of plasma TC concentrations.

Other techniques for measuring human lipid synthesis include kinetic approaches using stable or radiolabeled cholesterol. While kinetic studies with [^{14}C]-cholesterol provide detailed information on pool sizes, distribution and formation rates of cholesterol, these

studies require prolonged measurement intervals consistent with metabolic steady state (65).

More recent tracer approaches for measuring human cholesterol biosynthesis involve stable isotope techniques such as deuterium incorporation (DI) and mass isotopomer distribution analysis (MIDA). The MIDA technique, despite its advantages, is invasive, requiring indwelling catheters for delivery of isotope label, serial blood sampling, and lengthy data analysis with elaborate mathematical processing (96).

The DI method offers a safe and non-invasive alternative approach for the investigation of human lipid metabolism. Theoretically this technique is similar to the tritiated water incorporation method (65). Deuterated water enters cells readily and equilibrates quickly with intracellular water. Little unlabelled water is generated intracellularly, allowing the cell precursor pool enrichment to equal that of plasma (97,98). The calculated rate of cholesterol synthesis depends upon the rate of label incorporated per molecule of cholesterol. During the synthesis of cholesterol, hydrogen atoms from water are incorporated into the sterol molecule in 3 different ways, as depicted in Figure 2-4. Seven atoms of hydrogen are incorporated directly from water, 15 atoms from NADPH, and eventually hydrogen atoms from water are incorporated into the acetyl CoA pool, which can be used as a cholesterol precursor (98). If the assumptions are made that over a 48 hour period there is complete equilibrium of deuterated water with plasma water, and with NADPH, but not the acetyl CoA pool, then 81% of the hydrogen atoms per cholesterol carbon atoms, the H/C ratio, will be incorporated into the molecule from

deuterium (99). Other assumptions include (i) the amount of recycling of label into other pools, such as acetate, during the period of interest, (ii) the form of the mathematical equation, usually accepted as monoexponential over short periods of time, and (iii) the theoretical and actual maximum plasma cholesterol enrichment. However, when measurements are made over the short-term, the rate of D uptake is unaffected by flux rates of other, unlabeled material into the system and can be taken to represent a direct measure of synthesis. Procedurally, the major drawback of the DI method is the lengthy, multi-stage process that could lend itself to experimental error. Despite these limitations, the DI method has been shown to be a relatively quick and non-invasive alternative to the sterol balance (100) and MIDA (96) technique. The DI method has also been validated against plasma mevalonic acid concentration (88), however, a direct cross-comparison between the DI method and plasma levels of other commonly used precursors, such as squalene, desmosterol, lathosterol and lanosterol, has never been carried out.

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Table 2-1 Estimated Per Capita Consumption of Dietary *Trans* Fatty Acids In North America

Method of Assessment	Reference	Average Intake of *trans* fatty acids ($g.d^{-1}$)
Disappearance Data	Statistics Canada (5)	12.6
	Hunter and Applewhite (6)	7.6
	Hunter and Applewhite (7)	8.1
	Senti (8)	10.2
Availability Data	Enig et al (9)	13.3
Analysis of Self Selected Diets	Aitchison et al (10)	2.7
Diet Records	Innis et al (11)	6.9
	Van den Reek et al (12)	2.8
	Allison et al (13)	2.6
Food Frequency Questionnaire	Triosi et al (14)	4.0
	Willet et al (15)	3.4
	Ascherio et al (16)	3.8
	Lemaitre et al (17)	2.2
	Garland et al (18)	2.8
Adipose Tissue Biopsy	Chen et al (19) #	10.6
	Ratnayake and Chen (20)	8.4
	London et al (21)	3.4
	Hudgins et al (22)	4.5
	Garland et al (18)	6.1

Per capita consumption data available for women only

Table 2-2

Summary of Epidemiological Studies Evaluating the Relation Between *Trans* Fatty Acid Intake and Risk of Developing CVD

Reference	Study Design	Subject Characteristics	Method of *Trans* Fatty Acid Intake Assessment	Main Results
Troisi et al (14)	Cross-sectional	748 middle aged to elderly men from the Normative Aging Study	FFQ	Small but significant positive association between *trans* fatty acid intake and TC, LDL-C and TC/HDL-C concentrations A negative association was also observed between *trans* fatty acids and HDL-C concentrations
Willet et al (15)	Prospective cohort	85095 female nurses	FFQ	Intake of *trans* isomers significantly associated with CHD risk
Ascherio et al (16)	Case-control	239 case subjects with first MI and 282 control subjects from the Boston area	FFQ	Significant association between *trans* fatty acid intake and risk of first MI
Pietinen et al (29)	Prospective cohort	21930 adult Finnish smoking men from the ATBC trial	FFQ	Significant positive association between *trans* fatty acid intake and risk of coronary death for men in the top quintile of *trans* fatty acid intake compared with men in the lowest quintile of intake

Table 2.2 continued.....

Reference	Study Design	Subject Characteristics	Method of *Trans* Fatty Acid Intake Assessment	Main Results
Tzonou et al (30)	Case-control	329 case subjects with first evidence of CHD and 570 control subjects without CHD from Athens	Dietary history	Non-significant increase in CHD risk in subjects using margarine as the main cooking medium
Thomas et al (31)	Case-control	59 men with ECG evidence of ischemia and 61 men without such evidence from Britain	Adipose tissue concentration	No significant differences in level of *t*-C:16:1, *t*-C:18:1 or *t*-C:18:2 between cases and controls
Thomas and Winter (32)	Case-control	27 men who died of ischemic heart disease and 27 men who died of other causes from Britain	Adipose tissue concentration	*t*-C:16:1 but not *t*-C:18:1 was significantly higher in case subjects
Hudgins (22)	Cross-sectional	76 adult white men from the NY area	Adipose tissue concentration	No significant association was observed between *trans* fatty acid intake and serum lipid and lipoprotein concentrations

Table 2.2 continued.....

Reference	Study Design	Subject Characteristics	Method of *Trans* Fatty Acid Intake Assessment	Main Results
Aro et al (33)	Case-control	671 men with acute MI and 717 men without MI from Europe and Israel	Adipose tissue concentration	No significant association between *t*-C:18:1 concentrations and risk of MI
Roberts et al (25)	Case-control	66 deceased English men with CHD and 286 healthy living men	Adipose tissue concentration	*t*-C:18:2 concentrations were unrelated to risk of CHD. *t*-C:18:1 concentrations were significantly associated with lower risk of CHD
Fritsche et al (34)	Case-control	24 patients with angiographically documented CHD and 25 controls without CHD	Adipose tissue concentration	No significant difference observed between cases and controls with regard to adipose tissue total *trans* fatty acid content
Siguel and Leman (35)	Case-control	47 cases with angiographically documented CAD (35 men/ 12 women) and 56 controls (29 men/ 27 women) without CAD	Plasma concentration	Individual and total *trans* isomers were higher in cases than controls. *t*-C:16:1 was negatively associated with HDL-C, HDL-C/TC and positively associated with TG, TC, LDL-C and CAD

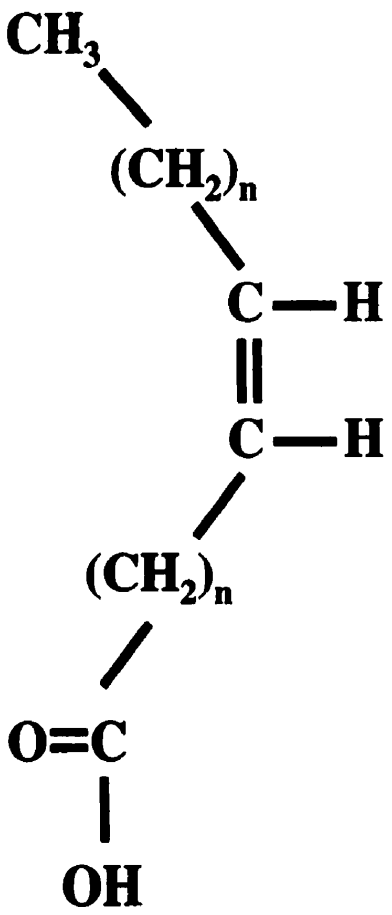
2.10 Figure Legends

Figure 2-1: Structure of a monounsaturated fatty acid with the double bond in the *cis* and *trans* configuration

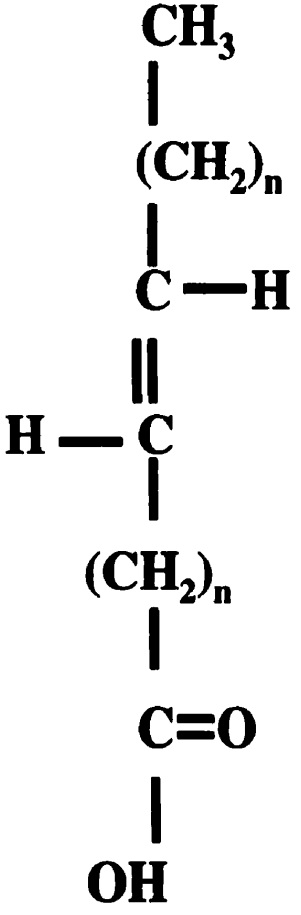
Figure 2-2: Consumption of visible fats in North America (1965-1995). Data are derived using per capita disappearance information provided by the International Trade Division, Statistics Canada and Economic Research Service, United States Department of Agriculture.

Figure 2-3: Three pool (M_1, M_2 and M_3) model of cholesterol turnover in man. These pools are denoted as 1,2 and 3; rate constants (d^{-1}) are denoted by k; and rate of transfer of cholesterol mass ($g.d^{-1}$) into or out of a pool is denoted as R.

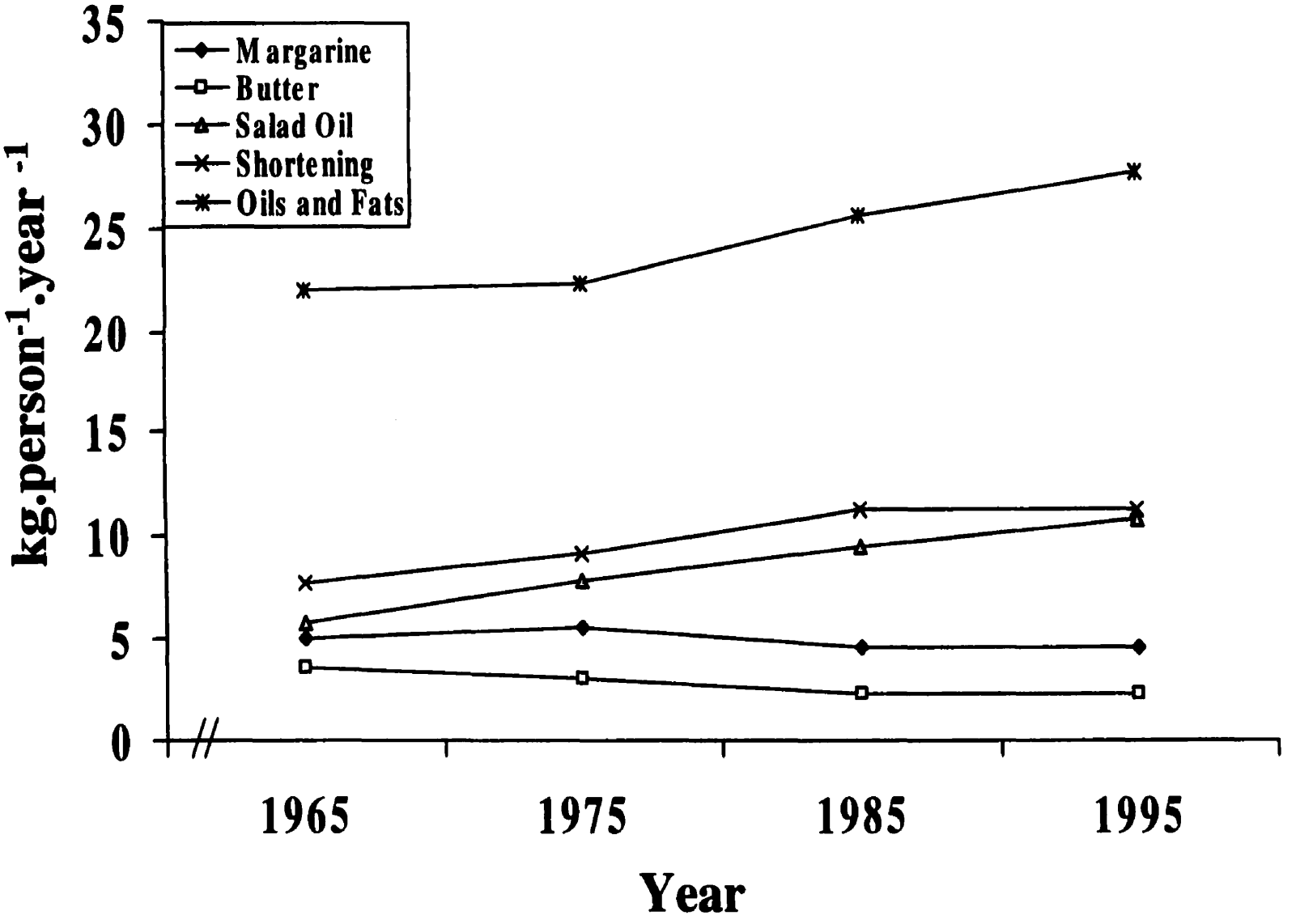
Figure 2-4: Origin of hydrogen species used in cholesterol biosynthesis and site of deuterium labeling within the first 48 hours of D_2O dosing. Numbers in brackets represent number of hydrogen atoms contributed by each source. D_{max}, the maximum number of deuteriums per molecule of cholesterol, is 22 over periods up to 48 hours.



cis monounsaturated fatty acid
(kink in molecule)



trans monounsaturated fatty acid
(straight molecule)


Margarine
Butter
Salad Oil
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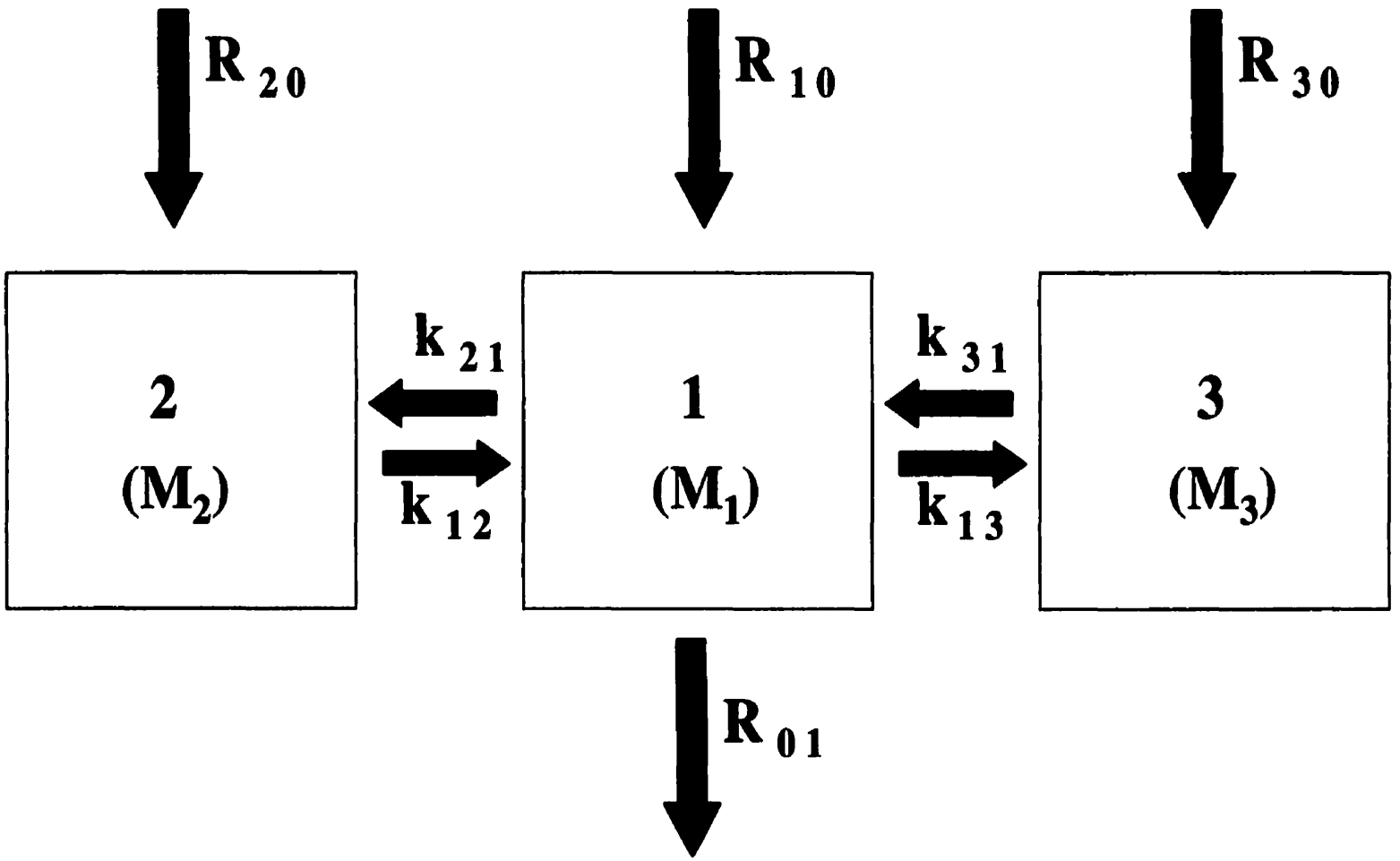
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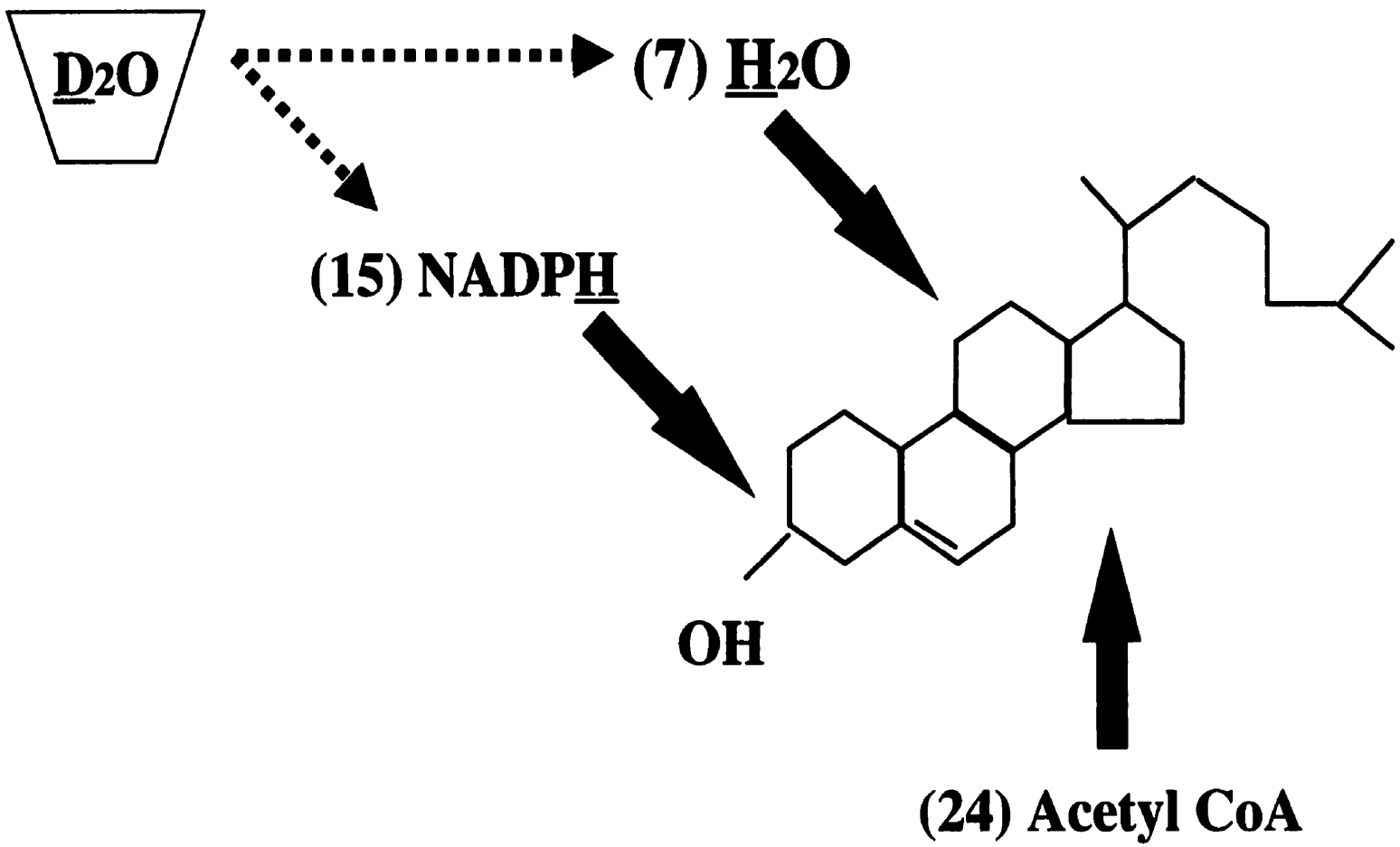
D2O
(7) H2O
(15) NADPH
OH
(24) Acetyl CoA

CHAPTER 3

DEUTERIUM UPTAKE AND PLASMA CHOLESTEROL PRECURSOR LEVELS CORRESPOND AS METHODS FOR MEASUREMENT OF ENDOGENOUS CHOLESTEROL SYNTHESIS IN HYPER-CHOLESTEROLEMIC WOMEN

Submitted for publication

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3.1 Abstract

To assess the validity of 2 techniques used to measure human cholesterol synthesis, the rate of uptake of deuterium (D) into plasma free cholesterol (FC), and plasma cholesterol precursor (squalene, lanosterol, desmosterol and lathosterol) levels were compared in 14 women (65-71 years with LDL-C $\geq$3.36 mmol.L^{-1}). Subjects consumed each of 6 diets for 5 week periods according to a randomized crossover design. The experimental diets included a baseline (BL) diet (39% energy as fat, 164 mg chol. 4.2 MJ^{-1}) and five reduced fat diets, where 2/3rd of the fat was either soybean oil (SO), squeeze (SQM), tub (TM), or stick (SM) margarines, or butter (BT). Fractional and absolute synthesis rates (FSR and ASR) of FC were determined using the deuterium incorporation (DI) method, while cholesterol precursor levels were measured using gas liquid chromatography. Data were pooled across diets for each variable and correlation coefficients calculated to determine if associations were present. There was good agreement among levels of the various cholesterol precursors. In addition, FSR in pools/day (p.d^{-1}) and ASR in grams/day (g.d^{-1}) were strongly associated with lathosterol (r=0.72 and 0.76, p=0.0001), desmosterol (r=0.75 and 0.75, p=0.0001), lanosterol (r=0.67 and 0.69) and squalene (r=0.69 and 0.69) when levels of the precursors were expressed as μmol.mmol^{-1} C. Significant but lower correlations were observed between the D uptake and plasma cholesterol precursor levels when the latter was expressed in absolute amounts (μmol.L^{-1}). The wide range of *trans* and fatty acid profiles of the experimental diets did not influence the degree of association between methods. In conclusion, the DI method and levels of some cholesterol precursors correspond as methods for short-term measurement of cholesterol synthesis.

3.2 Introduction

Cholesterol homeostasis is maintained by regulatory mechanisms that balance input and output so as to normally prevent net accumulation of cholesterol (1). Alterations in homeostasis resulting in elevated circulating cholesterol concentrations are associated with undesirable plasma lipid response that increase the risk of developing cardiovascular disease (CVD) (2,3). A factor known to influence *de novo* cholesterogenesis is dietary fat modification (4-6). Several techniques have been used to measure cholesterol synthesis in humans, including sterol balance (7-11), kinetic approaches (12,13), activity of the enzyme 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase (14,15), and cholesterol precursor levels (14,16-22). More recent approaches include the mass isotopomer distribution analysis (MIDA) (23-25), and deuterium incorporation (DI) methodology (26-31).

The sterol balance method provides an accurate value for biosynthesis once internal sterol pools have equilibrated, but has the disadvantage of being laborious, requiring complete stool collections and accuracy in recording food intake (32). Kinetic studies with [^{13}C]-cholesterol provide detailed information about pool sizes, distribution and formation rates of cholesterol, but require prolonged measurement intervals consistent with metabolic steady state (6). Determination of the activity of HMG-CoA reductase, the rate limiting enzyme in the synthetic pathway of cholesterol, provides an index of cholesterol synthesis over the short-term, but is restricted by the need for a liver biopsy (15). Sterol precursor levels in plasma including mevalonic acid (16,17), squalene (14,18), lanosterol (14,19), desmosterol (14,20) and lathosterol (14,21,22) are alternate

indicators of synthesis, however, the sensitivity of these techniques are still under investigation. The MIDA technique, despite its advantages, is invasive, requiring indwelling catheters for delivery of isotope label, serial blood sampling, and lengthy data analysis with elaborate mathematical processing (6).

Alternately, the deuterium (D) uptake method, capable of directly measuring the rate of incorporation of D from body water into newly formed cholesterol (26-31), offers a safe, non-invasive alternative for detecting changes in cholesterol synthesis rates over short periods of time. This method has been validated against levels of plasma mevalonic acid (33), sterol balance (32) and MIDA (34) techniques, however, a direct comparison between DI and the commonly used cholesterol precursor level assessment method has not been performed. Thus, the objective of the present investigation was to determine whether levels of some cholesterol precursors (squalene, lanosterol, desmosterol and lathosterol) correlate with the D uptake method for measurement of endogenous cholesterogenesis. The study was originally designed to assess the effect of different *trans* and fatty acid profiles on cholesterol synthesis. The specific effects induced by the diet change are reported elsewhere. Here, the focus of attention will be to determine whether an association exists between the 2 methods.

3.3 Methods

3.3.1 Subjects

Fourteen postmenopausal, middle aged to elderly, moderately hypercholesterolemic (LDL-C $\geq$ 3.36 mmol.L^{-1}) women (65-71 years) were recruited as previously described

(35). Subjects had no family history of premature CVD, nor showed any signs of hepatic, renal, thyroid, or intestinal disease. None of the subjects were taking any medication known to alter lipid metabolism such as lipid lowering drugs, β-blockers, diuretics or hormones. The protocol was reviewed and approved by the human-investigation review committee of New England Medical Center and Tufts University. All potential subjects were given a verbal and written description of the study prior to obtaining written consent.

3.3.2 Study Design and Diets

Subjects were given in a double blind fashion, each of six diets for 5 week periods, separated by washout periods ranging from 2 to 4 weeks. The experimental diets included a baseline (BL) diet designed to approximate that currently consumed in the United States (39% energy as fat, 164 mg chol. 4.2 MJ^{-1}), and 5 reduced fat diets (30 % of energy) where two-thirds of the total fat was provided by either soybean oil (SO) or soybean oil based margarines in the squeeze (SQM), tub (TM) or stick (SM) forms as well as butter (BT). All food and drink were provided by the Metabolic Research Unit of the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University to be consumed on site or packaged for take-out. Energy intakes of the subjects were tailored to individual requirements, as verified by the ability to maintain stable body weight. Analysis of the macronutrient, fatty acid and cholesterol content of the diets were carried out by Covance Laboratories (Madison, WI) and Best Foods Research and Engineering Center (Union, NJ). These compositions are given in

Table 3-1. Levels of the cholesterol precursors proposed to be measured in plasma were not detected in the different diets.

3.3.3 Protocol and Analyses

During the final week of each dietary phase, fasting blood samples were collected in tubes containing EDTA (0.15%). This was immediately followed by administration of an oral dose of deuterium oxide (1.2g per kg total body water). Twenty four hours post dose, another fasting blood sample was collected. Plasma was separated, aliquoted and frozen at -80 °C until further analysis.

3.3.3.1 Cholesterol Precursor Analyses

Plasma total cholesterol concentrations were analyzed using enzymatic procedures (36,37). The precursor sterols were quantified using GLC. An internal standard containing 150 μL of 5 α cholestane was added to 1 mL of plasma. The sample, in duplicate, was then saponified for 1 hour at 100 °C, with 0.5 M methanolic KOH in 15 mL teflon capped glass tubes. After saponification, samples were allowed to cool to room temperature, followed by the addition of 2.5 mL of distilled water and 3 mL petroleum ether to each tube. The tubes were vortexed, centrifuged at 1500g for 15 min and the upper layer containing the nonsaponifiable materials transferred into clean glass tubes. The extraction procedure was repeated twice. Combined extracts were then dried down under nitrogen and re-suspended in 1 mL of chloroform. A sample volume of 2 μL was injected into a GLC equipped with a flame ionization detector (HP 5890, Series II, Hewlett Packard, CA) using a 30-m capillary column (SAC-5, Supelco, Bellefonte,

PA). Injector and detector temperatures were 300 and 310 °C, respectively. A multi-ramp oven temperature program was used. The initial temperature was 80 °C, held for 1 min and then increased to 120 °C at a rate of 20 degrees/min. After 15 min, the temperature was further increased to 269 °C at a rate of 20 degrees/min and held for 25 min. The final temperature of 320 °C was reached using a rate of 20 degrees/min and held for 5 min. Peaks of interest were identified by comparison with authentic standards (Supelco, Bellefonte, PA).

3.3.3.2 Deuterium Uptake Analyses

Additional plasma aliquots were used to determine deuterium (D) enrichment in body water and FC as previously described (26-31). Briefly, lipids were extracted (38) and FC separated by thin layer chromatography using petroleum ether/ ethyl ether/ acetic acid (135:15:1.5 v/v/v). FC was eluted with hexane/ chloroform/ ethyl ether (5:2:1 v/v/v) and dried under a stream of nitrogen. The purified cholesterol was converted into water and carbon dioxide by combustion over cupric oxide and silver wire at 520°C for 2 hours. In addition, pre and post D_2O samples diluted 2-fold and 10-fold respectively to produce D enrichments within detectable ranges on the mass spectrometer were distilled into zinc containing tubes. This enabled measurement of D enrichment of plasma water. The combustion water from FC and plasma were then vacuum distilled into Pyrex™ tubes containing zinc reagent, and reduced to hydrogen deuterium gas by heating at 520°C for 30 minutes. D enrichment of the gas was analyzed by isotope ratio mass spectrometry (VG isomass 903 D, Cheshire, England). The instrument was calibrated daily using water standards of known isotopic composition. Values were

expressed relative to the enrichment of standard mean ocean water (SMOW) in parts per mil ($^{o}/_{oo}$). The per mil designation was used because of the relatively small enrichments encountered. Duplicate samples for each subject were analyzed concurrently against a single set of standards. Maximum acceptable precision for D was 5 $^{o}/_{oo}$ at enrichments over 500 $^{o}/_{oo}$ and 2 $^{o}/_{oo}$ at enrichments below 200 $^{o}/_{oo}$.

FSR-FC defined as the proportion of the central or M_1 pool replaced daily by newly synthesized cholesterol, was calculated as the change in product enrichment over time divided by the maximum possible enrichment, based on a linear rate of uptake of label into cholesterol over time (27,29). The equation used was:

$$\text{FSR-FC } (p.d^{-1}) = \frac{\delta \text{ cholesterol } (\%)}{\delta \text{ plasma water } (\%) \times 0.478}$$

where δ is the difference in D enrichment over 24 hours. Model parameters and assumptions underlying use of D_2O as tracer for FSR measurements have been described previously (27,29). ASR-FC, which is an approximation of the daily production of newly synthesized cholesterol expressed in grams/day ($g.d^{-1}$), was derived by multiplying the FSR-FC by M_1 pool size and a factor of 0.33. The M_1 pool size was calculated using Goodman's equation (39), which takes into account subjects' body weight, plasma TC and TG concentrations. The factor of 0.33 was included to account for the proportion of FC in the overall plasma TC pool.

3.4 Statistical Analysis

Prior to analyses, the distribution of each outcome variable was checked for normality (Proc Univariate, SAS version 6.12, SAS Institute Inc., Cary, NC) and if necessary the appropriate transformations were performed. Data were pooled across diets for each variable and Pearson's correlation coefficients were determined to test for associations between variables, with significance set at $p<0.05$ (Proc Corr, SAS version 6.12, SAS Institute Inc., Cary, NC). Untransformed data are presented in text and tables as means ± standard deviation (SD).

3.5 Results

This study was originally designed to determine the effect of changes in dietary fatty acid composition on endogenous cholesterol synthesis. However, it is the purpose of the present study to focus on the association between plasma levels of cholesterol precursors and D uptake as indicators of cholesterol synthesis. The results of the diet-induced changes in cholesterol synthesis have been reported elsewhere (40). Nevertheless, it was deemed appropriate to provide these values in summary form to be read only as background information. Table 3-2 lists the pooled mean (± SD) plasma concentrations of total cholesterol, its precursors, as well as fractional and absolute synthesis rates of cholesterol. The range of values for each variable is in good agreement with those previously reported for humans (14,20,22,41,42).

Table 3-3 summarizes the correlations between the various cholesterol precursors when expressed as $\mu mol.L^{-1}$ or $\mu mol.mmol^{-1}$ of cholesterol (C). In general, good agreement

was observed between concentrations of all 4 precursors (range from r=0.88 to 0.95). Figures 3-1 and 3-2 depict the relation between FSR ($p.d^{-1}$) obtained using the DI method and plasma concentrations of squalene, lanosterol, desmosterol and lathosterol, expressed as either $\mu mol.L^{-1}$ or $\mu mol.mmol^{-1}$ C respectively. Higher correlations (p=0.0001) were obtained between FSR and plasma concentrations of desmosterol (r=0.71 and 0.75) and lathosterol (r=0.68 and 0.72). Significant (p=0.0001) but lower associations were observed between FSR and squalene (r=0.67 and 0.69), and FSR and lanosterol (r=0.65 and 0.67). The total amount of cholesterol synthesized per day (ASR) was also computed and correlated with absolute ($\mu mol.L^{-1}$) or relative ($\mu mol.mmol^{-1}$ C) concentrations of the cholesterol precursors (Figure 3-3 and 3-4 respectively). Similar to the FSR data, we found that desmosterol (r=0.75 and 0.75) and lathosterol (r=0.71 and 0.76) were better correlated with ASR than were squalene (r=0.68 and r=0.69) and lanosterol (r=0.67 and 0.69). Overall, concentrations of the precursors were found to correlate better with FSR and ASR, when expressed relative to cholesterol ($\mu mol.mmol^{-1}$ C) than when expressed in absolute amounts ($\mu mol.L^{-1}$ of plasma).

3.6 Discussion

A variety of methods exist for measuring human cholesterol biosynthesis. These methods vary in the duration needed to attain equilibrium and thus represent either a short-term or longer-term picture into *de novo* cholesterogenesis (6). Among the short-term approaches, both the D uptake and cholesterol precursor assessment methods offer potential advantages over other indices of cholesterol formation rates. However, a direct cross-comparison of these techniques has not been carried out. We present new

evidence that the D uptake method corresponds well with levels of the cholesterol precursors- lathosterol, desmosterol, lanosterol and squalene. In addition, the degree of association was not influenced by the differing fatty acid composition of the experimental diets.

The rationale for using plasma levels of cholesterol precursors as indicators of cholesterol synthesis stems from the assumption that these compounds leak into plasma lipoproteins at rates proportional to their formation in the cholesterol synthetic pathway (14). On the other hand, the DI method is based on the incorporation of deuterated water tracer into *de novo* cholesterol from a precursor pool of body water, which is in equilibrium with NADPH (27). Labeled water equilibrates quickly between the intracellular site of cholesterogenesis and extracellular body fluid. Thus, newly synthesized cholesterol is derived from a pool of known enrichment that can be determined by measuring either urine, saliva or plasma. Despite the different assumptions of each method, both are considered valid tools to assess changes in endogenous cholesterogenesis under various physiological and pathological conditions (20,22,29,30,41).

Comparatively, plasma cholesterol precursor concentrations and the D uptake method each offer both advantages and disadvantages. The former method is rapid and easy, but values obtained are relative indicators of synthetic rates. In contrast, results obtained by the D uptake method yield a direct measure of net synthesis. However, this method has its drawbacks; both procedural and theoretical. Technically, the DI method is labor

intensive, requiring lengthy multi-stage sample preparation. Theoretically, the major concern is establishing the maximum number of D molecules (D_{max}) incorporated per molecule of newly synthesized cholesterol. Over the long term, D_{max} has been shown to increase, as the acetyl-CoA pool begins to incorporate D. However, over the short term it has been established that D_{max} fluctuates between the narrow range of 20 and 22 (n=7 hydrogen from water and 15 from NADPH). Thus, the D uptake method is considered a valid and reliable indicator of endogenous cholesterol synthesis when measurements are made around 24 hours.

Similarly, the cholesterol precursor assessment method has been criticized because differences in plasma levels of the precursors could be a mere consequence of varying number of lipoprotein acceptor particles in the plasma compartment (18). It has been suggested that expressing plasma levels of precursors relative to the levels of free or total cholesterol would be a more valid index of cholesterol synthesis, as it would account for these differences in number of lipoprotein acceptor particles (18). Indeed, we found stronger correlations between levels of cholesterol precursors and the D uptake method when precursor levels were calculated relative to plasma cholesterol concentrations. In addition, our results indicate that desmosterol and lathosterol were more closely associated with FSR and ASR, while squalene and lanosterol showed significant but smaller associations. Since a certain degree of accumulation of precursors must occur inside the cells before there is a significant leakage into circulation, and given that there is a high capacity to convert squalene and lanosterol into other cholesterol intermediates, it seems reasonable that these early intermediates in

the cholesterol synthetic pathway would be associated to a lesser degree than intermediates formed at later stages of the pathway.

Our results demonstrate that the D uptake method correlates well with some plasma cholesterol precursor levels. While this association demonstrates the strength of the relation between the two methods, it does not indicate the agreement between them. The Bland and Altman (43) technique is considered an accurate statistical method for assessing agreement between 2 methods as it plots the difference among the methods against their mean. Unfortunately, this type of analysis could not be performed in the present study given the different units of measurements for each method. However, using the above technique good agreement has been demonstrated between the DI method and sterol balance (32) and MIDA (34) techniques. In addition, plasma levels of some of the precursors have also been validated against HMG-CoA reductase (14) and sterol balance (21). Thus in their own right, the accuracy of the D uptake and plasma cholesterol precursor levels has been established. Consequently it is felt that the association observed in this study provides evidence that both methods are reliable and suitable for relatively uninvasive, short-term detection of cholesterol synthesis. Furthermore, these methodologies can be used to study factors known to influence human cholesterol metabolism and consequently the risk of developing CVD.

3.7 Acknowledgements

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Table 3-1. Composition of Test Diets as Determined by Chemical Analysis*

Constituent	Baseline (BL)	Soybean oil (SO)	Squeeze Margarine (SQM)	Tub Margarine (TM)	Stick Margarine (SM)	Butter (BT)
	percentage of total daily energy intake					
Protein	16.8	15.7	17.1	16.3	16.7	16.9
Carbohydrate	44.6	55.8	51.7	52.9	53.5	53.9
Fat	38.6	28.5	31.2	30.8	29.7	29.1
Saturated fatty acids	15.5	7.3	8.6	8.4	8.5	16.7
12:0	1.8	0.8	1.0	0.7	0.8	1.4
14:0	0.1	0.6	0.7	0.6	0.6	2.5
16:0	7.8	3.7	4.3	4.2	4.0	7.5
18:0	3.6	1.5	1.9	2.3	2.2	3.6
Monounsaturated fatty acids#	15.1	8.1	8.1	8.0	8.5	8.1
18:1	12.2	7.2	7.1	6.7	6.5	7.0
Polyunsaturated fatty acids#	7.0	12.5	13.5	11.1	6.3	2.4
18:2 (n-6)	5.9	10.7	12.1	10.0	5.6	2.1
18:3 (n-3)	1.0	1.7	1.4	1.1	0.7	0.3
Trans fatty acids	1.7	0.6	0.9	3.3	6.7	1.3
Cholesterol (mg.1000 $kcal^{-1}$)	163.8	65.9	68.0	70.3	66.5	121.0

* Because of rounding, percentages may not total 100

\# Only *cis* isomers are included

Table 3-2. Pooled mean values for total cholesterol, its precursors, FSR and ASR*

Variable	Concentration Range	Mean ± SD
Total Cholesterol		
$mmol.L^{-1}$	4.32-8.25	6.28 ± 0.84
Squalene		
$\mu mol.L^{-1}$	0.01-2.77	0.74 ± 0.58
$\mu mol.mmol^{-1\#}$	0.13-45.37	12.11 ± 10.05
Lanosterol		
$\mu mol.L^{-1}$	0.39-5.36	1.78 ± 1.08
$\mu mol.mmol^{-1\#}$	5.98-103.13	28.78 ± 19.36
Desmosterol		
$\mu\mu mol.L^{-1}$	0.41-6.09	2.47 ± 1.45
$\mu mol.mmol^{-1\#}$	7.18-110.74	40.08 ± 24.62
Lathosterol		
$\mu mol.L^{-1}$	6.12-32.53	13.63 ± 4.47
$\mu mol.mmol^{-1\#}$	81.52-625.93	220.82 ± 84.37
FSR		
$p.d^{-1}$	0.01-0.22	0.07 ± 0.03
ASR		
$g.d^{-1}$	0.06-1.71	0.61 ± 0.28

* Data was pooled across diets for all subjects (n=14)

Relative to concentration of total cholesterol

Table 3-3. Correlation among various cholesterol precursors expressed as absolute ($\mu mol.L^{-1}$) or relative concentrations ($\mu mol.mmol^{-1}$)

Cholesterol Precursor*	Correlation Coefficient ($r^{\#}$)	
	$\mu mol.L^{-1}$	$\mu mol.mmol^{-1\#\#}$
Squalene vs Lanosterol	0.92	0.93
Squalene vs Desmosterol	0.94	0.95
Squalene vs Lathosterol	0.90	0.90
Lanosterol vs Desmosterol	0.88	0.89
Lanosterol vs Lathosterol	0.91	0.93
Desmosterol vs Lathosterol	0.89	0.90

* Data was pooled across diets for all subjects (n=14)

\# $p<0.0001$

\#\# Relative to concentration of total cholesterol

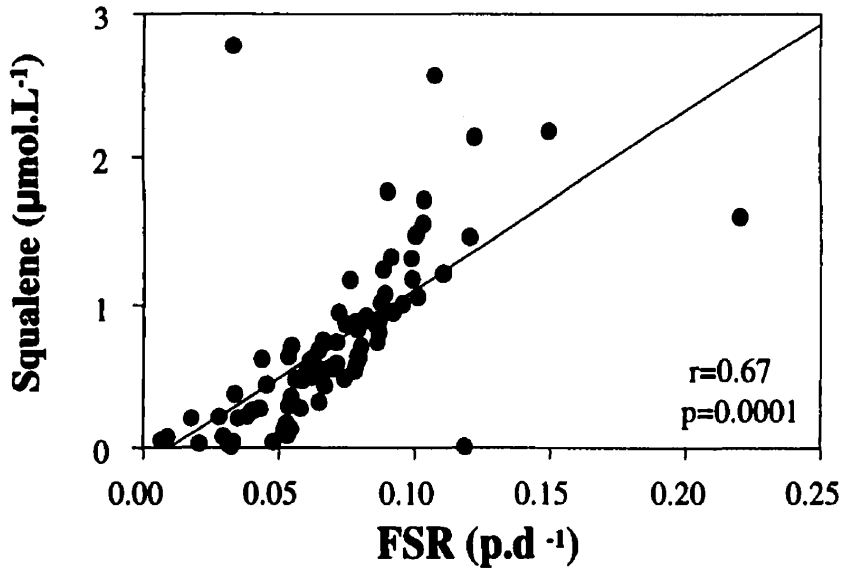
3.9 Figure Legends

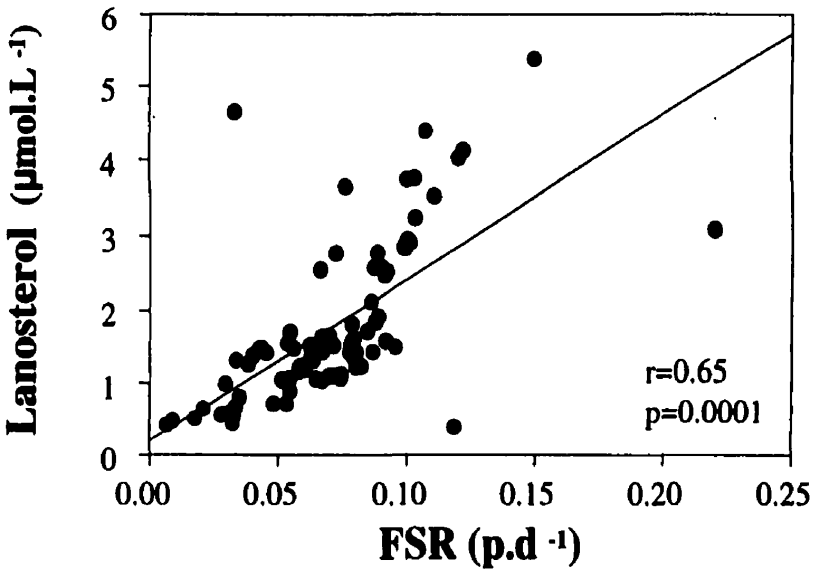
Figure 3-1: Correlation between FSR ($p.d^{-1}$) and concentrations of different precursors to cholesterol expressed as $\mu mol.L^{-1}$. Data were pooled across diets for each variable.

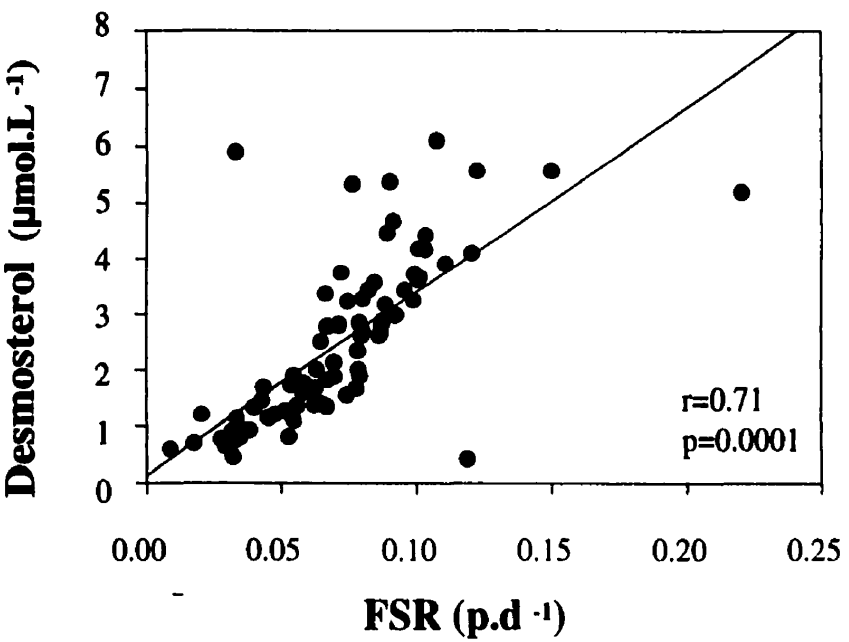
Figure 3-2: Correlation between FSR ($p.d^{-1}$) and concentrations of different precursors to cholesterol expressed as $\mu mol.mmol^{-1}$ of cholesterol (C). Data were pooled across diets for each variable.

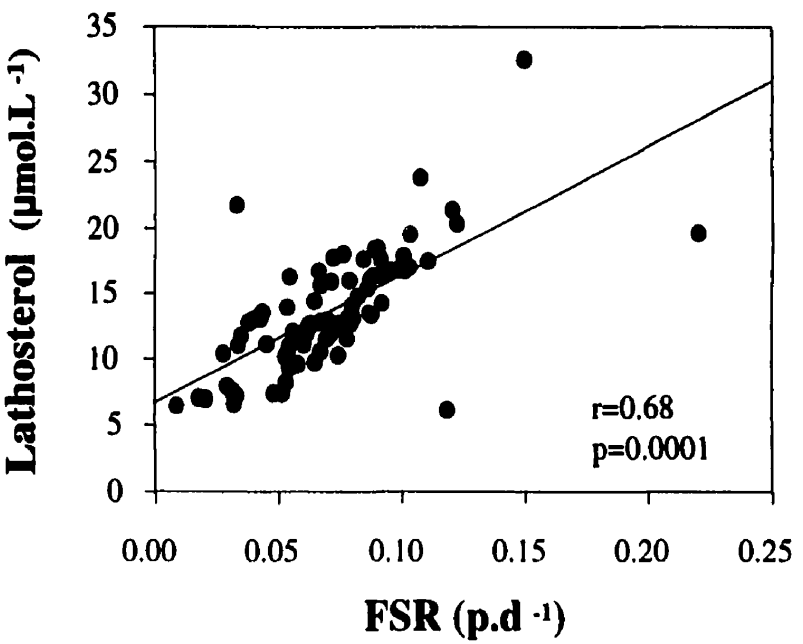
Figure 3-3: Correlation between ASR ($g.d^{-1}$) and concentrations of different precursors to cholesterol expressed as $\mu mol.L^{-1}$. Data were pooled across diets for each variable.

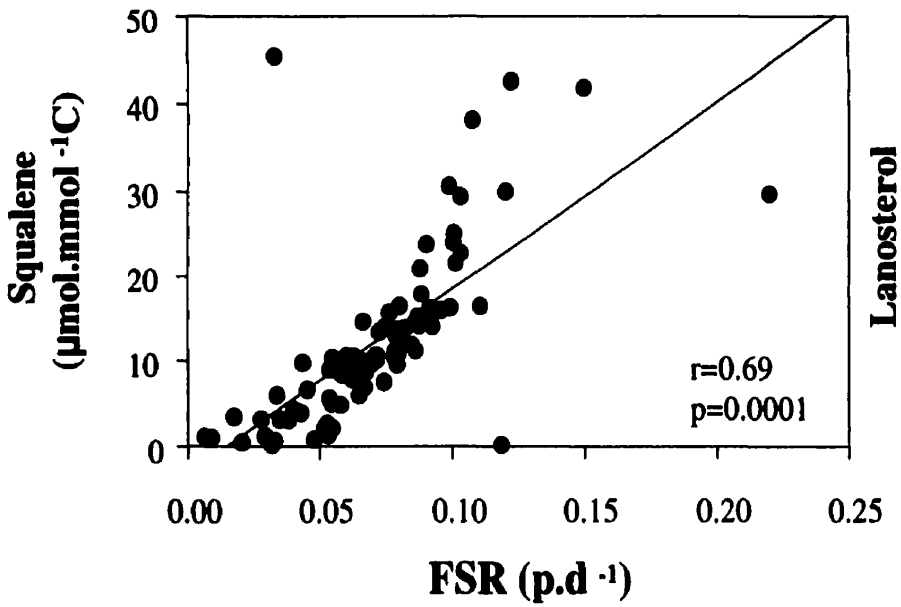
Figure 3-4: Correlation between ASR ($g.d^{-1}$) and concentrations of different precursors to cholesterol expressed $\mu mol.mmol^{-1}$ of cholesterol (C). Data were pooled across diets for each variable.

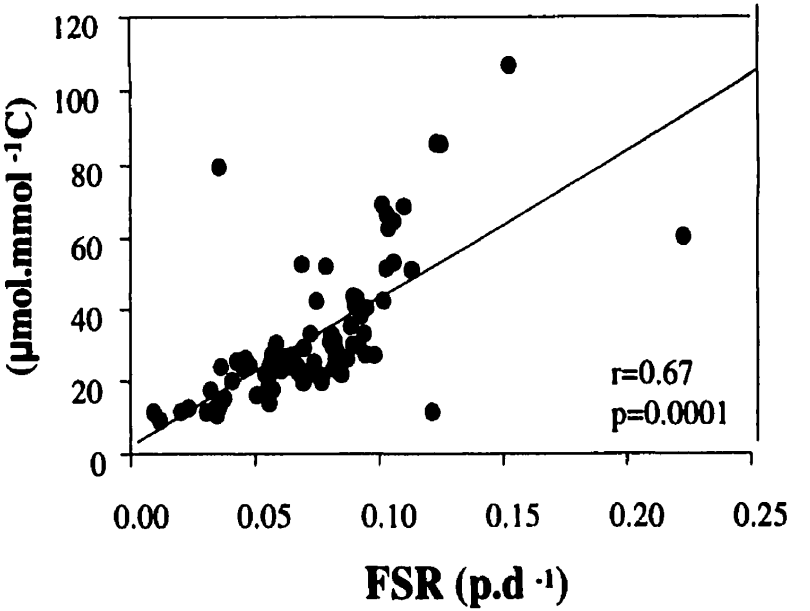

Squalene (µmol.L-1)
FSR (p.d -1)
r=0.67
p=0.0001

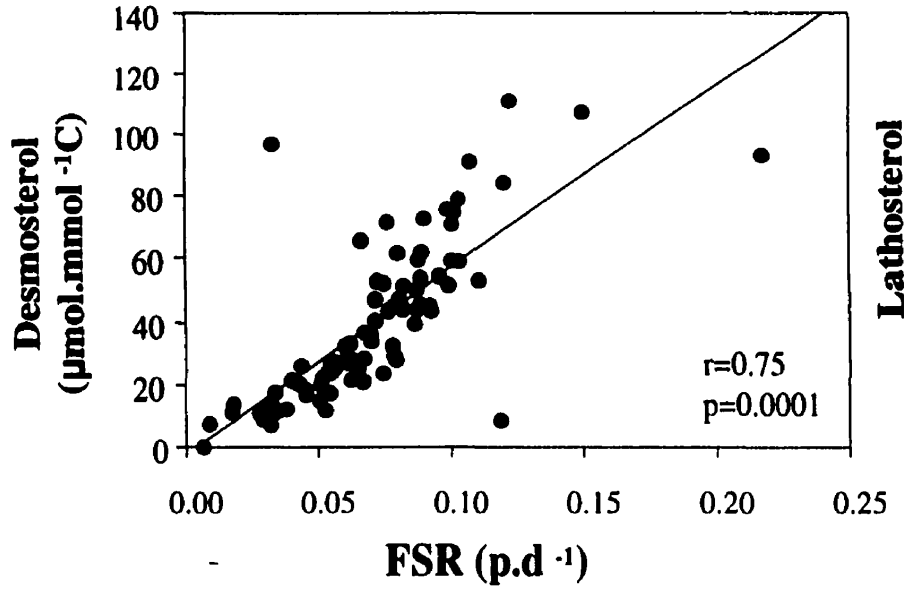

Lanosterol (µmol.L -1)
FSR (p.d -1)
r=0.65
p=0.0001

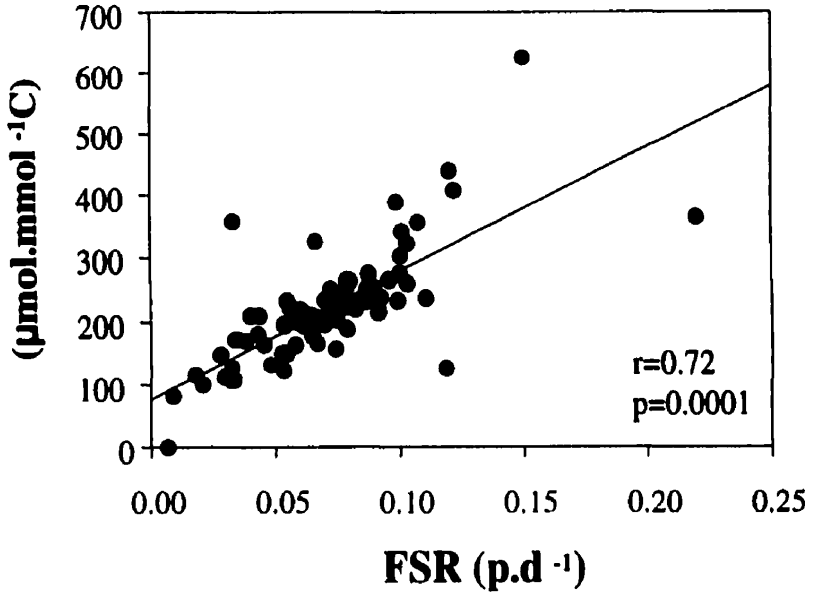

Desmosterol (µmol.L -1)
FSR (p.d -1)
r=0.71
p=0.0001


Lathosterol (µmol.L -1)
FSR (p.d -1)
r=0.68
p=0.0001


Squalene (µmol.mmol -1C)
50
40
30
20
10
0
0.00
0.05
0.10
0.15
0.20
0.25
FSR (p.d -1)
r=0.69
p=0.0001


Lanosterol (µmol.mmol -1C)
120
100
80
60
40
20
0
0.00
0.05
0.10
0.15
0.20
0.25
FSR (p.d -1)
r=0.67
p=0.0001


Desmosterol (µmol.mmol -1C)
140
120
100
80
60
40
20
0
0.00
0.05
0.10
0.15
0.20
0.25
FSR (p.d -1)
r=0.75
p=0.0001


Lathosterol (µmol.mmol -1C)
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600
500
400
300
200
100
0
0.00
0.05
0.10
0.15
0.20
0.25
FSR (p.d -1)
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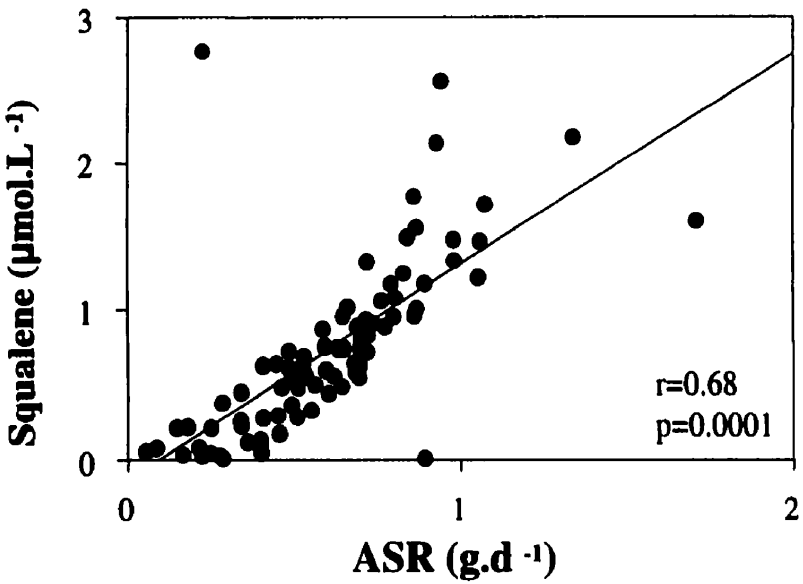
p=0.0001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ASR (g.d -1)
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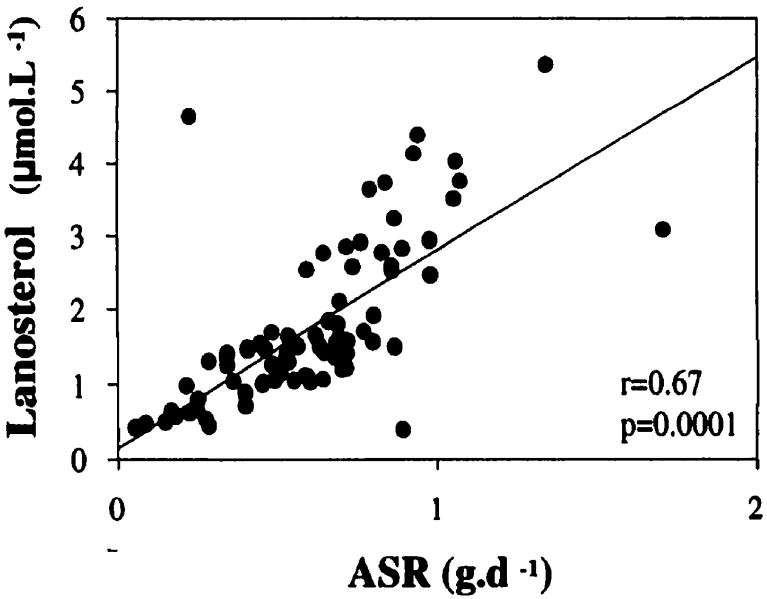
2
3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p=0.0001
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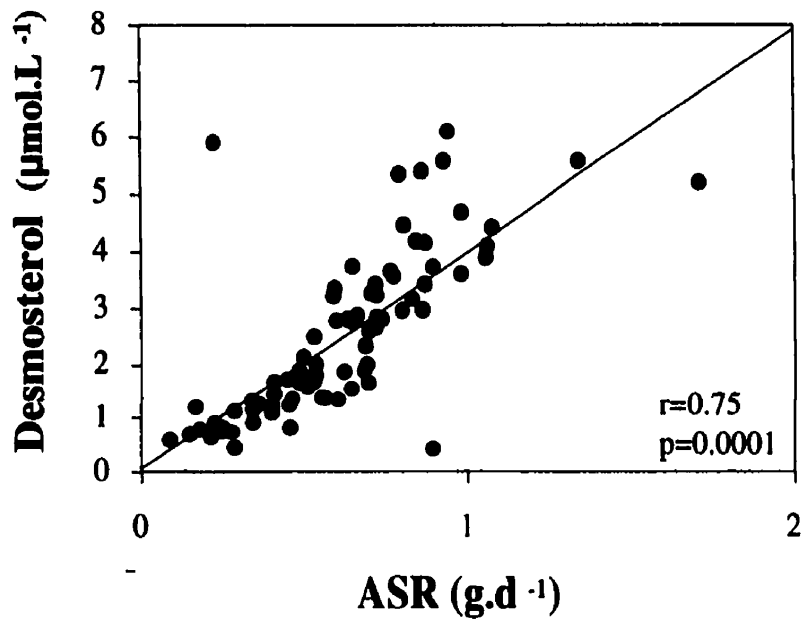
3
4
5
6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2
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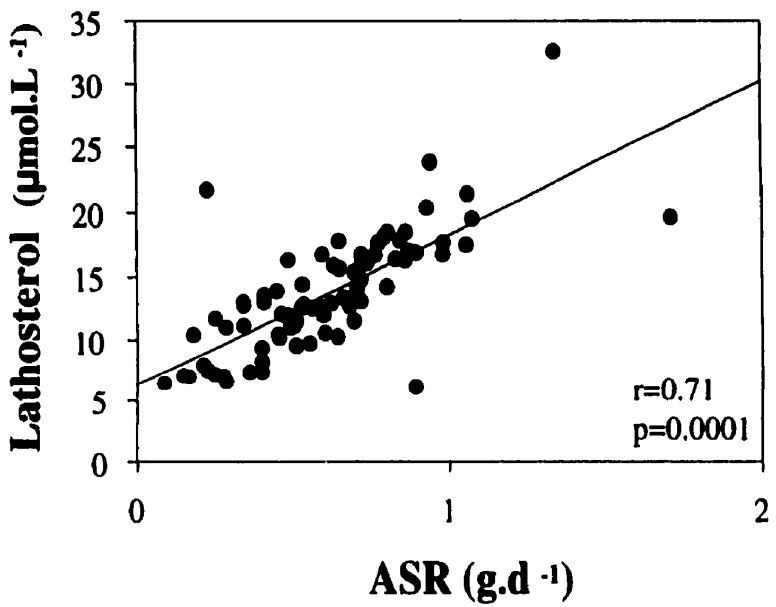
4
5
6
7
8


Lathosterol (µmol.L -1)
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p=0.0001
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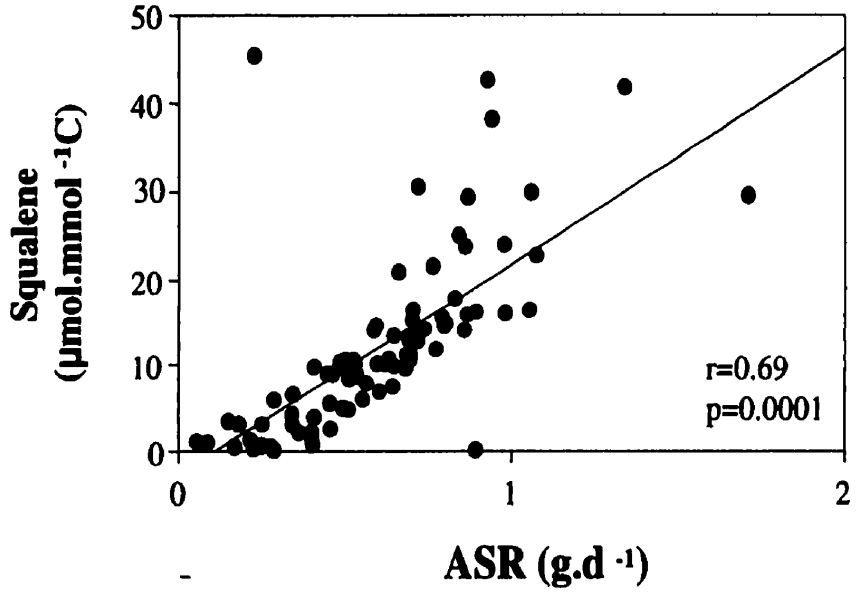
5
10
15
20
25
30
35


Squalene (µmol.mmol -1C)
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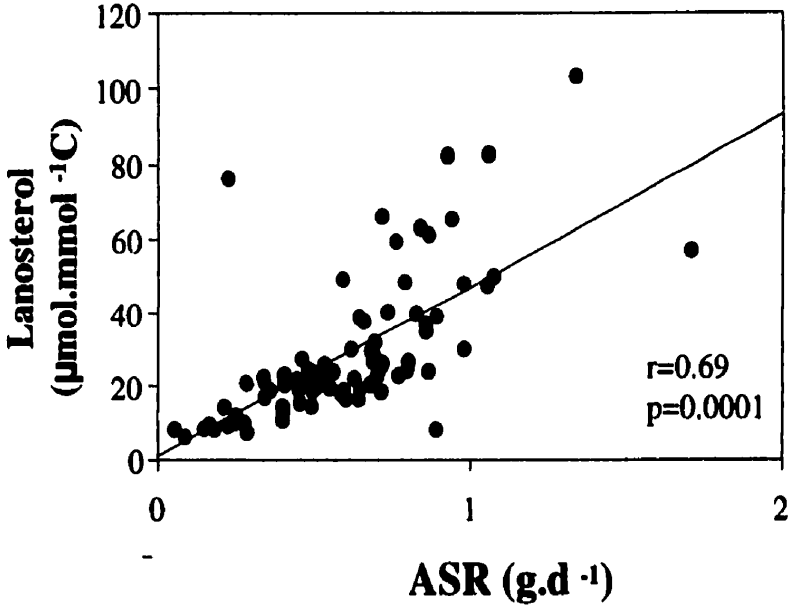
40
30
20
10
0
0
1
2
ASR (g.d -1)
r=0.69
p=0.0001


Lanosterol (µmol.mmol -1C)
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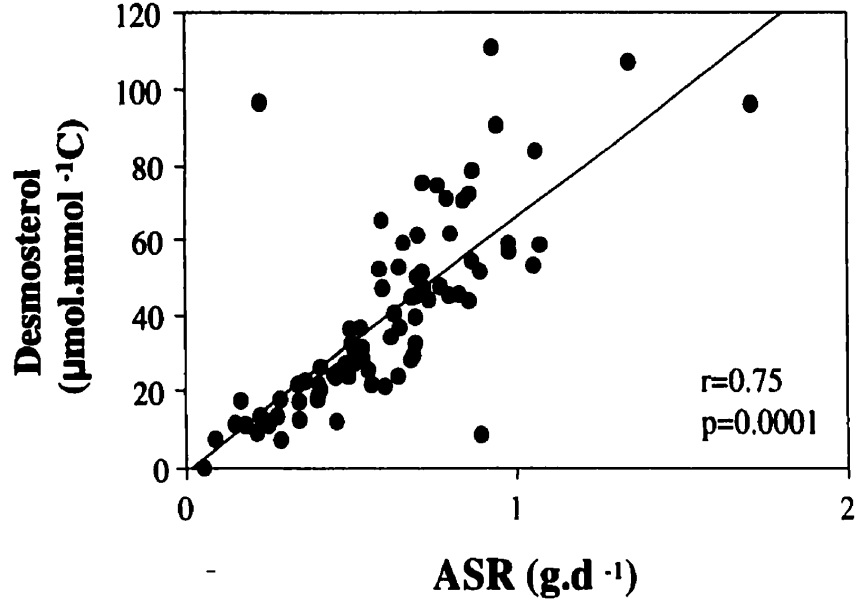
60
40
20
0
0
1
2
ASR (g.d -1)
r=0.69
p=0.0001


Desmosterol (µmol.mmol -1C)
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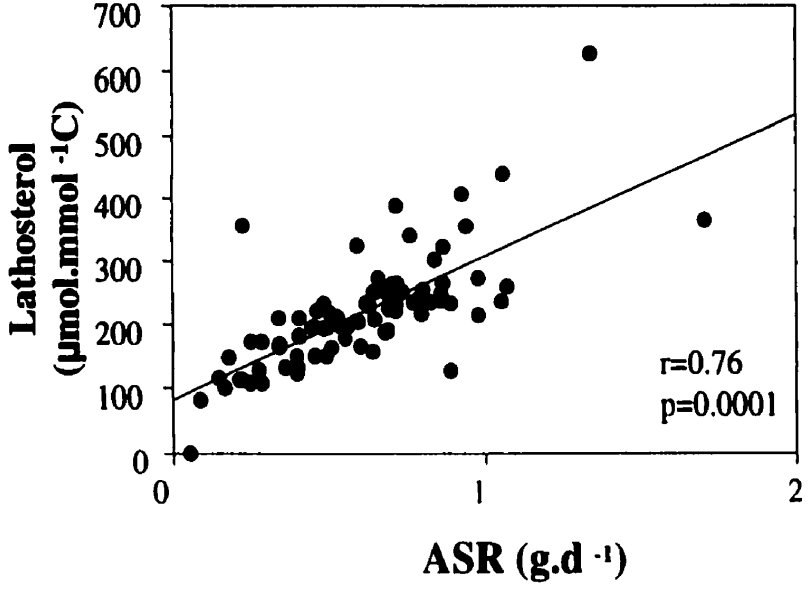
100
80
60
40
20
0
0
1
2
ASR (g.d -1)
r=0.75
p=0.0001


Lathosterol (µmol.mmol -1C)
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100
0
0
1
2
ASR (g.d -1)
r=0.76
p=0.0001

BRIDGE

Endogenous cholesterol synthesis contributes substantially to whole body input, and has been shown to be influenced by dietary fatty acid composition. However, its regulation has been difficult to assess due to methodological limitations. Newer techniques such as the deuterium incorporation (DI) method offer advantages over existing methods, but the appropriateness for application under different dietary conditions has not been validated against the commonly used cholesterol precursor assessment method.

Our results from manuscript 1 indicate that plasma cholesterol precursor (squalene, lanosterol, desmosterol and lathosterol) concentrations, as well as the D uptake method are suitable for measurement of short-term cholesterol synthesis rates in humans. In addition, the DI method offers the advantage of being a direct measure of net synthesis compared to cholesterol precursor levels, which are relative indices of synthesis.

Having demonstrated that our choice of methodology is technically sound and applicable under different dietary situations, our next objective was to determine the effect of hydrogenated fat consumption on endogenous cholesterol synthesis rates. Although evidence suggests that *trans* fatty acids from hydrogenated fats increase plasma cholesterol concentrations and subsequent risk of CVD, it has not yet been defined if the alterations in circulating cholesterol levels are due to perturbations in rates of endogenous cholesterol synthesis.

CHAPTER 4

HYDROGENATED FAT CONSUMPTION AFFECTS CHOLESTEROL SYNTHESIS IN MODERATELY HYPECHOLESTEROLEMIC WOMEN

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4.1 Abstract

To determine mechanisms by which hydrogenated fat influences plasma lipid levels, 14 women (65-71 yrs with LDL-C $\geq$ 130 mg.dl^{-1}) consumed for 5 week periods each a baseline (BL) diet (39% kcal fat, 164 mg chol.1000 kcal^{-1}) and reduced fat diets (30% kcal) where 2/3rd of the fat was either soybean oil (SO), low *trans* squeeze (SQM), medium *trans* tub (TM), or high *trans* stick (SM) margarines, or butter (BT). Plasma lipid levels were analyzed at the end of each phase. Fractional synthetic rates (FSR) in pools/day (p.d^{-1}) and absolute synthetic rates (ASR) in grams/day (g.d^{-1}) of free cholesterol (FC) were measured using the deuterium incorporation methodology. Plasma total ($p<0.01$) and low density lipoprotein ($p<0.05$) cholesterol levels increased with increasing degree of hydrogenation or saturated fat intake. High density lipoprotein cholesterol levels ($p<0.05$) were lowest on the SM diet when compared to the BT diet. Low *trans* SQM (0.081 $\pm$ 0.019 p.d^{-1}) and medium *trans* TM (0.086 $\pm$ 0.029 p.d^{-1}) diets elicited similar responses as the SO (0.078 $\pm$ 0.024 p.d^{-1}) diet, whereas high *trans* SM (0.053 $\pm$ 0.029 p.d^{-1}) diet mimicked the BT (0.062 $\pm$ 0.017 p.d^{-1}) and high fat BL (0.053 $\pm$ 0.023 p.d^{-1}) diet in its suppression ($p<0.05$) of FSR-FC. ASR-FC, which is an approximation of the daily production of newly synthesized cholesterol, showed a similar trend as the FSR-FC data. These results indicate that reduced synthesis is not responsible for the higher plasma TC levels seen with consumption of the SM, BT and BL diets, and suggest that another mechanism, possibly impairment of the catabolic pathway of cholesterol is involved.

4.2 Introduction

Trans fatty acids are geometrical isomers of the naturally occurring *cis* fatty acids. They are produced as a result of biohydrogenation in the ruminant fat of animals or by commercial hydrogenation of vegetable oils (1). Majority of dietary *trans* fatty acids in Western diets is contributed from commercial hydrogenation. Hence it is not surprising that *trans* fatty acid intake mirrors consumption of margarines, baked products and commercially prepared foods, which has steadily increased since the beginning of this century (2). Their consumption has come under scrutiny in view of recent studies indicating adverse health effects with *trans* fatty acids relative to their *cis* counterparts (3-5) or unhydrogenated oils (6-10). These effects include elevated total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C) and triglycerides (TG), as well as decreased high density lipoprotein cholesterol (HDL-C) levels (3-10). This is of concern because dietary counseling aimed at lowering plasma lipid levels usually encourages substitution of vegetable oils and its products such as margarines and shortenings for those of animal origin (11). In addition, over the past few years there has been a proliferation of new products on the market that vary in their degree of hydrogenation. These include softer margarines in semi-liquid (squeeze) or soft (tub) forms intended to substitute for hard margarines in stick form. Whether the consumption of these products has a similar or less dramatic effect relative to butter on measures of CVD risk was recently evaluated (12). Findings revealed that consumption of products low in *trans* fatty acids like soybean oil, squeeze and tub margarines had a beneficial effect on the serum lipid profile compared to diets enriched in stick margarine, shortening (hydrogenated soybean oil) or butter. However the

mechanisms responsible for the adverse effects on circulating lipid levels seen after consumption of the latter diets remain to be elucidated.

One possible mechanism involves alterations in endogenous cholesterol synthesis. In humans biosynthesis of cholesterol accounts for 60 to 80% of whole body cholesterol input (13,14), and has been shown to be influenced by dietary fatty acid composition. Saturated fats tend to suppress, whereas polyunsaturated fats tend to increase cholesterol synthesis rates (15-17). Cuchel and colleagues (18) have shown that feeding subjects corn oil margarine in stick form as compared to a corn oil diet, increased plasma lipid and lipoprotein levels in spite of a trend toward decreased *de-novo* cholesterol synthesis rates. However, the issue remained unresolved since this declining trend in fractional synthetic rate of cholesterol (FSR-FC) did not reach statistical significance, possibly due to sample size limitations and high variability in synthesis among the male and female subjects. With the exception of the above study, the effect of hydrogenation, specifically degree of hydrogenation on cholesterogenesis remains unknown. Thus, the present study was designed to evaluate the impact of consumption of commonly available sources of dietary fats subjected to different degrees of hydrogenation on endogenous synthesis rate of free cholesterol (FC) using the deuterium incorporation (DI) methodology.

4.3 Methods

4.3.1 Subjects

Fourteen postmenopausal middle aged to elderly women (65-71 yrs) were selected to participate in this study from an original study population of 18 female subjects as previously described (12). Subjects had LDL-C levels ≥130 mg/dl, were free from

chronic illness and were not taking any medication known to affect lipid metabolism (lipid lowering drugs, β-blockers, diuretics or hormones). Subjects who smoked or consumed ≥2 alcoholic drinks per day were also excluded from the study. The protocol was reviewed and approved by the human-investigation review committee of New England Medical Center and Tufts University. All potential subjects were given a verbal and written description of the study prior to obtaining consent. However, during the study period, subjects were blinded to the dietary phase assignments. A portion of the data focusing on plasma lipid and lipoprotein parameters has been published previously (12).

4.3.2 Experimental Design and Diets

Subjects consumed each of six diets according to a randomized cross-over design. Each dietary phase had a duration of 5 weeks and was separated by a washout period ranging from 2 to 4 weeks, during which subjects consumed their habitual diets. All diets were isocaloric. The soybean oil diet was formulated to meet NCEP 2 dietary guidelines, providing 15% energy as protein, 55% as carbohydrate and 30% as fat (≤ 7% SFA, 10-15% MUFA and ≤ 10% PUFA) and less than 85 mg cholesterol per 1000 kcal. The soybean oil component comprised 20% of energy. In subsequent diets, the soybean oil was replaced by the experimental fat, so that the effect of consuming diets enriched in hydrogenated fats could be assessed within the general context of current recommendations for individuals with elevated plasma lipid levels. The specific fats investigated were soybean oil (SO) and soybean oil based margarines in the squeeze (SQM), tub (TM) and stick (SM) form as well as butter (BT). These fats were especially chosen because as a group they represent a broad range of *trans* and fatty acid profiles. The baseline diet was included in order to access the biochemical parameters proposed

for each individual on a defined diet with a fatty acid composition similar to that currently consumed in North America to best characterize the study population. All food and drink were provided by the Metabolic Research Unit of the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University to be consumed on site or packaged for take-out. Energy intakes of the subjects were tailored to individual requirements, as verified by the ability to maintain stable body weight. Analysis of the macronutrient, fatty acid and cholesterol contents of the diets were carried out by Covance Laboratories (Madison, WI) and Best Foods Research and Engineering Center (Union, NJ).

4.3.3 Protocol and Analyses

During week 5 of each dietary phase a fasting blood sample was drawn followed by administration of a bolus oral dose of deuterium oxide (1.2g D_2O per kg total body water). Another fasting blood sample was taken 24 hours after dosing. Blood samples were centrifuged at 3000 rpm at 4°C and plasma was separated, aliquoted and frozen at -80 °C until further analysis. VLDL was isolated from plasma by ultracentrifugation at 39,000 rpm for 18 hours at 4 °C (19). Serum and the 1.006 $g.ml^{-1}$ infranatant fraction were assayed for TC and TG with an Abbott Diagnostic (North Chicago, IL) spectrum CCX biochromatic analyzer using enzymatic reagents as previously described (20). HDL-C levels were measured after precipitation of the apolipoprotein B containing lipoproteins by a dextran-magnesium sulphate procedure (21). Lipid assays were standardized through the Lipid Standardization Program at the Center for Disease Control (Atlanta, GA).

Additional plasma aliquots were used to determine deuterium (D) enrichment in body water and FC as previously described (12,22). Briefly, lipids were extracted (23) and the FC band was separated by thin layer chromatography using petroleum ether/ ethyl ether/ acetic acid (135:15:1.5 v/v/v). FC was eluted with hexane/ chloroform/ ethyl ether (5:2:1 v/v/v) and dried under a stream of nitrogen. The purified cholesterol was converted into water and carbon dioxide by combustion over cupric oxide and silver wire at 520°C for 2 hours. In addition, pre and post D_2O samples diluted 2-fold and 10-fold respectively to produce D enrichments within detectable ranges on the mass spectrometer were distilled into zinc containing tubes. This enabled measurement of D enrichment of plasma water. The combustion water from FC and plasma were then vacuum distilled into pyrex™ tubes containing zinc reagent, and reduced to hydrogen deuterium gas by heating at 520°C for 30 minutes. D enrichment of the gas was analyzed by isotope ratio mass spectrometry (VG isomass 903 D, Cheshire, England). The instrument was calibrated daily using water standards of known isotopic composition. Values were expressed relative to the enrichment of standard mean ocean water (SMOW) in parts per mil. The per mil designation was used because of the relatively small enrichments encountered. Duplicate samples for each subject were analyzed concurrently against a single set of standards.

4.3.4 Calculation of Cholesterol Synthesis Rates

FSR-FC defined as the proportion of the central or M_1 pool replaced daily by newly synthesized cholesterol, was calculated as the change in product enrichment over time divided by the maximum possible enrichment, based on a linear rate of uptake of label into cholesterol over time (12). The equation used was:

$$\text{FSR-FC (pools/day)} = \frac{\delta \text{ cholesterol (\%)}}{\delta \text{ plasma water (\%)} \times 0.478}$$

where δ is the difference in D enrichment over 24 hours. Model parameters and assumptions underlying use of D_2O as tracer for FSR measurements have been described previously (13,22). ASR-FC which is an approximation of the daily production of newly synthesized cholesterol expressed in grams/day ($g.d^{-1}$) was derived by multiplying the FSR-FC by M_1 pool size and a factor of 0.33. The M_1 pool size was calculated using Goodman's equation (24) which takes into account the subjects body weight, plasma TC and TG concentrations. The factor of 0.33 was included to account for the proportion of FC in the overall plasma TC pool.

4.4 Statistical Analysis

One way analyses of variance with the main effect of diet and subject as the repeated measure was employed on each outcome variable using a SAS general linear model program (SAS version 6, SAS Institute Inc, Cary, NC). Group means were separated by Tukey's test at a significance level of $p<0.05$. Since the TG, VLDL-C, FSR-FC and ASR-FC data were not normally distributed, logarithmic transformations were performed prior to statistical testing. Untransformed data are presented in text and tables as means ± standard deviation (SD).

4.5 Results

Baseline characteristics at the time of screening are shown in Table 4-1. By design, all subjects had LDL-C concentrations ≥ 130 $mg.dl^{-1}$ indicating that they were in the borderline high risk or high-risk categories for CVD as defined by the Adult Treatment

Panel (25). Mean (± SD) TC, LDL-C, HDL-C, VLDL-C and TG concentrations were 258 ± 31, 172 ± 30, 54 ± 12, 31 ± 13 and 158 ± 75 $mg.dl^{-1}$ respectively. The average body mass index (BMI) was 26.5 ± 2.4 $kg.m^{-2}$, which is characteristic of a group of moderately hypercholesterolemic middle aged and elderly women.

The composition of the experimental diets based on chemical analyses is shown in Table 4-2. Excluding the BL diet, all other reduced fat diets had similar protein, fat and carbohydrate contents. Dietary *trans* fatty acid levels ranged from 0.6 to 6.7 % of total energy. This was accompanied by lower PUFA levels, which are consistent with effects of the hydrogenation process. The cholesterol content of the butter diet was approximately twice that of the other diets. The option to compensate for this endogenous cholesterol content was not proposed because cholesterol is an inseparable component of butter and it would be artificial to assess the impact of the fatty acid composition of the product on various physiological parameters without including the cholesterol.

Mean (± SD) plasma lipid and lipoprotein concentrations during the 6 dietary phases are shown in Table 4-3. Plasma TC levels were elevated with consumption of the BL, BT and SM diets when compared to the SO, SQM and TM diets ($p<0.05$ for BT vs SO, SQM, TM; BL vs SO, SQM; and SM vs SO). The LDL-C levels followed a similar pattern, with consumption of the BT, BL and SM diets resulting in higher ($p<0.05$) levels relative to the SO diet. Switching from the BL to all five reduced fat diets resulting in a significant ($p<0.05$) lowering of HDL-C. Among the reduced fat diets, plasma HDL-C concentrations were lowest on the SM diet (45 ± 9 $mg.dl^{-1}$) and highest on the BT diet

(50 ± 10 mg.dl^{-1}). A trend toward higher plasma VLDL-C and TG levels was observed with SM feeding, but values did not reach statistical significance

With respect to the cholesterol kinetic data, FSR-FC rates (Figure 4-1) were higher ($p<0.05$) on the SO (0.078 ± 0.024 p.d^{-1}), SQM (0.081 ± 0.019 p.d^{-1}) and TM (0.086 ± 0.029 p.d^{-1}) diets when compared to the SM (0.053 ± 0.029 p.d^{-1}) and BL (0.053 ± 0.023 p.d^{-1}) diets. These rates represent the proportion of the rapidly turning over pool of cholesterol synthesized per day. In order to 4-2) rates were calculated. The ASR-FC data mimicked the FSR-FC data with rates being lower ($p<0.05$) on the low fat high *trans* SM (0.450 ± 0.258 g.d^{-1}) and high fat BL (0.458 ± 0.195 g.d^{-1}) diets when compared to the SO and other two margarine diets (0.661 ± 0.234, 0.689 ± 0.192 and 0.739 ± 0.273 g.d^{-1} respectively). The BT diet also resulted in lower FSR-FC (0.062 ± 0.017 p.d^{-1}) and ASR-FC (0.557 ± 0.193 g.d^{-1}) rates, but differences were significant only when compared to the TM (0.086 ± 0.029 p.d^{-1} and 0.739 ± 0.273 g.d^{-1} respectively) diet.

4.6 Discussion

Considerable interest has been focussed on the mechanism by which dietary fatty acids influence plasma TC and LDL-C concentrations because elevated levels are associated with a greater risk of developing coronary heart disease (26,27). One specific class of dietary fatty acids that has received growing attention is *trans* fatty acids. These fatty acids are known to cause changes in plasma lipid and lipoprotein cholesterol profiles (3-10,12), but the mechanisms involved are unclear. Thus the present study was conducted

to determine whether endogenous cholesterogenesis changes in response to shifts in dietary *trans* and fatty acid profile, and consequently influences circulating lipid levels.

Using the DI method to measure *de-novo* cholesterol synthesis, we have demonstrated for the first time that FSR-FC and ASR-FC rates were lower with stick margarine and high fat BL feeding when compared to the oil and softer margarine diets. The BT diet was intermediate in its effects on cholesterol synthesis relative to the other diets. However, the reverse was seen with regard to the plasma lipid and lipoprotein data, with consumption of the former diets producing the most favorable profile and the latter diets producing the least desirable profile. Since circulating cholesterol levels reflect changes in synthesis and clearance, it can be speculated that the enhanced biosynthesis seen with SO, SQM and TM feeding was compensated by an even greater rate of clearance, which could, in part, explain the lower lipid levels seen after consumption of these diets. This hypothesis is consistent with the findings of increased net fecal neutral and acidic sterol excretion seen with corn oil feeding compared to stick margarine or beef tallow (28). Conversely, despite marked suppression of synthesis, the higher plasma TC and LDL-C levels seen on the SM, BT and BL diets suggest some impairment in the catabolic pathway of cholesterol. One mechanism (29) proposed involves interference with LDL receptor mediated clearance of cholesterol rich lipoproteins. Spady and Dietschy (30), using a hamster model, have demonstrated that dietary saturated fatty acids (SFA) affect LDL cholesterol levels through changes in the activity of the hepatic LDL receptor. The liver enzyme- acyl-CoA: cholesterol acyltransferase (ACAT) that converts free cholesterol to its esterified form has a lower affinity for saturated than unsaturated fatty acids. In fact, Woollett and colleagues (31) have demonstrated in animals that the

selectivity of ACAT for unsaturated fatty acids is lost when the double bond is in the *trans* configuration (specifically *t*-C18:1n-9). The resulting accumulation of hepatic free cholesterol could lead to down regulation of the LDL receptor, causing accumulation of LDL particles in plasma and increased formation of LDL from its precursor-VLDL. An alternate mechanism, supported by *in vitro* studies (32-34) involves enrichment of cell membrane phospholipids with *trans* and saturated fatty acids. The resultant alterations in membrane fluidity could reduce binding or internalization of circulating LDL, thus accounting for the higher plasma cholesterol levels despite lowered synthesis.

These findings suggest that *trans* and SFA rich diets elevate TC and LDL-C levels by similar mechanisms. However, among the reduced fat diets, the BT diet, despite its higher SFA and cholesterol content, produced a similar reduction in endogenous cholesterol synthesis when compared to the SM diet. This finding is not readily explainable but could relate to the fatty acid profile of the BT diet. It has been reported (35) that consumption of diets high in 16:0 but low in linoleic acid (18:2 n-6), which is similar to the fatty acid profile of the BT diet, does not alter endogenous cholesterol synthesis rates relative to either a low 16:0/ high 18:2 n-6 diet or a high 16:0/high 18:2 n-6 diet. Thus, the high 16:0 content of the BT diet could attenuate the 12:0 + 14:0, and exogenous cholesterol induced reduction in synthesis, and account for the observed modest but not marked suppression of FSR-FC and ASR-FC rates.

A limitation of the present study is the difficulty in partitioning out effects of *trans* from other fatty acids in the experimental diets. However, our study does allow assessment of the altered fatty acid profile resulting from hydrogenation, and consequently the impact

of consuming various hydrogenated products. Secondly, the validity of the DI method has been questioned because there is no physiological basis for a model based on linear regression (36). While this may be true, the initial, short term deuterium-oxide incorporation rate is linear (13,22), unaffected by flux rates of other unlabeled substances into the system and thus can be taken to represent a direct measure of synthesis independent of total whole body production rate. In addition, the DI method has been compared and validated against the classic sterol balance technique (28). More recently, Di Buono et al (37), measured fractional and absolute synthesis rates of cholesterol simultaneously by the DI and mass isotopomer distribution analysis (MIDA) methods. The authors concluded that both techniques yield comparable rates of cholesterogenesis in humans when measurements are made over 24 hours. Consequently, we feel that this method offers a safe, reliable and non-invasive tool for accurate assessment of human cholesterol biosynthesis.

In summary, cholesterol synthesis rates were lower with consumption of the high *trans* SM and high fat BL diets when compared to the SO, SQM and TM diets, with rates after subjects consumed the BT diet being intermediate. However, plasma lipid and lipoprotein concentrations were higher on the former than on the latter diets. This clearly demonstrates that the elevations in circulating levels of cholesterol are not due to a rise in cholesterol synthesis, thereby supporting the hypothesis of an impairment in the catabolic pathway of cholesterol rich lipoproteins. While these results support present dietary guidelines to reduce the SFA content of diets they also suggest the need to specify optimal ratios of individual fatty acids classes including *trans* fatty acid contributions from hydrogenated fats.

4.7 Acknowledgements

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Table 4-1. Baseline Characteristics of Study Subjects (n = 14)

Characteristic	Mean ± SD
Age (yrs)	68.1 ± 2.5
Body Mass Index ($kg.m^{-2}$)	26.5 ± 2.4
Serum Cholesterol ($mg.dl^{-1}$) *	
Total	258.3 ± 30.8
LDL-C	171.5 ± 29.8
HDL-C	53.9 ± 12.2
VLDL-C	30.9 ± 13.2
Serum Triglyceride ($mg.dl^{-1}$) #	158.1 ± 75.2

*To convert values for cholesterol to millimoles per liter, divide by 38.67

#To convert values for triglycerides to millimoles per liter, divide by 88.54

Table 4-2. Composition of Test Diets as Determined by Chemical Analysis*

Constituent	Baseline (BL)	Soybean oil (SO)	Squeeze Margarine (SQM)	Tub Margarine (TM)	Stick Margarine (SM)	Butter (BT)
	percentage of total daily energy intake					
Protein	16.8	15.7	17.1	16.3	16.7	16.9
Carbohydrate	44.6	55.8	51.7	52.9	53.5	53.9
Fat	38.6	28.5	31.2	30.8	29.7	29.1
Saturated fatty acids	15.5	7.3	8.6	8.4	8.5	16.7
12:0	1.8	0.8	1.0	0.7	0.8	1.4
14:0	0.1	0.6	0.7	0.6	0.6	2.5
16:0	7.8	3.7	4.3	4.2	4.0	7.5
18:0	3.6	1.5	1.9	2.3	2.2	3.6
Monounsaturated fatty acids#	15.1	8.1	8.1	8.0	8.5	8.1
18:1	12.2	7.2	7.1	6.7	6.5	7.0
Polyunsaturated fatty acids#	7.0	12.5	13.5	11.1	6.3	2.4
18:2 (n-6)	5.9	10.7	12.1	10.0	5.6	2.1
18:3 (n-3)	1.0	1.7	1.4	1.1	0.7	0.3
Trans fatty acids	1.7	0.6	0.9	3.3	6.7	1.3
Cholesterol (mg.1000 $kcal^{-1}$)	163.8	65.9	68.0	70.3	66.5	121.0

* Because of rounding, percentages may not total 100

\# Only *cis* isomers are included

Table 4-3 : Serum Lipid and Lipoprotein Profile at the End of Each Phase (n=14)

Variable	Baseline (BL)	Soybean Oil (SO)	Squeeze Margarine (SQM)	Tub Margarine (TM)	Stick Margarine (SM)	Butter (BT)
$mg.dl^{-1}$			mean ± SD			
TC	252.2±31.9^{ab}	229.5±28.3^{d}	232.8±28.1^{cd}	239.9±29.9^{bcd}	247.3±33.5^{abc}	255.9±33.3^{a}
LDL-C	175.5±30.8^{a}	156.5±32.4^{b}	157.5±22.4^{bc}	165.2±29.3^{ab}	171.9±30.3^{ac}	176.8±28.7^{a}
HDL-C	51.7±12.0^{a}	46.5± 9.2^{bc}	46.5±10.8^{bc}	47.2± 9.7^{bc}	45.3± 9.4^{c}	50.2± 9.6^{ab}
VLDL-C	24.1±7.2	27.1±11.2	26.8±7.9	26.6± 7.5	29.6± 13.5	28.8±11.1
TG	131.9±44.6	136.9±52.8	131.2±53.5	140.5±55.8	154.5±64.2	139.1±45.6

Within a row, values with different superscripts are significantly different ($p<0.05$)

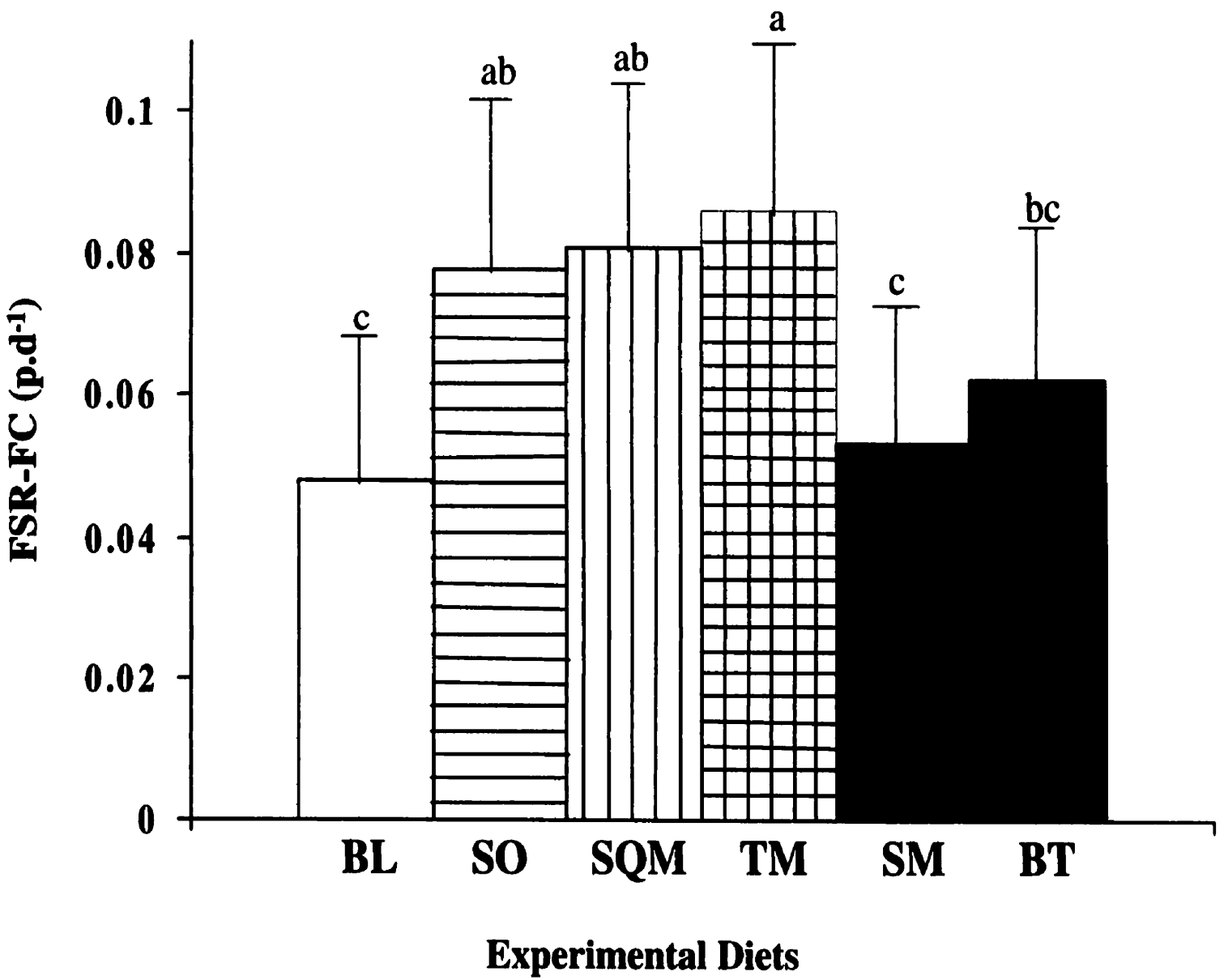
To convert values for cholesterol to millimoles per liter, divide by 38.67

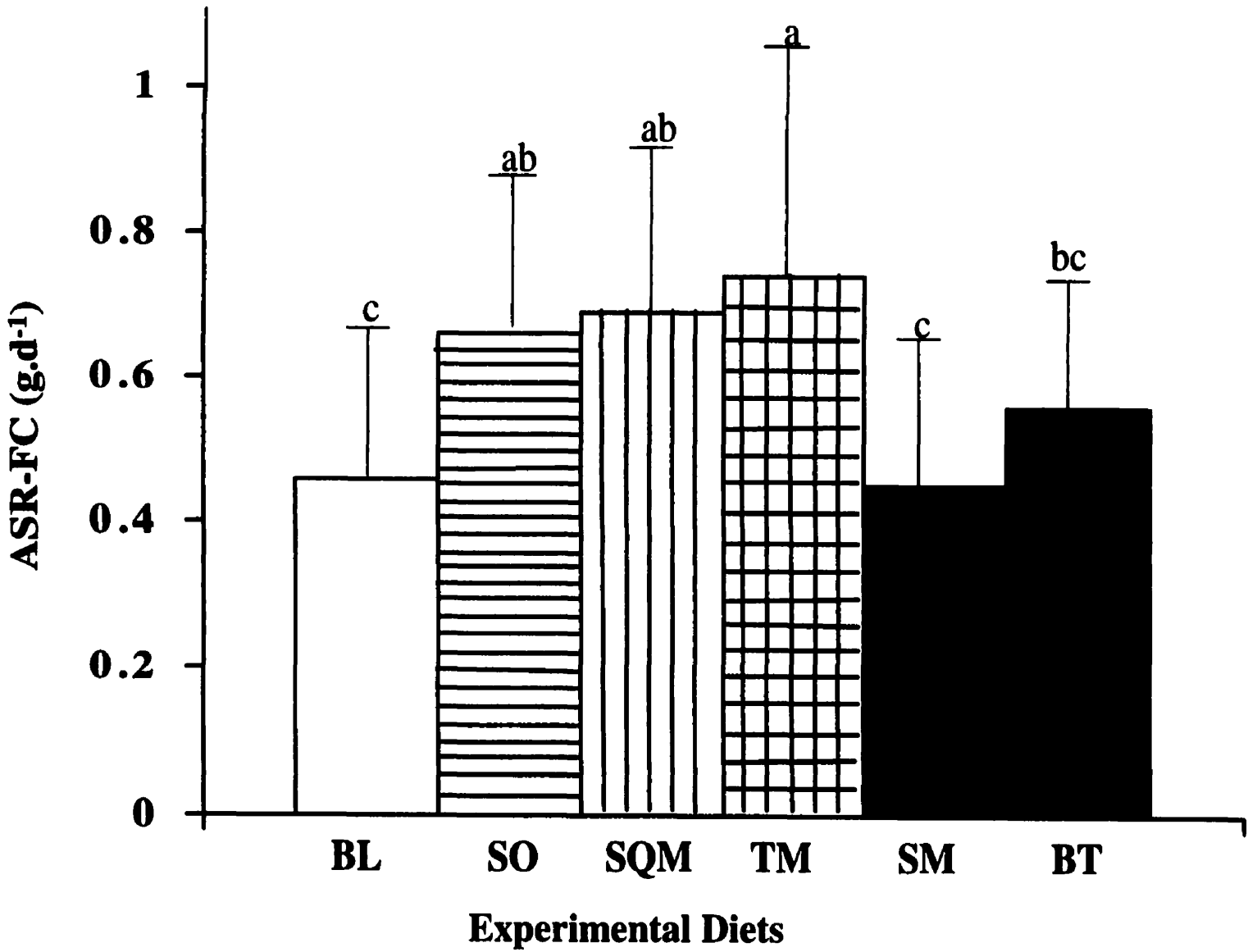
To convert values for triglycerides to millimoles per liter, divide by 88.54

4.9 Figure Legends

Figure 4-1: Fractional synthesis rates of free cholesterol (FSR-FC) at the end of each dietary phase. Data are presented as means ± SD, n =14. Diet abbreviations are as follows; BL: baseline, SO: soybean oil, SQM: squeeze margarine, TM: tub margarine, SM: stick margarine, BT: butter. Columns with different letters are significantly different ($p<0.05$).

Figure 4-2: Absolute synthesis rates of free cholesterol (ASR-FC) at the end of each dietary phase. Data are presented as means ± SD, n =14. Diet abbreviations are as follows; BL: baseline, SO: soybean oil, SQM: squeeze margarine, TM: tub margarine, SM: stick margarine, BT: butter. Columns with different letters are significantly different ($p<0.05$).


FSR-FC (p.d-1)
0.1
0.08
0.06
0.04
0.02
0
c
ab
ab
a
c
bc
BL
SO
SQM
TM
SM
BT
Experimental Diets


ASR-FC (g.d-1)
1
0.8
0.6
0.4
0.2
0
c
ab
ab
a
c
bc
BL
SO
SQM
TM
SM
BT
Experimental Diets

BRIDGE

Results from manuscript 2 indicate that cholesterol synthesis rates were higher after consumption of diets enriched with SO, SQM and TM, compared to diets containing the high *trans* SM and elevated fat BL diets, with rates being intermediate after consumption of the diet enriched in BT. Reciprocally, plasma lipid concentrations were higher on the latter than on the former diets. This suggests that an impairment in the catabolic pathway of cholesterol rich lipoproteins, rather than a rise in cholesterol synthesis are responsible for the elevations in circulating cholesterol (TC, LDL-C and VLDL-C) concentrations.

However, this explanation does not account for the lowering of HDL-C levels, a key feature that distinguishes *trans* from saturated fatty acids. The majority of cholesterol esterification in plasma occurs during the maturation and transformation of HDL particles, and is proportional to the activity of the esterification enzyme LCAT. *In vitro* evidence suggests that *trans* fatty acids are poor substrates for the LCAT reaction. It is possible that the discrimination against *trans* fatty acids by esterification enzymes could alter the fractional esterification rate of cholesterol, thereby accounting for the lowered HDL-C levels seen after consumption of hydrogenated fats. Thus, the next objective was to determine whether fractional and absolute rates of cholesterol esterification, measured using the DI method, are influenced by consumption of different forms of hydrogenated fats. Furthermore, the model for measuring cholesterol esterification rates was revised to account for the input of newly synthesized cholesterol of the free cholesterol pool esterified daily and corrected for pool size differences.

CHAPTER 5

DEGREE OF HYDROGENATION AFFECTS CHOLESTEROL ESTERIFICATION RATES IN HYPERCHOLESTEROLEMIC WOMEN

To be submitted for publication

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5.1 Abstract

To determine whether the rates of cholesterol esterification in moderately hypercholesterolemic subjects are affected by consumption of different forms of hydrogenated fat, 14 women (65-71 years) consumed each of 6 diets for 5 week periods according to a randomized crossover design. The experimental diets included a baseline (BL) diet (39% kcal fat) and 5 reduced fat diets (30% kcal fat) where 2/3rd of the fat was either soybean oil (SO), low *trans* squeeze (SQM), medium *trans* tub (TM), high *trans* stick (SM) margarines, or butter (BT). Subjects were given a dose of deuterium oxide at the end of each dietary phase to determine the fractional synthesis rates of free and esterified cholesterol (FSR-FC and FSR-CE). Absolute esterification rates (AER) were calculated as the product of FSR-CE and estimated cholesterol pool size. The esterification ratio (ER) was computed as the proportion of newly synthesized cholesterol of the central pool that is esterified daily and corrected for FC pool input. Plasma TC ($p<0.01$) and LDL-C ($p<0.05$) levels increased with increasing degree of hydrogenation or saturated fat intake. HDL-C levels ($p<0.05$) were lowest on the SM diet when compared to the BT diet. LCAT activity was reduced with SM (23 ± 4 nmol.hr^{-1}.d^{-1}), BL (18 ± 2 nmol.hr^{-1}.d^{-1}) and TM (19 ± 8 nmol.hr^{-1}.d^{-1}) feeding relative to the BT (25 ± 6 nmol.hr^{-1}.d^{-1}) phase. However CETP levels were found to be lowest ($p<0.05$) on the BT diet when compare to all other diets. Consumption of the SM diet (0.017 ± 0.009 p.d^{-1}) resulted in lowering ($p<0.05$) of FSR-CE relative to the SO (0.038 ± 0.029 p.d^{-1}) and SQM (0.032 ± 0.019 p.d^{-1}) diets. No differences were seen across other diets. AER showed a similar trend as the FSR-CE data. The ER was lowest ($p<0.05$) after consumption of the SM (0.111 ± 0.062 p.p^{-1}) diet and highest after consumption of the

SQM (0.216 ± 0.123 p.p^{-1}) diet. In addition, ER were positively correlated with LCAT activity ($r=0.32$, $p<0.05$). The association between ER and LCAT were strongest on the SM diet ($r=0.73$, $p<0.0001$). Esterification rates on the SM diet were also found to positively correlate with HDL-C and the HDL_2 subfraction ($r=0.24$ and 0.51 respectively, $p<0.05$), while VLDL-C ($r=0.60$, $p<0.05$) and TG ($r=0.59$, $p<0.05$) levels were negatively correlated with HDL-C. These results indicate a lowering of cholesterol esterification with high *trans* SM feeding, which could explain the decrease observed in HDL-C and corresponding increase in circulating cholesterol levels.

5.2 Introduction

High density lipoprotein cholesterol (HDL-C) is an important negative risk factor for coronary heart disease (1-4). It is well recognized that HDL-C levels are affected by dietary modifications, generally with an increase during diets high in saturated fat and cholesterol, and a decrease during replacement of dietary fat with carbohydrates (5-7). Recent metabolic studies have also shown that *trans* fatty acids from hydrogenated fats decrease HDL-C levels, in addition to the increases in total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C) and triglycerides (TG) (8-17). Thus *trans* fatty acids behave like saturated fatty acids (SFA) in terms of their ability to increase plasma cholesterol concentrations, yet are unlike SFA in terms of their tendency to depress HDL-C, affirming a striking difference between these two type of fatty acids. Several mechanisms have been proposed for the changes in lipid and lipoprotein profile induced by hydrogenated fat consumption.

One possible mechanism that could account for the increase in LDL-C and decrease in HDL-C concentrations is an increased transfer of cholesteryl esters from HDL-C to VLDL-C and LDL-C. Abbey and Nestel (18) have reported significantly higher cholesterol ester transfer protein (CETP) activity in the plasma of subjects fed *trans* fatty acid rich diets as opposed to subjects fed oleic acid rich diets. Similar results of higher CETP activity with *trans* fatty acids relative to linolenic and stearic (19) or *cis* fatty acids (20) have also been reported.

Another potential mechanism responsible for the lowering of HDL-C levels could be an impairment in cholesterol esterification. Most of cholesterol esterification in plasma occurs in HDL-C and is proportional to the activity of the esterification enzyme lecithin: cholesterol acyltransferase (LCAT), which in turn is closely correlated with plasma TG and HDL-C concentration (21). Dietschy and colleagues (22) have demonstrated that SFA intake decreases the rate of esterification, with simultaneous increases in LDL production rates. The converse is true for unsaturated fatty acids. Mazier and Jones (23) using the deuterium incorporation methodology, have reported higher fractional esterification rates (FER) when subjects consumed polyunsaturated (PUFA) versus either monounsaturated (MUFA) or SFA containing diets. The lowering of cholesterol esterification rates with SFA diets has been attributed to the preference of esterification enzymes for PUFA and MUFA. Recently, Subbaiah et al (24) using an *in vitro* model have demonstrated that *trans* fatty acids, despite being unsaturated, are also poor substrates for the LCAT reaction. It is possible that this discrimination against *trans* fatty acids by esterification enzymes could alter esterification of cholesterol, which in turn could explain the lower HDL-C levels observed after consumption of hydrogenated fats. However to date no study has documented this effect in humans. Thus the objective of the present study was to determine whether rates of cholesterol esterification, determined using the deuterium incorporation method, are influenced by consumption of different forms of hydrogenated fats.

5.3 Methods

5.3.1 Subjects

Fourteen postmenopausal middle aged to elderly (65-71 years), moderately hypercholesterolemic (LDL-C levels $\geq$130 mg.dl^{-1}) women were recruited to participate in this study from an original study population of 18 female subjects as previously described (8). Sample losses during shipping and preparation precluded generation of complete data sets for 4 subjects, and hence they could not be included in the present study. Subjects were free from chronic illness and were not taking any medication known to affect lipid metabolism (lipid lowering drugs, β-blockers, diuretics or hormones). Subjects who smoked or consumed $\geq$2 alcoholic drinks per day were also excluded from the study. The protocol was reviewed and approved by the human-investigation review committee of New England Medical Center and Tufts University. All potential subjects were given a verbal and written description of the study prior to obtaining consent. However, during the study period, subjects, investigators and laboratory personnel were blinded to the dietary phase assignments. A portion of the data focusing on plasma lipid and lipoprotein parameters has been published previously (8).

5.3.2 Experimental Design and Diets

Subjects consumed each of six diets according to a randomized cross-over design. Each dietary phase had a duration of 5 weeks and was separated by a washout period ranging from 2 to 4 weeks, during which subjects consumed their habitual diets. The soybean oil diet was formulated to meet NCEP 2 dietary guidelines, providing 15% energy as protein, 55% as carbohydrate and 30% as fat ($\leq$ 7% SFA, 10-15% MUFA and $\leq$10% PUFA) and

less than 85 mg cholesterol per 1000 kcal. The soybean oil component comprised 20% of energy. In subsequent diets, the soybean oil was replaced by the experimental fat, so that the effect of consuming diets enriched in hydrogenated fats could be assessed within the general context of current recommendations for individuals with elevated plasma lipid levels. The specific fats investigated were soybean oil (SO) and soybean oil based margarines in the squeeze (SQM), tub (TM) and stick (SM) form as well as butter (BT). These fats were especially chosen because as a group they represent a broad range of *trans* and fatty acid profiles. The baseline diet was included in order to access the biochemical parameters proposed for each individual on a defined diet with a fatty acid composition similar to that currently consumed in North America to best characterize the study population. All food and drink were provided by the Metabolic Research Unit of the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University to be consumed on site or packaged for take-out. Subjects were required to consume all food provided to them and not to supplement their diet with any other food or drink except water and non-caloric beverages. Initial energy intakes of the subjects were calculated using the Harris-Benedict equation, and adjustments made when necessary to maintain body weight. Analysis of the macronutrient, fatty acid and cholesterol contents of the diets were carried out by Covance Laboratories (Madison, WI) and Best Foods Research and Engineering Center (Union, NJ).

5.3.3 Protocol and Analyses

During week 5 of each dietary phase a fasting blood sample was drawn followed by administration of a bolus oral dose of deuterium oxide (1.2g D_2O per kg total body

water). Another fasting blood sample was taken 24 hours after dosing. Blood samples were centrifuged at 3,000 rpm at 4°C and plasma was separated, aliquoted and frozen at -80 °C until further analysis. VLDL was isolated from plasma by ultracentrifugation at 39,000 rpm for 18 hours at 4 °C (25). Serum and the 1.006 $g.ml^{-1}$ infranatant fraction were assayed for TC and TG with an Abbott Diagnostic (North Chicago, IL) spectrum CCX biochromatic analyzer using enzymatic reagents as previously described (26). HDL-C levels were measured after precipitation of the apolipoprotein B containing lipoproteins by a dextran-magnesium sulphate procedure (27). Lipid assays were standardized through the Lipid Standardization Program at the Center for Disease Control (Atlanta, GA). LCAT activity was measured using prepared ethanolosomes and plasma substrates labelled with ^{3}H-C as described by Dobiasova and Frohlich (28). Plasma CETP activity was measured using the CETP mass method described by Groener et al (29).

Additional plasma aliquots were used to determine deuterium (D) enrichment in body water, free cholesterol (FC) and cholesterol ester (CE) fractions as previously described (22,30,31). The procedure was as follows: lipids were extracted (32) and the FC and CE band was separated by thin layer chromatography using petroleum ether/ ethyl ether/ acetic acid (135:15:1.5 v/v/v). The FC band was eluted from silica with hexane/ chloroform/ ethyl ether (5:2:1 v/v/v) and dried under a stream of nitrogen. The CE band was saponified with 5ml methanolic potassium hydroxide, capped tightly and boiled at 100 °C for 55 minutes. After cooling, 2ml of distilled water and 13 ml of petroleum ether were added per tube, which was shaken for 5 minutes, and then centrifuged at 1500g for 15 minutes. The upper ether phase was separated, and the extraction procedure repeated two more times, omitting the addition of water. Combined ether phases were dried down

under a stream of nitrogen and then plated to separate the cholesterol from free fatty acids. The resultant de-esterified cholesterol (d-CE) was purified from silica as described above. The FC and d-CE were then converted into water and carbon dioxide by combustion over cupric oxide and silver wire at 520°C for 2 hours. In addition, pre and post D_2O samples diluted 2-fold and 10-fold respectively to produce D enrichments within detectable ranges on the mass spectrometer were distilled into zinc containing tubes. This enabled measurement of D enrichment of plasma water. The combustion water from FC, d-CE and plasma were then vacuum distilled into pyrex™ tubes containing zinc reagent, and reduced to hydrogen deuterium gas by heating at 520°C for 30 minutes. D enrichment of the gas was analyzed by isotope ratio mass spectrometry (VG isomass 903 D, Cheshire, England). The instrument was calibrated daily using water standards of known isotopic composition. Values were expressed relative to the enrichment of standard mean ocean water (SMOW) in parts per mil ($^{o}/_{oo}$). The per mil designation was used because of the relatively small enrichments encountered. Duplicate samples for each subject were analyzed concurrently against a single set of standards. Maximum acceptable precision for D was 5 $^{o}/_{oo}$ at enrichments over 500 $^{o}/_{oo}$ and 2 $^{o}/_{oo}$ at enrichments below 200 $^{o}/_{oo}$.

5.3.4 Calculation of Fractional and Absolute Cholesterol Esterification Rates, and Esterification Ratio

FSR values were calculated as cholesterol D enrichment in FC and CE in relation to that of the precursor body water pool adjusted for the fraction of hydrogens of cholesterol derived from labeled substrate (30). The equation used was

$$FSR\ (p.d^{-1}) = \frac{\delta\ \text{cholesterol}\ (\%)}{\delta\ \text{plasma water}\ (\%) \times 0.478}$$

where δ is the difference in D enrichment over 24 hours. Model parameters and assumptions underlying use of D_2O as tracer for endogenous cholesterol measurements have been described previously (22,30,31). The AER expressed in grams/day ($g.d^{-1}$) was derived by multiplying the FSR-CE by M_1 pool size and a factor of 0.67. The M_1 pool size was calculated using Goodman's equation (33) which takes into account the subjects body weight, plasma TC and TG concentrations. The factor of 0.67 was included to account for the proportion of CE in the overall plasma TC pool. Given that newly synthesized cholesterol initially appears into the FC pool, the ER expressed as pools/pools ($p.p^{-1}$), was computed to reflect the appearance of label in cholesteryl ester from the FC pool after correcting for pool size differences (Figure 5-1). The equation used was:

$$ER = (p.p^{-1}) \quad \frac{\text{FSR-CE/ CE-}M_1\ \text{pool}}{\text{FSR-FC/ FC-}M_1\ \text{pool}}$$

The size of the FC- M_1 pool was calculated in the manner similar to that described for CE, with replacement of the factor of 0.67 by 0.33 to account for the contribution of FC to the TC pool.

5.4 Statistical Analysis

One way analyses of variance with the main effect of diet and subject as the repeated measure was employed on each outcome variable using a SAS general linear model program (SAS version 6, SAS Institute Inc, Cary, NC). Differences in group means were

identified using Tukey's test at a significance level of $p<0.05$. Correlation coefficients were calculated to determine if associations existed between variables of interest. Since the FSR-CE, AER and CETP data were not normally distributed, logarithmic transformations were performed prior to statistical testing. Untransformed data are presented in text and tables as means ± standard deviation (SD).

5.5 Results

The composition of the experimental diets based on chemical analyses is shown in Table 5-1. Excluding the BL diet, all other reduced fat diets had similar protein, fat and carbohydrate contents. Among the reduced fat diets, the SFA and MUFA contents were relatively similar. However increasing degree of hydrogenation resulted in higher *trans* fatty acid and lower PUFA levels. The cholesterol content of the butter diet was approximately twice that of the other diets. The option to compensate for this endogenous cholesterol content was not acted upon because cholesterol is an inseparable component of butter and it would be artificial to assess the impact of the fatty acid composition of the product on various physiological parameters without including the cholesterol.

Mean (± SD) plasma lipid and lipoprotein concentrations, LCAT and CETP activity levels during the 6 dietary phases are depicted in Table 5-2. Plasma TC levels were elevated with consumption of the BL, BT and SM diets when compared to the SO, SQM and TM diets ($p<0.05$ for BT vs SO, SQM, TM; BL vs SO, SQM; and SM vs SO). A similar pattern of response was observed with LDL-C levels, with consumption of the BL and BT diets resulting in higher ($p<0.05$) LDL-C levels relative to the SO and SQM diets. Switching from the BL to all five reduced fat diets resulting in a significant ($p<0.05$)

lowering of HDL-C. Among the reduced fat diets, plasma HDL-C concentrations were lowest on the SM diet (45 ± 9 $mg.dl^{-1}$) and highest on the BT diet (50 ± 10 $mg.dl^{-1}$). Plasma concentrations of HDL_2 and HDL_3 were higher on the SFA containing diets compared to the SO and margarine based diets. LCAT activity was lower ($p<0.05$) after consumption of SM (22.6 ± 4.4 $nmol.hr^{-1}.d^{-1}$), BL (17.7 ± 1.5 $nmol.hr^{-1}.d^{-1}$) and TM (18.9 ± 8.4 $nmol.hr^{-1}.d^{-1}$) diets, and higher on the BT (25.4 ± 6.3 $nmol.hr^{-1}.d^{-1}$) diet. Conversely, CETP activity was lowest on the BT diet relative to all other diets.

With respect to the cholesterol kinetic data, FSR-FC rates (Figure 5-2A) were higher ($p<0.05$) on the SO (0.078 ± 0.024 $p.d^{-1}$), SQM (0.081 ± 0.019 $p.d^{-1}$) and TM (0.086 ± 0.029 $p.d^{-1}$) diets when compared to the SM (0.053 ± 0.029 $p.d^{-1}$) diet. Consumption of the BL (0.053 ± 0.023 $p.d^{-1}$) diet resulted in suppressed ($p<0.05$) synthesis when compared to the SQM and TM diets. No differences in FSR-FC rates were observed between the BT (0.074 ± 0.044 $p.d^{-1}$) and other diets. FSR-CE (Figure 5-2B) was higher ($p<0.05$) on the SO (0.038 ± 0.029 $p.d^{-1}$) and SQM (0.032 ± 0.019 $p.d^{-1}$) diets and lower on the SM (0.017 ± 0.009 $p.d^{-1}$) diet, with esterification rates on the TM (0.024 ± 0.012 $p.d^{-1}$), BT (0.027 ± 0.020 $p.d^{-1}$), and BL (0.053 ± 0.023 $p.d^{-1}$) diets being intermediate. The AER (Figure 5-3A) data was similar, with consumption of the SM (0.292 ± 0.165 $g.d^{-1}$) diet resulting in a significant ($p<0.05$) reduction of esterification relative to the SO (0.648 ± 0.506 $g.d^{-1}$) and SQM (0.537 ± 0.324 $g.d^{-1}$) diets. AER did not differ among the other diets (0.417 ± 0.390, 0.458 ± 0.319 and 0.451 ± 0.364 $g.d^{-1}$ for TM, BT and BL respectively). The ER data (Figure 5-3B), which corrects for input of D label from the FC

pool, was lower on the SM (0.111 ± 0.062 $p.p^{-1}$) diet relative to the SQM (0.216 ± 0.123 $p.p^{-1}$) diet, but differences among other diets were no longer observed.

When the ER data determined using the D uptake method was correlated against LCAT activity (Figure 5-4) a small but significant association was observed ($r=0.32$, $p<0.05$). When correlation's were performed per diet, we found that the strongest association between ER and LCAT (Figure 5-5A) was on the SM diet ($r=0.73$, $p<0.001$). ER was also positively associated with HDL-C (Figure 5-5B) and HDL_2 levels (Figure 5-5C) on the SM diet ($r=0.24$ and 0.51, $p<0.05$). An inverse association was observed on the SM diet between HDL-C levels (Figure 5-6A and 5-6B) and VLDL ($r=0.60$, $p<0.05$) and TG ($r=0.59$, $p<0.05$) levels.

5.6 Discussion

High density lipoprotein cholesterol levels have been implicated as a factor which protects against the development of cardiovascular disease (1-4). Thus, the lowering of HDL-C levels, seen with consumption of *trans* fatty acids from hydrogenated fats, has raised concern, and several investigators have attempted to understand the mechanisms for this effect. One possible mechanism involves impaired cholesterol esterification, majority of which occurs in plasma during maturation and formation of HDL-C particles (21). Consequently, the present study was conducted to determine if fractional and absolute esterification rates of cholesterol, directly measured using the DI method, respond to shifts in dietary *trans* and fatty acid profile, and consequently influences circulating HDL-C levels.

Our results provide evidence of lowered cholesterol esterification on the higher *trans* fatty acid containing SM diet relative to the SQM diet. Given that HDL-C is the predominant site of cholesterol esterification in plasma, it can be speculated that the reduction in esterification rates seen with SM feeding are due to alterations in the activity of this enzyme. The above hypothesis is consistent with the findings of Subbaiah et al (24), who demonstrated *in vivo* that both human and rat LCAT activity was significantly lowered (-37 % to 50 %) with synthetic phosphatidylcholine (PC) containing C 18:1*t* and C 18:2*t*, when compared to PC containing the corresponding *cis* isomers. The authors concluded that inhibition of LCAT could explain the lowering of HDL-C by *trans* fatty acids. Studies have also documented that among the HDL subfractions, HDL_3 acts as a substrate for the LCAT reaction, while HDL_2 inhibits this process (34,35). Therefore inhibition of LCAT activity by *trans* fatty acids, would be expected to decrease cholesterol esterification rates and consequently HDL_2 levels. We did in fact observe such an association on the SM diet. Additionally, the lowering of cholesterol esterification rates could result in an expanded M_1 pool of unesterified cholesterol when hydrogenated and saturated fat diets are consumed, which is thought to contribute to the down regulation of the LDL receptor and subsequently increase circulating cholesterol levels (22).

Several studies (22,23) have also shown that SFA rich diets tend to decrease rates of cholesterol esterification. Although a trend toward lower FER and AER were observed in our study after consumption of the SFA rich BT and BL diets, differences did not attain statistical significance, possibly due to the high variability among subjects. However, if

SFA diets lower esterification rates, then one would expect the lowering of HDL-C levels with SFA to parallel those seen with *trans* fatty acid feeding, but the reverse is usually observed (9,14). This paradox is not readily explained but is presumed to occur via alterations in the positional specificity of LCAT by different fatty acid species, and the subsequent substrate available for the CETP reaction (24). Saturated fatty acids are exclusively incorporated into the sn-1 position of PC, whereas *trans* fatty acids can be incorporated into either sn-1 or sn-2 positions of PC (36). When a *trans* fatty acid occupies the sn-2 position, it is generally paired with a SFA in sn-1, which results in the formation of a more saturated cholesterol ester, given the structural similarities between *trans* and SFA. These saturated cholesterol esters are better substrates for CETP (37) when compared to cholesterol esters containing PUFA in sn-2 and SFA in sn-1. The resultant increase in CETP activity with *trans* diets, would result in greater transfer of cholesterol esters for TG and consequently a further lowering of HDL-C levels, with concomitant increases in VLDL-C and LDL-C levels. The above theory is supported by the lower levels of CETP on the BT and BL diets relative to the SO and other margarine diets. In addition, increased CETP and decreased HDL during replacement of palmitic by elaidic acid (*t*-C18:1), has also been reported by Khosla and colleagues (38).

In the present study, cholesterol esterification rates were measured using LCAT activity levels as well as the D uptake methodology. The *in vitro* LCAT assay has been widely used to estimate esterification rates following dietary manipulation but results have been conflicting. For example several investigators (22,39) have demonstrated decreased LCAT activity during periods of high SFA intake compared to PUFA and MUFA

containing diets, however, the reverse has also been reported (40,41). This discrepancy among studies could be due to differences in the incubation period of plasma with labeled substrate. In addition, prolonged incubation would result in lower esterification and transfer rates of cholesterol, due to feedback inhibition of accumulated CE in the medium (42). By contrast, *in vivo*, such an accumulation is presumably relieved by clearance of CE rich lipoproteins into the liver and other sites. Thus, the applicability of the *in vitro* LCAT assay to the *in vivo* situation is questionable.

Alternately, the D uptake method represents a direct *in vivo* measure of esterification. Mazier and Jones (23) using the DI method, calculated AER rates as the product of FSR-CE and M_1 pool size, the latter derived from a decay curve following injection of [4-^{14}C] cholesterol. The authors demonstrated that AER were higher after consumption of a PUFA versus MUFA or SFA containing diets, however this trend was not statistically significant, possibly due to the small sample size (n=9) used in the study. Given the invasive nature of the above technique to estimate M_1 pool size, we opted to use the Goodman's equation, which is considered an accurate estimate M_1 pool size. We also observed lower AER not only on the SFA rich diets but also after consumption of the high *trans* containing SM diet when compared to the SO and SQM diets. However the AER calculation does not account for movement of FC into the ester pool. Consequently, the ER model was devised. Our revised model assumes that free cholesterol migrates rapidly between lipoproteins, erythrocytes and other cellular structures (33,43). Thus, D enrichment of plasma FC represents that of free sterol within the rapidly exchanging M_1 pool found in the liver, intestine and circulation. Turnover rates for CE are slower (41),

thus, present data likely reflect events occurring within the plasma, rather than the overall CE pool within the M_1 compartment. For periods longer than 24-48 hours, gradual turnover of cholesterol between the rapidly exchangeable and slow turnover pools, as well as a certain amount of de-esterification would result in the input of unlabeled cholesterol into the M_1 pool, thereby altering FSR and consequently ER. However, over the short term, esterification rates obtained by the D uptake method are considered reliable and valid.

In summary, cholesterol esterification rates were shown to be influenced by dietary *trans* and fatty acid profile, being lower when subjects were fed the SM diet versus the SQM diet. These results provide a possible explanation not only for the lowered HDL-C levels, but also increase plasma cholesterol levels, typically observed after consumption of hydrogenated fats.

5.7 Acknowledgements

This work was supported by a grant from the National Institute of Health (Grant number: HL-54727) and Medical Research Council of Canada.. We acknowledge the technical assistance of Sarah McGladdery and Matti Jauhiainen for performing the LCAT and CETP assays respectively. We are indebted to the staff of the Metabolic Research Unit for the expert care provided to the study subjects. We would also like to acknowledge the cooperation of the study subjects without whom this investigation would not be possible.

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Table 5-1. Composition of Test Diets as Determined by Chemical Analysis*

Constituent	Baseline (BL)	Soybean oil (SO)	Squeeze Margarine (SQM)	Tub Margarine (TM)	Stick Margarine (SM)	Butter (BT)
			percentage of total daily energy intake			
Protein	16.8	15.7	17.1	16.3	16.7	16.9
Carbohydrate	44.6	55.8	51.7	52.9	53.5	53.9
Fat	38.6	28.5	31.2	30.8	29.7	29.1
Saturated fatty acids	15.5	7.3	8.6	8.4	8.5	16.7
12:0	1.8	0.8	1.0	0.7	0.8	1.4
14:0	0.1	0.6	0.7	0.6	0.6	2.5
16:0	7.8	3.7	4.3	4.2	4.0	7.5
18:0	3.6	1.5	1.9	2.3	2.2	3.4
Monounsaturated fatty acids#	15.1	8.1	8.1	8.0	8.5	8.1
18:1	12.2	7.2	7.1	6.7	6.5	7.0
Polyunsaturated fatty acids#	7.0	12.5	13.5	11.1	6.3	2.4
18:2 (n-6)	5.9	10.7	12.1	10.0	5.6	2.1
18:3 (n-3)	1.0	1.7	1.4	1.1	0.7	0.3
Trans fatty acids	1.7	0.6	0.9	3.3	6.7	1.3
Cholesterol (mg. 4.2 MJ^{-1})	163.8	65.9	68.0	70.3	66.5	121.0

* Because of rounding, percentages may not total 100

\# Only cis isomers are included

Table 5-2 : Serum Lipid and Lipoprotein Profile, LCAT and CETP Activity Levels at the End of Each Phase

Variable	Baseline (BL)	Soybean Oil (SO)	Squeeze Margarine (SQM)	Tub Margarine (TM)	Stick Margarine (SM)	Butter (BT)
			mean ± SD			
TC ($mg.dl^{-1}$)	252 ± 33^{ab}	230 ± 28^{d}	233 ± 28^{cd}	240 ± 30^{bcd}	247 ± 34^{abc}	256 ± 33^{a}
LDL-C ($mg.dl^{-1}$)	176 ± 31^{a}	157 ± 32^{b}	158 ± 22^{bc}	165 ± 29^{ab}	172 ± 30^{ac}	177 ± 29^{a}
HDL-C ($mg.dl^{-1}$)	52 ± 12^{a}	47 ± 9^{bc}	47 ± 11^{bc}	47 ± 10^{bc}	45 ± 9^{c}	50 ± 10^{ab}
TG ($mg.dl^{-1}$)	132 ± 45	137 ± 53	131 ± 54	141 ± 56	155 ± 64	139 ± 46
LCAT ($nmol.hr^{-1}.d^{-1}$)	18 ± 1^{b}	22 ± 2^{ab}	23 ± 4^{ab}	19 ± 8^{b}	17 ± 5^{b}	25 ± 6^{a}
CETP ($nmol.ml^{-1}.hr^{-1}$)	14 ± 4^{a}	15 ± 7^{a}	19 ± 6^{a}	14 ± 5^{a}	16 ± 6^{a}	9 ± 3^{b}

Within a row, values with different superscripts are significantly different ($p<0.05$), n =14

To convert values for cholesterol to millimoles per liter, divide by 38.67

To convert values for triglycerides to millimoles per liter, divide by 88.54

5.9 Figure Legends

Figure 5-1: Proposed model for measurement of cholesterol esterification rates. Abbreviation are as follows: FC : free cholesterol; CE: cholesteryl ester. Input of cholesterol into the FC pool is from endogenous synthesis and diet, while the major output is into the CE pool, with a small amount of elimination via other pools.

Figure 5-2: FSR-FC (**A**) and FSR-CE (**B**) of cholesterol at the end of each dietary phase. Data are presented as means ± SD, n =14. Diet abbreviations are as follows; SO: soybean oil, SQM: squeeze margarine, TM: tub margarine, SM: stick margarine, BT: butter, BL: baseline. Columns with different letters are significantly different ($p<0.05$).

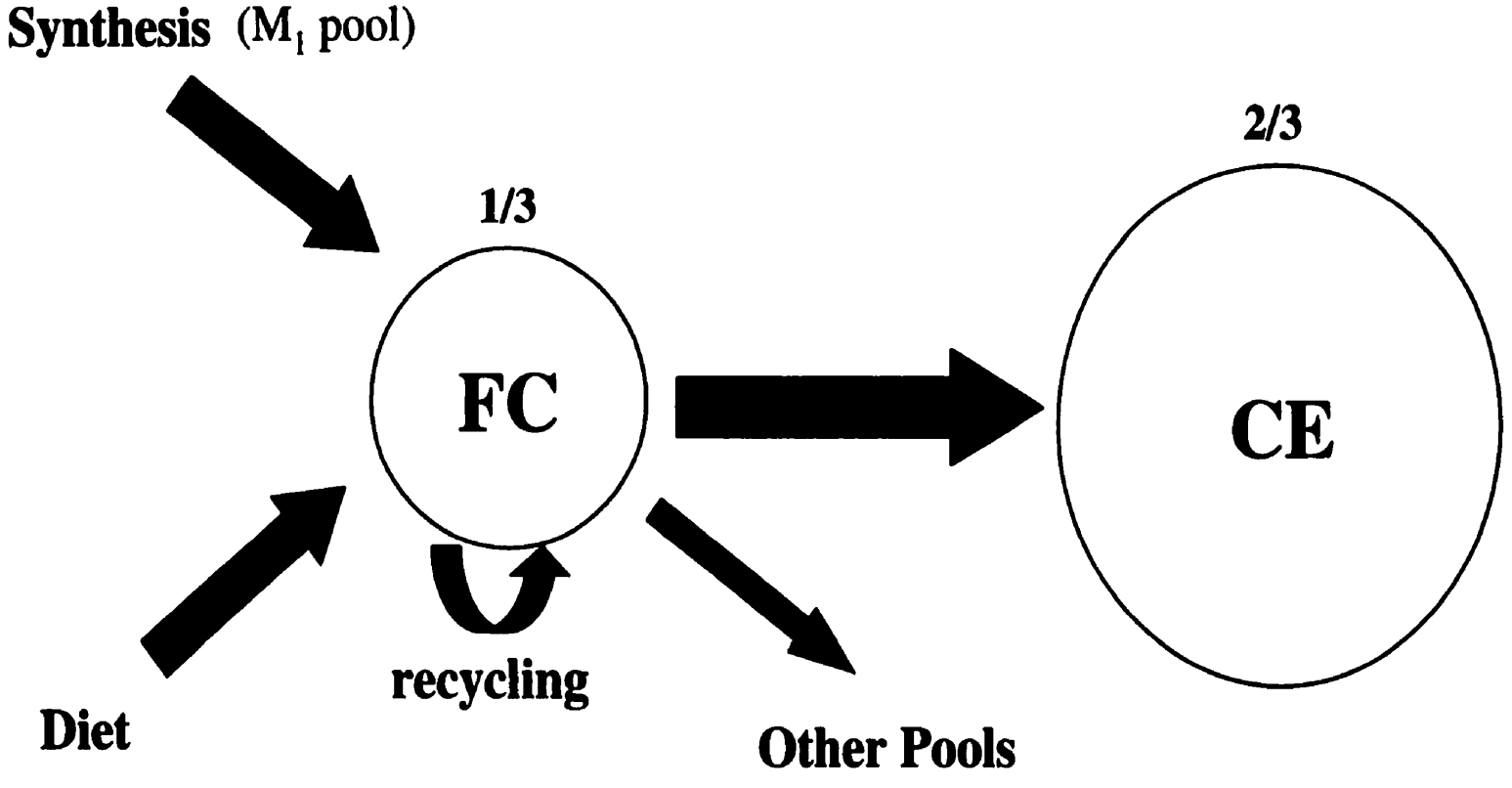
Figure 5-3: AER (**A**) and ER (**B**) of cholesterol at the end of each dietary phase. Data are presented as means ± SD, n =14. Diet abbreviations are as follows; SO: soybean oil, SQM: squeeze margarine, TM: tub margarine, SM: stick margarine, BT: butter, BL: baseline. Columns with different letters are significantly different ($p<0.05$).

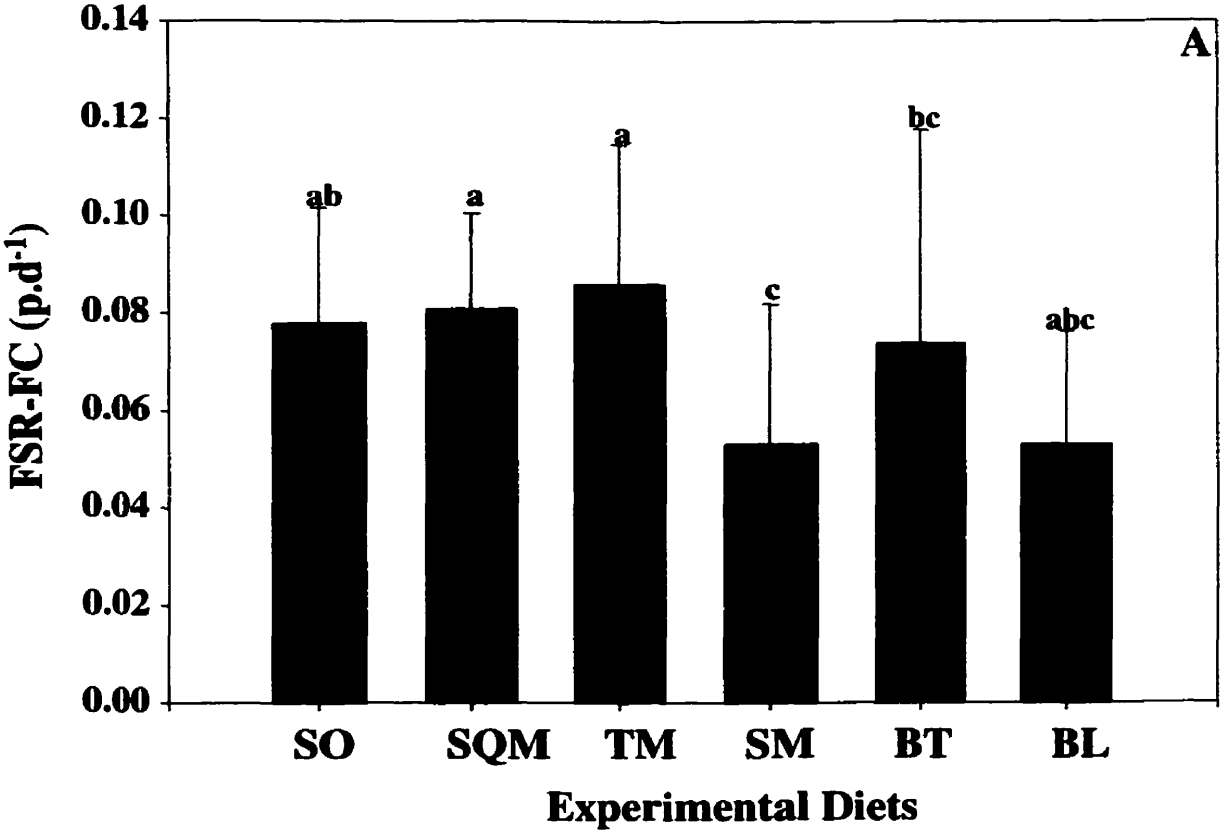
Figure 5-4: Association ER ($p.p^{-1}$) and plasma LCAT levels ($nmol.hr^{-1}.d^{-1}$). Data are presented for 14 subjects after consumption of the six experimental diets.

Figure 5-5: Association between ER ($p.p^{-1}$) and (**A**) plasma LCAT levels ($nmol.hr^{-1}.d^{-1}$), (**B**), plasma HDL-C levels ($mg.dl^{-1}$) and (**C**) plasma HDL_2 levels ($mg.dl^{-1}$). Data are presented for 14 subjects after consumption of the stick margarine (SM) diet.

Figure 5-6: Association between HDL-C ($mg.dl^{-1}$) and (**A**) plasma VLDL-C ($mg.dl^{-1}$) levels and (**B**) TG ($mg.dl^{-1}$) levels. Data are presented for 14 subjects after consumption of the stick margarine (SM) diet.

PLASMA CHOLESTEROL COMPARTMENT

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0.04
0.02
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FSR-FC (p.d-1)
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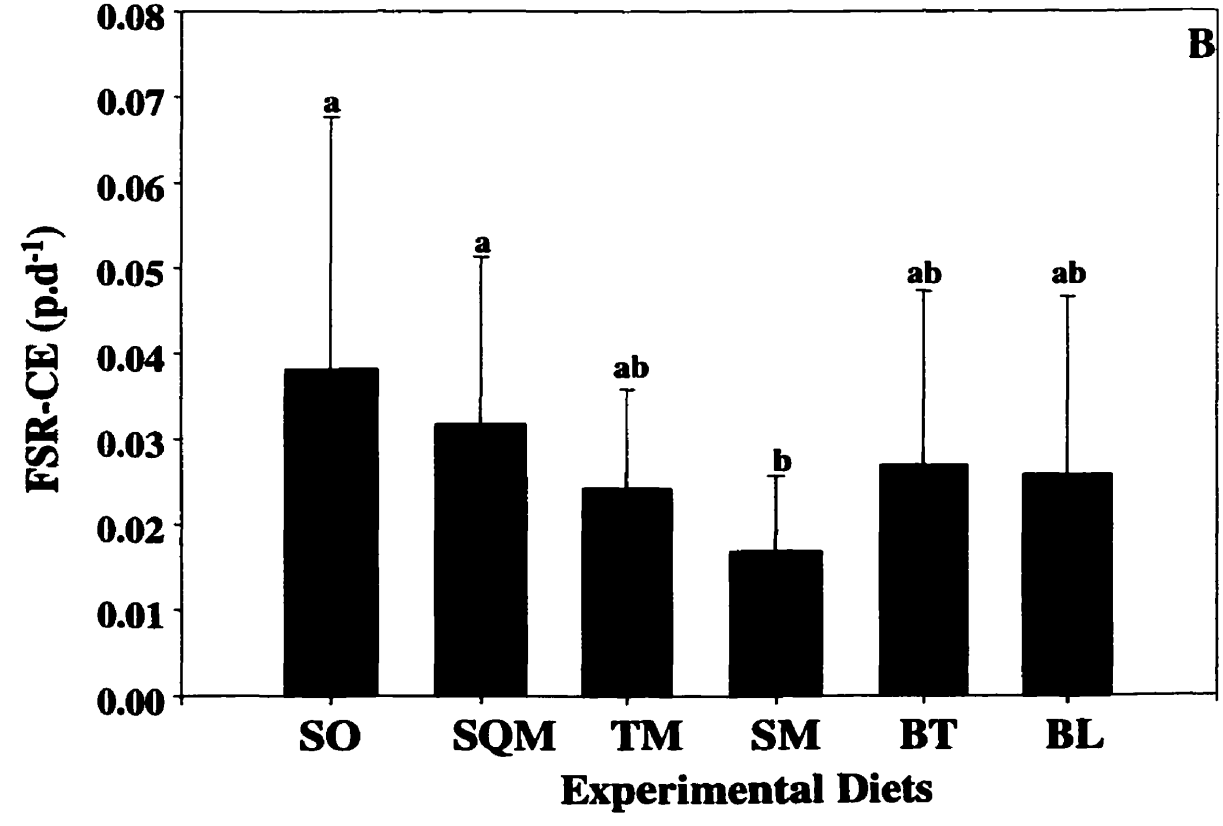
a
a
c
bc
abc
SO
SQM
TM
SM
BT
BL
Experimental Diets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0.03
0.02
0.01
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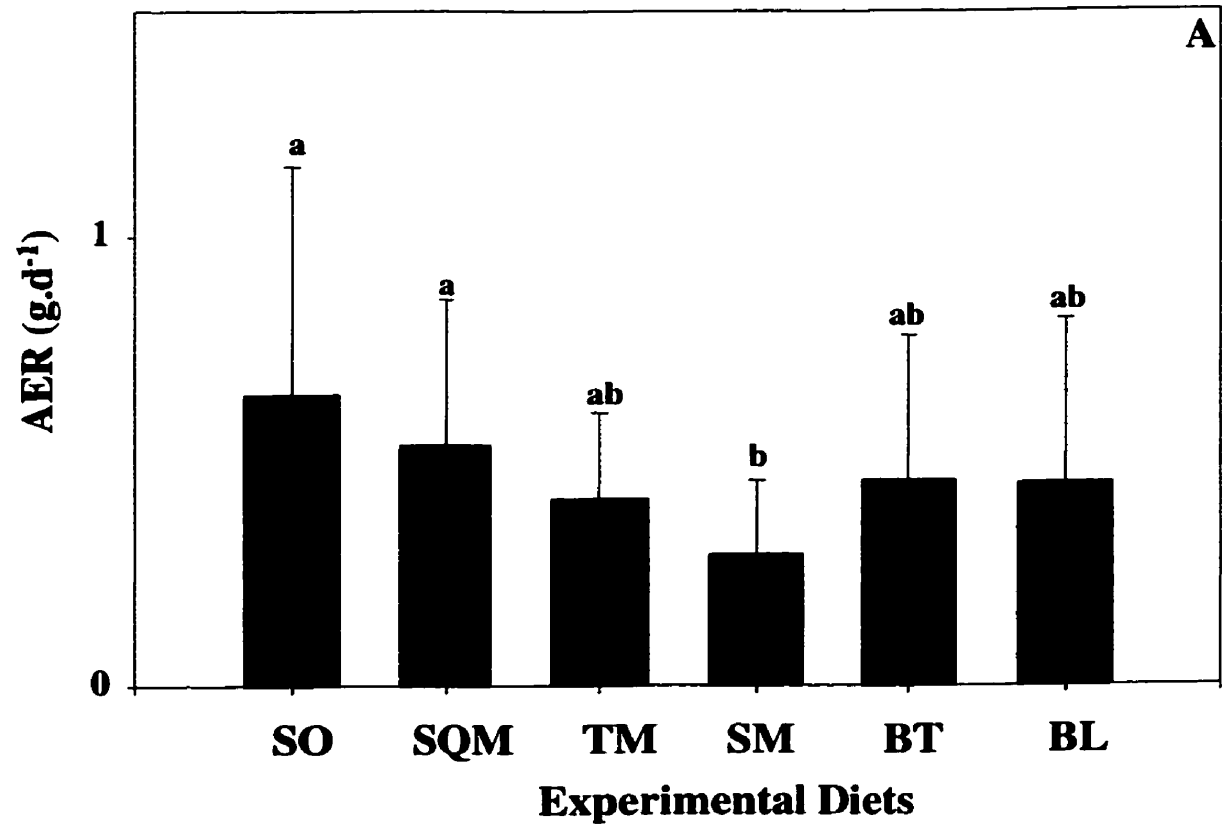
a
ab
b
ab
ab
SO
SQM
TM
SM
BT
BL
Experimental Diets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ab
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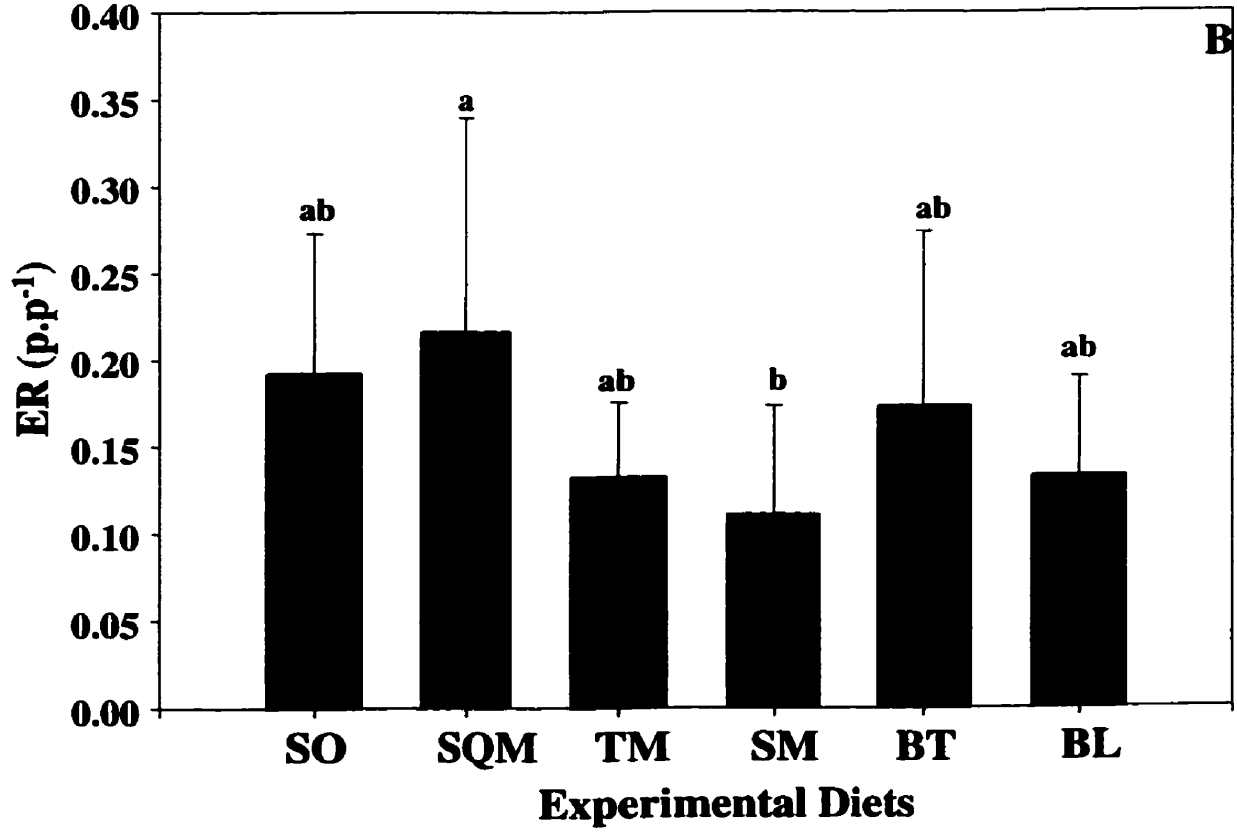
SQM
TM
SM
BT
BL
Experimental Diets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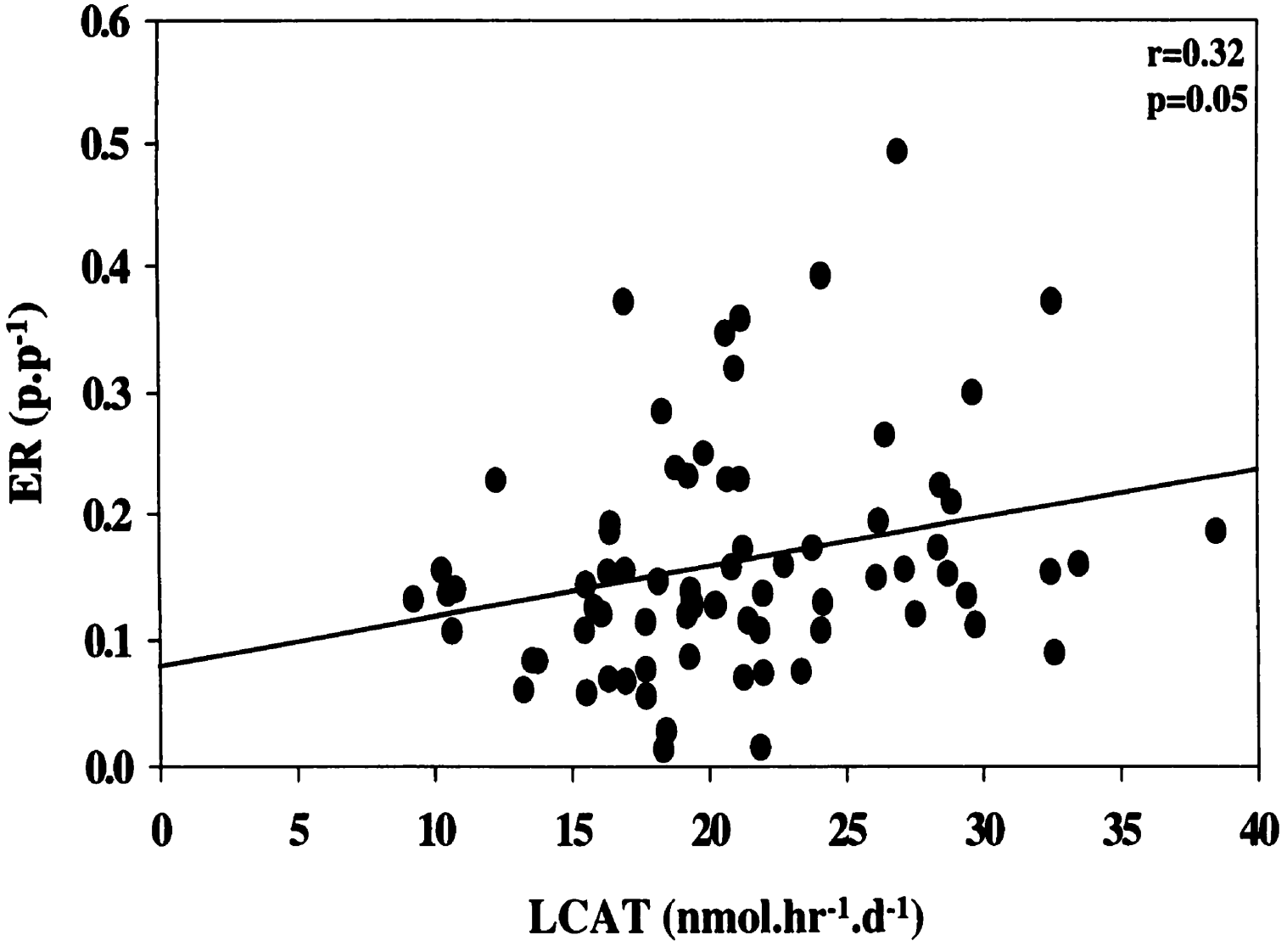
ab
SO
SQM
TM
SM
BT
BL
Experimental Diets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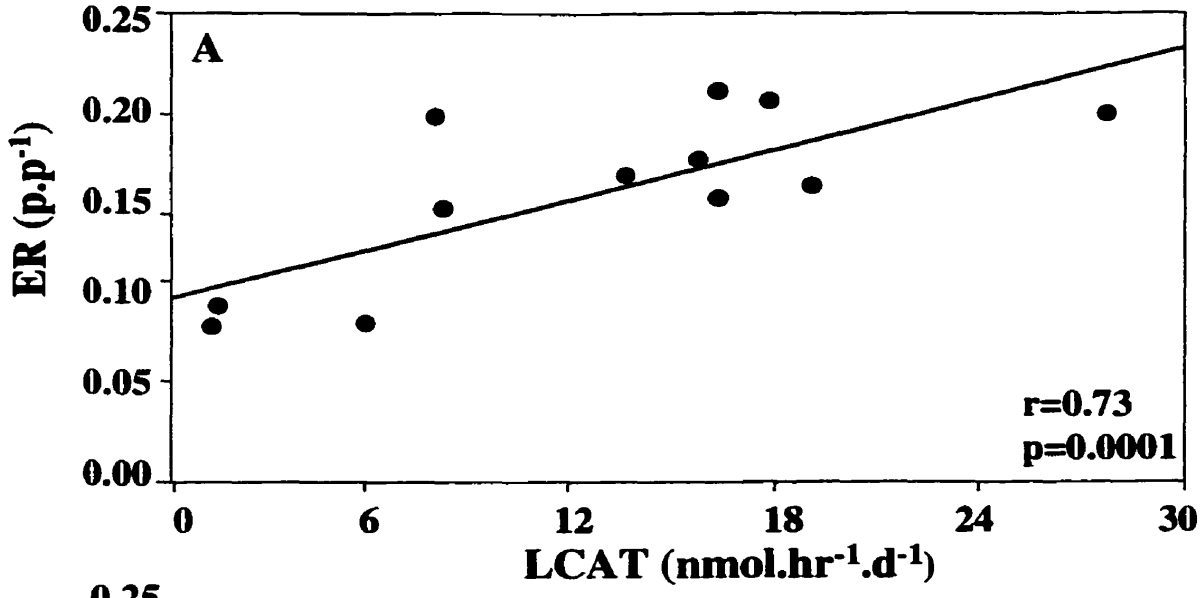
0.6
0
5
10
15
20
25
30
35
40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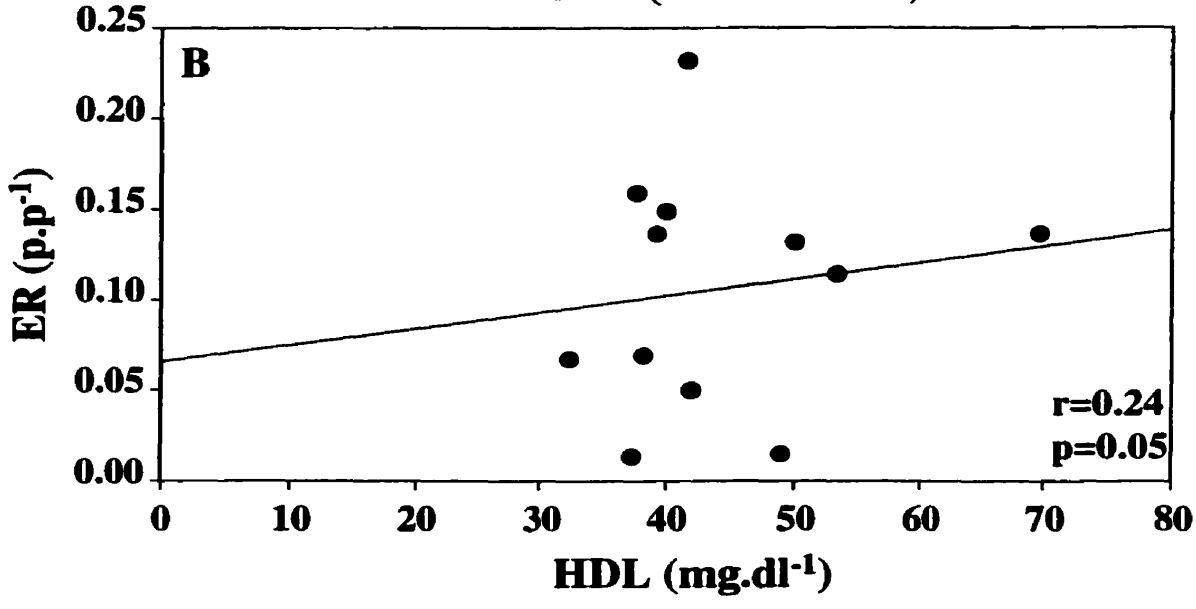
LCAT (nmol.hr-1.d-1)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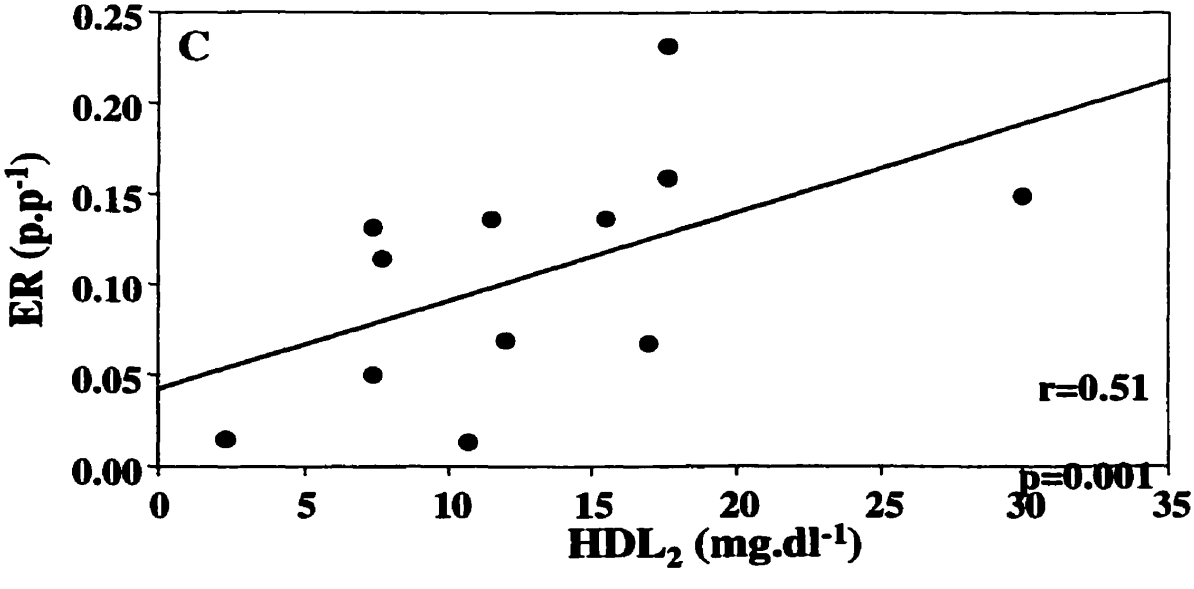
40
50
60
70
80
HDL (mg.dl-1)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0.10
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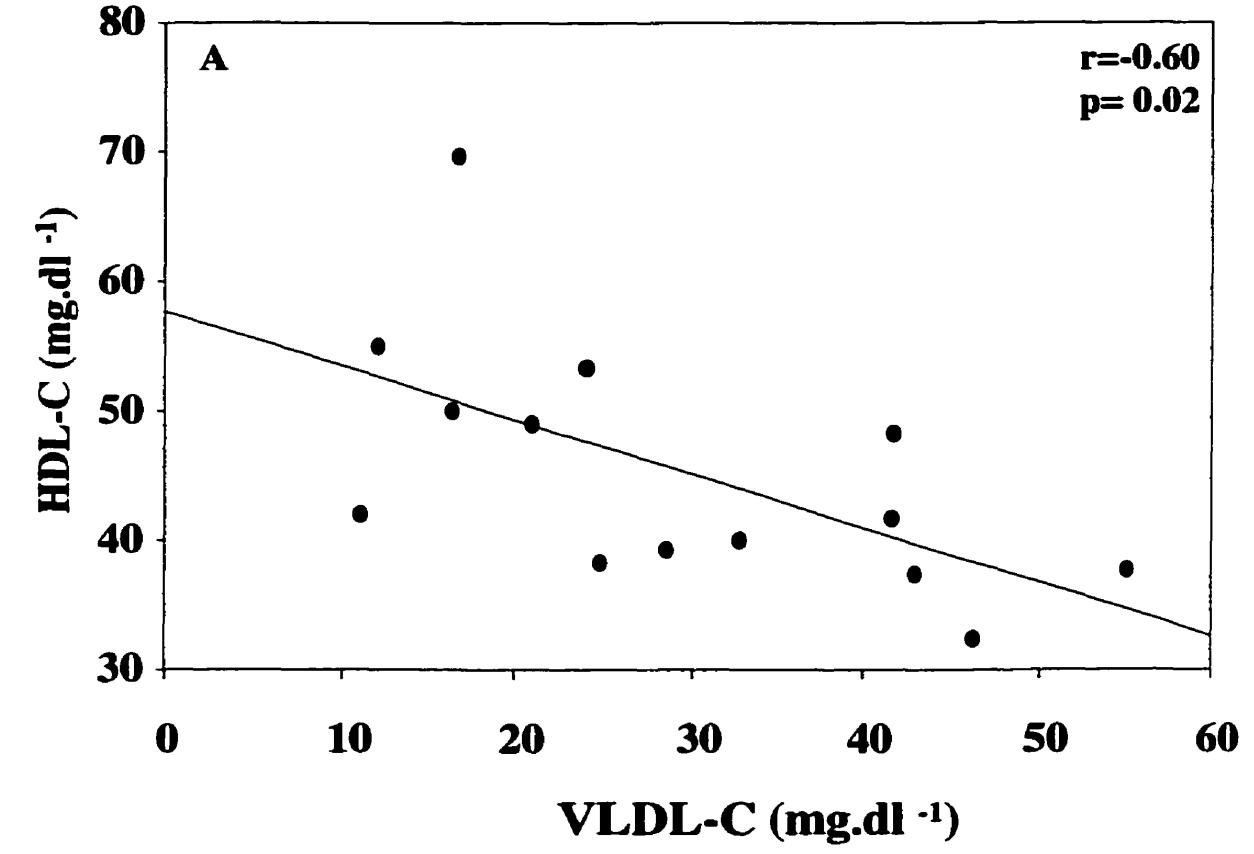
10
15
20
25
30
35
HDL2 (mg.dl-1)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HDL-C (mg.dl -1)
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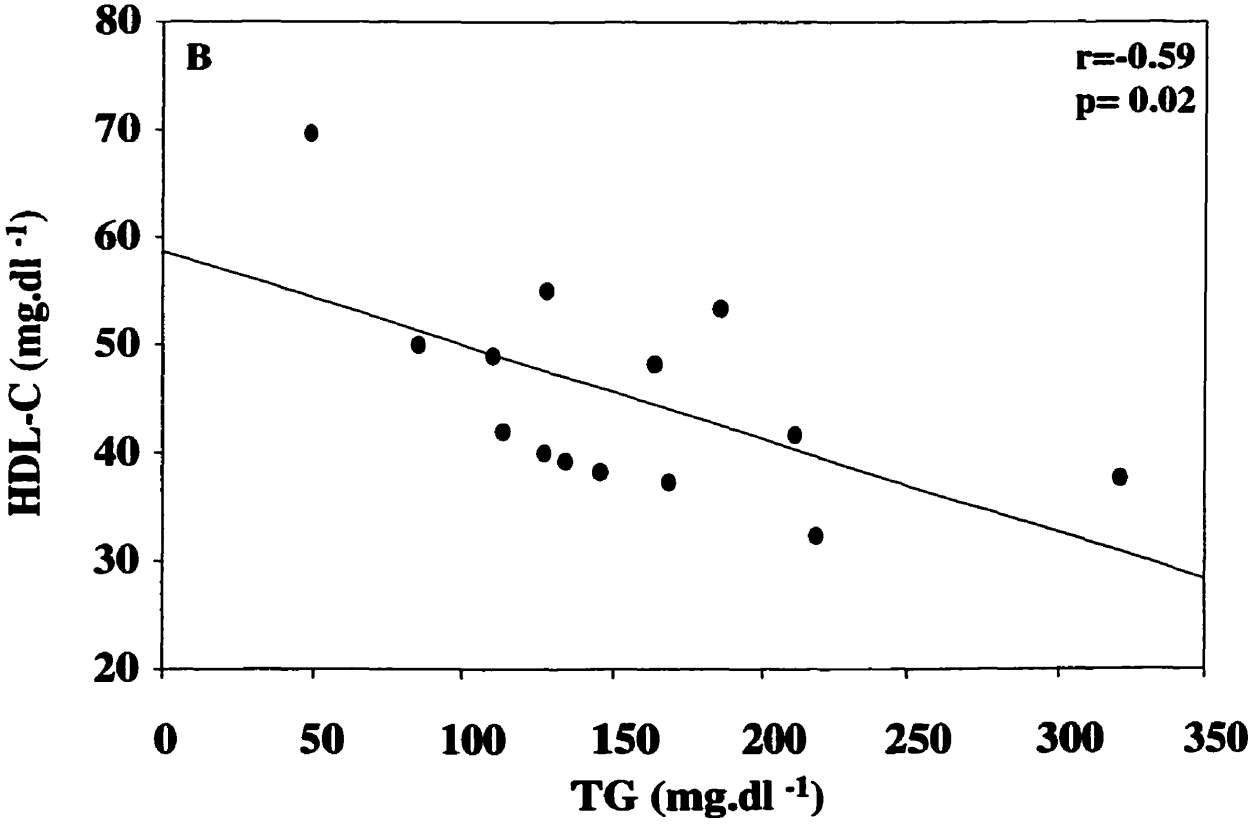
30
40
50
60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TG (mg.dl -1)
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70
60
50
40
30
20
0
50
100
150
200
250
300
350

BRIDGE

The overall goal of the thesis was to explore potential mechanisms that could explain the alterations in circulating cholesterol levels induced by hydrogenated fat consumption. Consequently, in manuscripts 2 and 3, the effect of consumption of different forms of hydrogenated fats on metabolism of free and esterified cholesterol was determined. Recently, it has been suggested that abnormalities in TG metabolism could also be the underlying cause for several putative lipid risk factors for coronary vascular disease (CVD), such as reduced levels of HDL-C and elevated levels of chylomicron and VLDL remnants, IDL and small dense LDL.

The ASP pathway is a key determinant of the rate of fatty acid re-esterification and TG synthesis in the adipocyte. The activity of the ASP pathway is determined by the level of, and cellular responsiveness to plasma ASP. Alterations in plasma ASP levels have been documented in subjects with coronary artery disease. Additionally, in mice lacking ASP, consumption of oral fat loads resulted in delayed postprandial clearance and increased VLDL and LDL cholesterol and TG concentrations. Given that the ASP pathway is modulated by dietary intake, the question arose as to whether consumption of diets differing in *trans* and fatty acid profile, could also alter functioning of the ASP pathway, which in turn would affect lipoprotein profile. To answer this question, the effect of consuming different forms of hydrogenated fats on key components of the ASP pathway, specifically plasma ASP, free fatty acid, glucose and insulin levels, were measured and results correlated with lipid and lipoprotein profile.

CHAPTER 6

HYDROGENATED FAT CONSUMPTION AFFECTS PLASMA ACYLATION STIMULATING PROTEIN LEVELS AND CONSEQUENTLY TRIACYLGLYCEROL AND CHOLESTEROL METABOLISM IN HYPERCHOLESTEROLEMIC WOMEN

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6.1 Abstract

Background: Hydrogenated fat consumption has been shown to negatively influence plasma lipids, however, the mechanisms responsible for this effect are unclear. A possibility involves alterations in the acylation stimulating protein (ASP) pathway, which modulates the rate of *de novo* triacylglycerol (TG) synthesis in adipose tissue.

Objectives: To determine if hydrogenated fat consumption alters the ASP pathway and consequently influences circulating lipid and lipoprotein levels

Design: Fourteen women (65-71 years with LDL-C $\geq$3.36 mmol.L^{-1}) each consumed for 5 weeks a baseline (BL) diet (39% energy as fat, 164 mg chol. 4.2 MJ^{-1}) and reduced fat diets, where 2/3rd of the fat was either soybean oil (SO), low *trans* squeeze (SQM), medium *trans* tub (TM), or high *trans* stick (SM) margarines, or butter (BT). Plasma ASP concentrations were measured using an ELISA sandwich assay, while plasma free fatty acids (FFA), glucose and insulin levels were analyzed using standard kits.

Results: Plasma ASP concentrations were lowest ($p<0.05$) on the SM (33.4 $\pm$12.7 nmol.L^{-1}) compared to SQM (50.7 $\pm$ 15.7 nmol.L^{-1}) diet. Conversely, FFA were highest ($p<0.05$) on the SM diet (0.86 $\pm$ 0.45 mmol.L^{-1}) relative to SQM (0.53 $\pm$ 0.23 mmol.L^{-1}), BT (0.53 $\pm$ 0.25 mmol.L^{-1}) and SO (0.49 $\pm$ 0.19 mmol.L^{-1}) diets. ASP levels were found to be negatively correlated with TC ($r=-0.25$, $p<0.03$), LDL-C ($r=-0.31$, $p<0.01$) and TG ($r=-0.34$, $p=0.03$), while FFA was positively correlated with apo B ($r=0.28$, $p=0.02$), VLDL-C and TG ($r=0.52$ and 0.64, respectively, $p=0.0001$) and negatively correlated with HDL-C ($r=-0.61$, $p=0.0001$). No differences were observed in plasma glucose and insulin concentrations among diets.

Conclusion: These data suggest an impairment of the ASP pathway with high *trans* SM feeding. The resultant increased FFA flux to the liver could be responsible for the higher secretion of hepatic B 100 containing particles, thus explaining the elevated cholesterol levels.

6.2 Introduction

The ability of dietary *trans* fatty acids formed as a result of commercial hydrogenation or by biohydrogenation in the ruminant fat of animals to influence plasma lipid and lipoprotein profile has generated much debate and scrutiny in the scientific community. Available data from human studies (1-10) indicate that *trans* fatty acids and/or hydrogenated fat tend to increase total (TC) and low density lipoprotein cholesterol (LDL-C), and at relatively high concentrations decrease high density lipoprotein cholesterol (HDL-C) levels. Several investigators have also reported modest but significant elevations in plasma triacylglycerol (TG) concentrations (1,4,5,8,9). We have previously reported (11) that alterations in endogenous cholesterol synthesis are not responsible for the adverse effects on circulating lipid levels seen after consumption of different forms of hydrogenated fats. Thus, the mechanisms responsible for the alterations in lipid profile still remain to be elucidated.

In humans, all *trans* fatty acids are diet derived, thus their incorporation into chylomicrons represent the first step in the cascade of metabolic events linking intake and subsequent lipoprotein profile. It has been suggested that abnormalities in TG metabolism could be the underlying cause for several putative lipid risk factors for coronary vascular disease (CVD), such as reduced levels of HDL-C and elevated levels of chylomicron and VLDL remnants, IDL and small dense LDL (12-15). However, to comprehend this relationship, the basic mechanism involved in TG synthesis must be understood. In this regard, recent studies (16-20) have shown that the acylation stimulating protein (ASP) pathway is a determinant of the rate of adipocyte TG synthesis and fatty acid re-

esterification. A small basic protein isolated from human plasma, ASP is generated through the interaction of the complement factors B and D (adipsin) with C3 (precursor protein), all of which are synthesized and secreted from human adipocytes (16). *In vivo* (17) and *in vitro* (18) evidence suggest a role for plasma ASP that markedly increase adipose tissue TG synthesis by increasing activity of diacylglycerol acyltransferase (DGAT), the last enzyme involved in formation of the TG molecule. ASP action is achieved through interaction with the cell membrane, which results in stimulation of the second messenger diacylglycerol (20). Diacylglycerol then mediates stimulation and translocation of protein kinase C producing a downstream stimulation of TG synthesis (20). It has also been reported that ASP increases specific membrane transport of glucose via translocation of glucose transporters from intracellular storage sites to the plasma membrane. The effect on glucose uptake has been shown to be independent and additive to that of insulin (21,22). In addition, Cianflone *et al* (23) recently documented alterations in plasma ASP levels in a number of dyslipoproteinemic states that suggest a relationship between the ASP pathway and other determinants of lipoprotein metabolism. One such determinant is the rate of synthesis and subsequent metabolism of TG rich lipoproteins, which in turn depends, in part, on the amount and type of fatty acid consumed. Thus the objective of the present study was to explore the impact of consumption of commonly available sources of dietary fats subjected to different degrees of hydrogenation on the ASP pathway, specifically plasma ASP, FFA, glucose and insulin levels, and consequently lipid and lipoprotein metabolism.

6.3 Methods

6.3.1 Subjects

Eighteen postmenopausal, moderately hypercholesterolemic (LDL-C ≥ 3.36 mmol.L^{-1}) women who were over the age of 60 years were recruited as previously described (1). Sample losses during shipping and preparation precluded generation of complete data sets for 4 subjects, consequently samples from only 14 women could be included in the present study. At the time of screening, subjects had normal liver, kidney, thyroid, and cardiac function, as well as normal fasting glucose levels. Subjects did not smoke and were not taking any medication known to affect lipid metabolism such as lipid lowering drugs, β-blockers, diuretics or hormones. The protocol was reviewed and approved by the human-investigation review committee of New England Medical Center and Tufts University. All potential subjects were given verbal and written descriptions of the study prior to obtaining written consent. However, during the study period, the subjects, investigators and laboratory personnel were blinded to the dietary phase assignments. A portion of the data focussing on plasma lipid and lipoprotein parameters has been published previously (1,11).

6.3.2 Experimental Design and Diets

Subjects consumed each of six diets according to a randomized cross-over design. Each dietary phase had a duration of 5 weeks, and was separated by washout periods ranging from 2 to 4 weeks, during which subjects consumed their habitual diets. The baseline (BL) diet was designed to approximate that currently consumed in the United States (39% energy as fat, 164 mg chol. 4.2 MJ^{-1}). All other diets were formulated to provide

30 % of energy as fat. Two-thirds of the total fat was replaced by the experimental fat or oil, so that the effect of consuming diets enriched in hydrogenated fats could be assessed within the context of current recommendations for individuals with elevated plasma lipid levels. The specific fats investigated were soybean oil (SO) and soybean oil based margarines in the squeeze (SQM), tub (TM) and stick (SM) forms as well as butter (BT). These fats were especially chosen because as a group they represent a broad range of *trans* and fatty acid profiles. All food and drink were provided by the Metabolic Research Unit of the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University to be consumed on site or packaged for take-out. Energy intakes of the subjects were tailored to individual requirements, as verified by the ability to maintain stable body weight. Analysis of the macronutrient, fatty acid and cholesterol content of the diets (Table 6-1) were carried out by Covance Laboratories (Madison, WI) and Best Foods Research and Engineering Center (Union, NJ). Increasing degrees of hydrogenation resulted in *trans* fatty acid levels ranging from 0.6 to 6.7 % of total energy, which was accompanied by decreased PUFA levels. The cholesterol content of the butter diet was approximately twice that of the other diets. The option to deplete the endogenous cholesterol content of butter was not acted on because cholesterol is an inseparable component of this product. It was felt inappropriate to assess the impact of the fatty acid composition of the product on various physiological parameters without including the cholesterol.

6.3.4 Protocol and Analyses

During the final week of each dietary phase, fasting blood samples were collected in tubes containing EDTA (0.15%) and centrifuged at 3000 rpm at 4°C. Plasma was separated, aliquoted and frozen at -80 °C until further analysis. VLDL-C was isolated from plasma by ultracentrifugation at 39,000 rpm for 18 hours at 4 °C (24). Plasma and the 1.006 $g.mL^{-1}$ infranatant fraction were assayed for TC and TG with an Abbott Diagnostic (North Chicago, IL) spectrum CCX biochromatic analyzer using enzymatic reagents as previously described (25). HDL-C levels were measured after precipitation of the apolipoprotein B (apo B) containing lipoproteins by a dextran-magnesium sulphate procedure (26). Lipid assays were standardized through the Lipid Standardization Program at the Center for Disease Control (Atlanta, GA). Plasma free fatty acid (FFA) was analyzed using an enzymatic method (WAKO NEFA kit, Alpha Laboratories, Eastleigh, UK). Plasma glucose concentrations were determined using an enzymatic colorimetric assay (Roche Laboratories, New Jersey, NY). Plasma insulin levels were determined using a human-insulin specific competitive binding radioimmunoassay (Linco Research Inc, St Louis, MO).

Additional plasma aliquots were used for plasma ASP determination by an ELISA sandwich immunoassay (27). The procedure is as follows: a murine (in house) monoclonal antibody was used as the capture antibody. This antibody was coated at 7 $\mu g.mL^{-1}$ in PBS (100 µg per well in a 96 well plate) overnight at 4°C and blocked with 1% ovalbumin for 2 hours. The plate was washed 3 times with wash solution (0.5% Tween-20 in 0.9% NaCl) between every step. Standard solutions (0.78-27.46 $ng.mL^{-1}$) of

ASP, purified as described previously (27), as well as the test and control plasma samples (precipitated and diluted appropriately) were added at 100 μL per well. The plate was incubated for 1 hour at 37°C and washed, followed by an incubation for 1 hour at 37°C with 100 μL of rabbit antiserum to human ASP (raised against holoprotein), diluted appropriately (1:2000) in PBS with 0.05% Tween-20. The plate was then incubated for 30 minutes at 37°C with 100 μL of goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000, Sigma) diluted in PBS with 0.05% Tween-20. Following the final wash, the color reaction was initiated with 100 μL of O-phenylenediamine (1 $mg.mL^{-1}$) in 100 $mmol.L^{-1}$ sodium citrate and 0.05% Tween-20. After visual development the reaction was stopped with 50 μL of 4N H_2SO_4, and the absorbance was read at 490 nm. ASP concentration versus absorbance was graphed and calculated by linear regression. The within and between assay coefficient of variation was less than 4%.

6.4 Statistical Analysis

Prior to analyses, the distribution of each outcome variable was checked for normality (Proc Univariate, SAS version 6, SAS Institute Inc., Cary, NC) and when violations were noted, logarithmic transformations were performed. One way analyses of variance with the main effect of diet and subject as the repeated measure was employed on each outcome variable using a SAS general linear model program (Proc GLM, SAS version 6, SAS Institute Inc., Cary, NC). Differences in group means were identified using Tukey's test at a significance level of $p<0.05$. Pearson's correlations were performed to test for associations between variables. Untransformed data are presented in text and tables as means ± standard deviation (SD).

6.5 Results

Mean (± SD) plasma lipid and lipoprotein concentrations during the 6 dietary phases are shown in Table 2. The average (± SD) plasma TC and LDL-C levels after consumption of the BL diet were 6.5 ± 0.8 and 4.5 ± 0.8 mmol.L^{-1} respectively. The TC levels were reduced by 9%, 8%, 5% and 2%, respectively, after subjects consumed the SO, SQM, TM and SM diets, relative to the BL diet ($p<0.05$ for BL vs SO, SQM; BT vs SO, SQM, TM; and SM vs SO). The LDL-C concentrations were reduced by 11%, 10%, 6% and 2% for the same order of diets ($p<0.05$ for BL and BT vs SO, SQM; and SM vs SO). Switching from the BL to all five reduced fat diets resulted in a significant lowering of HDL-C. Among the reduced fat diets, plasma HDL-C concentrations were lowest on the SM diet (1.2 ± 0.2 mmol.L^{-1}) and highest on the BT diet (1.3 ± 0.3 mmol.L^{-1}). As a result the ratio of TC to HDL-C was highest after consumption of the SM diet. A trend toward elevated plasma VLDL-C and TG levels was observed with the SM diet, but these differences did not attain statistical significance. Differences in plasma glucose and insulin concentrations were small and statistically indistinguishable among the various diets (data not shown).

Plasma ASP levels (Figure 6-1) were lower after consumption of the SM (33.4 ± 12.7 nmol.L^{-1}) and BT (37.5 ± 15.2 nmol.L^{-1}) diets relative to the SO (48.7 ± 17.0 nmol.L^{-1}) and SQM (50.7 ± 15.7nmol.L^{-1}) diets ($p<0.05$ for SM vs SO, SQM; and BT vs SQM). Reciprocally, plasma FFA levels (Figure 6-2) were significantly elevated with SM feeding when compared to the soybean oil, other 2 margarine, BT and BL diets. Correlation coefficients were calculated between plasma ASP, FFA and serum lipid and

lipoprotein concentrations to determine the presence of any associations. Plasma TC and LDL-C (Figure 6-3A and 6-3B) were found to negatively correlate with ASP levels. Plasma TG concentrations were negatively correlated ($r = -0.34$, $p = 0.03$) with plasma ASP (Figure 6-3C), but positively correlated ($r = 0.64$, $p = 0.0001$) with plasma FFA (Figure 6-4A). A positive association ($r = 0.52$, $p = 0.0001$) was also observed between plasma FFA and VLDL-C levels (Figure 6-4B). By contrast, plasma HDL-C concentrations were inversely related to FFA (Figure 6-4C), VLDL-C and TG (r=-0.61, -0.45 and -0.66 respectively, p=0.0001) concentrations.

6.6 Discussion

ASP is a key player in the newly characterized regulatory system which modulates the rate of triacylglycerol synthesis (23). Additionally, *in vivo* studies (17) indicate that ASP is produced postprandially in the adipose tissue milieu after a fat load, and is associated with increased clearance of postprandial TG levels. Given that alterations in TG metabolism are an integral part of overall lipoprotein metabolism, the present study was conducted to determine if the ASP pathway responds to shifts in dietary *trans* and fatty acid profile and consequently influences lipid and lipoprotein concentrations.

Our study documents, for the first time, lower plasma ASP and higher FFA levels following high *trans* SM feeding, which suggest an impairment in the functioning of the ASP pathway. Consequently, it is speculated that this dysfunction could diminish TG lipolysis by peripheral tissues due to product inhibition of lipoprotein lipase (LPL) by fatty acids. The excessive proportion of dietary fatty acids would be diverted to the liver,

which, having a limited capacity to oxidize or incorporate them into other biomolecules would shunt them into TG synthesis. The resulting excess TG's within the liver would be secreted as an increased number of hepatic B 100 particles (27-28). This would result in an increased rate of VLDL secretion, leading in turn, not only to an increased rate of LDL production, but also to a greater rate of core lipid exchange and lower HDL-C levels, thus explaining the adverse lipid and lipoprotein profile seen after consumption of the SM diet when compared to the SO, SQM and TM diets. In the present study, lower ASP levels were associated with higher plasma TC, LDL-C and TG concentrations, while plasma FFA levels were positively correlated with VLDL-C and TG, and negatively associated with HDL-C concentrations, which is consistent with the above hypothesis. The above hypothesis is also supported by a recent report by Murray and colleagues (29), who have demonstrated a delay in postprandial TG clearance in mice lacking ASP, reflected as an increased circulating free fatty acid level, with corresponding increases in plasma VLDL-C, LDL-C and TG.

The exact mechanism by which dietary fatty acids affect the ASP pathway remains to be fully elucidated. Chylomicrons are known to be potent stimulators of ASP production (30). An active component of chylomicrons which is responsible for increase in both C3 and ASP, has been recently identified (31) as transthyretin (TTR). TTR is a plasma protein that binds to retinol binding protein and complexes thyroxine and retinol. It has been proposed (31) that chylomicron associated TTR may help transport retinoic acid and thyroid hormone, both involved in gene expression, to the adipocyte. As the chylomicron docks with LPL, the TTR complex dissociates and initiates secretion of C3 (precursor

protein of ASP). We speculate that incorporation of *trans* and SFA into structural lipids of the chylomicron membrane could induce alterations in membrane permeability, transporter and receptor function (32,33), which in turn could interfere with docking of TTR to the chylomicron surface, and thereby affect C3 and consequently ASP production.

Another possible explanation involves fatty acid mediated alterations in chylomicron production at the level of the intestine. It has been reported (34) that feeding rats diets rich in PUFA results in the production of large chylomicron particles, whereas SFA feeding produced smaller chylomicron particles. Furthermore, the large chylomicron particles were cleared from plasma at a faster rate than small chylomicrons. Given the structural similarities between *trans* and SFA, it can be speculated that consumption of *trans* fatty acids from hydrogenated fats could result in the production of smaller chylomicrons that are inefficiently hydrolyzed. In fact, Van Greevenbroek et al (35), using an *in vitro* model of the human enterocyte, have demonstrated an increase in TG secretion by *trans* C18:1 n-9 (elaidic acid) compared to *cis* C18:1 n-9 (oleic acid). However, increase in apo B 48 secretion were similar between the *cis* and *trans* C18:1 n-9 fatty acids. Based on these results, the investigators propose that the TG richer chylomicrons induced by *trans* C18:1 n-9 are incompletely lipolyzed causing delayed postprandial clearance. In our study, the consumption of the SM diet, in addition to interfering with TTR formation, may have altered chylomicron composition. The combination of these effects may have been sufficient to significantly impair the production of ASP, and consequently TG clearance.

Unquestionably, further research will be required to delineate the effects of *trans* from other fatty acids in the hydrogenated diets. However, the present data suggests that the differing fatty acid profiles produced as a result of increasing degree of hydrogenation, impairs functioning of the ASP pathway. The metabolic cascade of events following disruption of this pathway, provides a possible novel mechanistic explanation for the adverse plasma lipid profile seen with consumption of the high *trans* and SFA rich diets.

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Table 6-1. Composition of Test Diets as Determined by Chemical Analysis*

Constituent	Soybean oil (SO)	Squeeze Margarine (SQM)	Tub Margarine (TM)	Stick Margarine (SM)	Butter (BT)	Baseline (BL)
	percentage of total daily energy intake					
Protein	15.7	17.1	16.3	16.7	16.9	16.8
Carbohydrate	55.8	51.7	52.9	53.5	53.9	44.6
Fat	28.5	31.2	30.8	29.7	29.1	38.6
Saturated fatty acids	7.3	8.6	8.4	8.5	16.7	15.5
12:0	0.8	1.0	0.7	0.8	1.4	1.8
14:0	0.6	0.7	0.6	0.6	2.5	0.1
16:0	3.7	4.3	4.2	4.0	7.5	7.8
18:0	1.5	1.9	2.3	2.2	3.4	3.6
Monounsaturated fatty acids#	8.1	8.1	8.0	8.5	8.1	15.1
18:1	7.2	7.1	6.7	6.5	7.0	12.2
Polyunsaturated fatty acids#	12.5	13.5	11.1	6.3	2.4	7.0
18:2 (n-6)	10.7	12.1	10.0	5.6	2.1	5.9
18:3 (n-3)	1.7	1.4	1.1	0.7	0.3	1.0
Trans fatty acids	0.6	0.9	3.3	6.7	1.3	1.7
Cholesterol (mg.1000 $kcal^{-1}$)	65.9	68.0	70.3	66.5	121.0	163.8

* Because of rounding, percentages may not total 100

\# Only *cis* isomers are included

Table 6-2 : Serum Lipid and Lipoprotein Profile at the End of Each Dietary Phase

Variable	Soybean Oil (SO)	Squeeze Margarine (SQM)	Tub Margarine (TM)	Stick Margarine (SM)	Butter (BT)	Baseline (BL)
$mmol.L^{-1}$			mean ± SD			
TC	5.94 ± 0.73^{d}	6.02 ± 0.73^{cd}	6.20 ± 0.77^{bcd}	6.40 ± 0.87^{abc}	6.62 ± 0.87^{a}	6.52 ± 0.82^{ab}
LDL-C	4.05 ± 0.84^{b}	4.10 ± 0.58^{bc}	4.27 ± 0.76^{ab}	4.44 ± 0.78^{ac}	4.58 ± 0.75^{a}	4.54 ± 0.80^{a}
HDL-C	1.20 ± 12^{bc}	1.20 ± 0.28^{bc}	1.22 ± 0.25^{bc}	1.17 ± 0.24^{c}	1.27 ± 0.26^{ab}	1.34 ± 0.31^{a}
VLDL-C	0.69 ± 0.19	0.69 ± 0.20	0.70 ± 0.29	0.77 ± 0.35	0.75 ± 0.29	0.69 ± 0.30
TG	1.55 ± 0.60	1.48 ± 0.60	1.59 ± 0.63	1.74 ± 0.72	1.57 ± 0.52	1.49 ± 0.50
TC/HDL-C	0.13 ± 0.03^{b}	0.13 ± 0.03^{b}	0.14 ± 0.03^{ab}	0.15 ± 0.03^{a}	0.14 ± 0.03^{ab}	0.13 ± 0.03^{b}

Within a row, values with different superscripts are significantly different ($p<0.05$), n=14

To convert values for cholesterol to milligram per deciliter, multiply by 38.67

To convert values for triacylglycerol to milligram per deciliter, multiply by 88.54

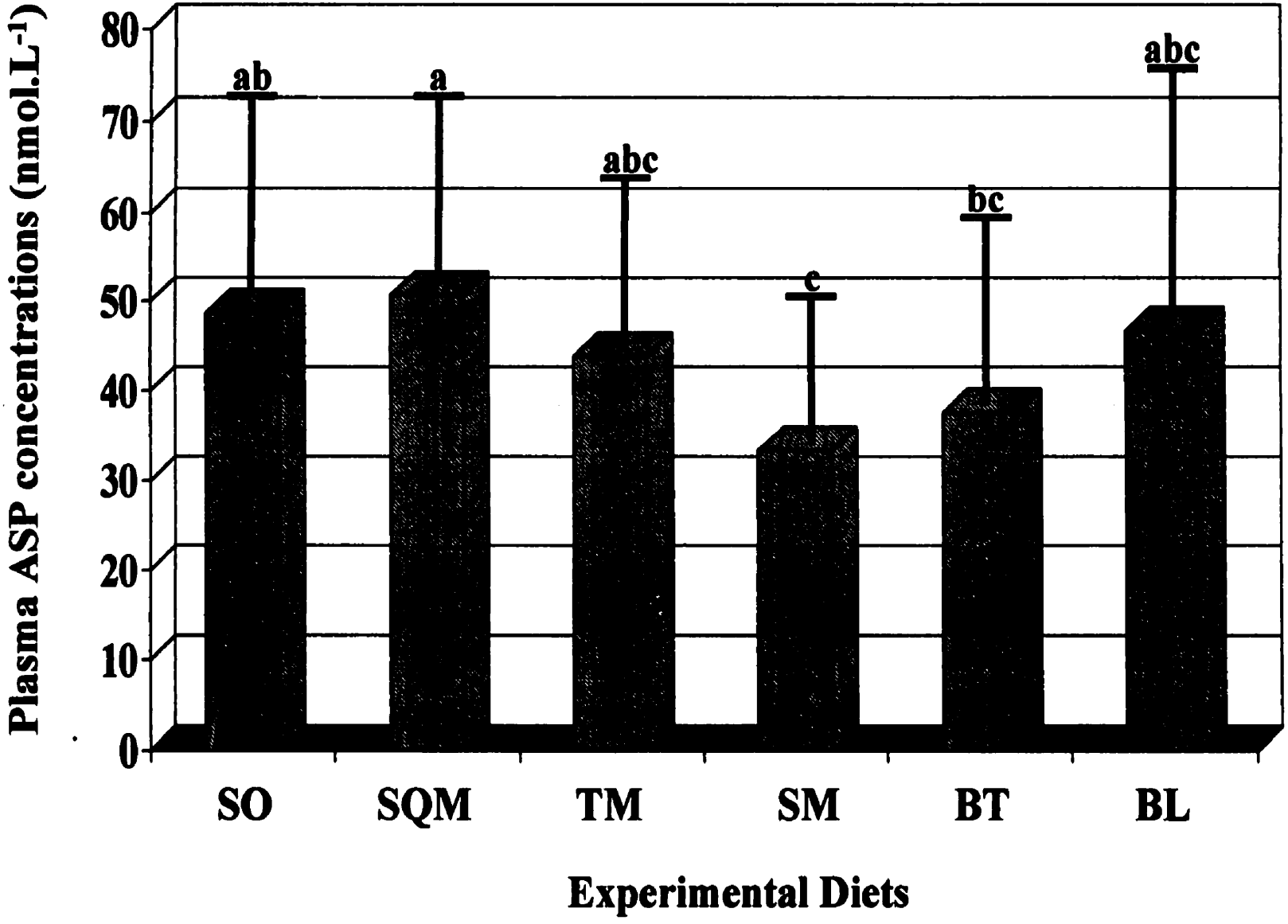
6.9 Figure Legends

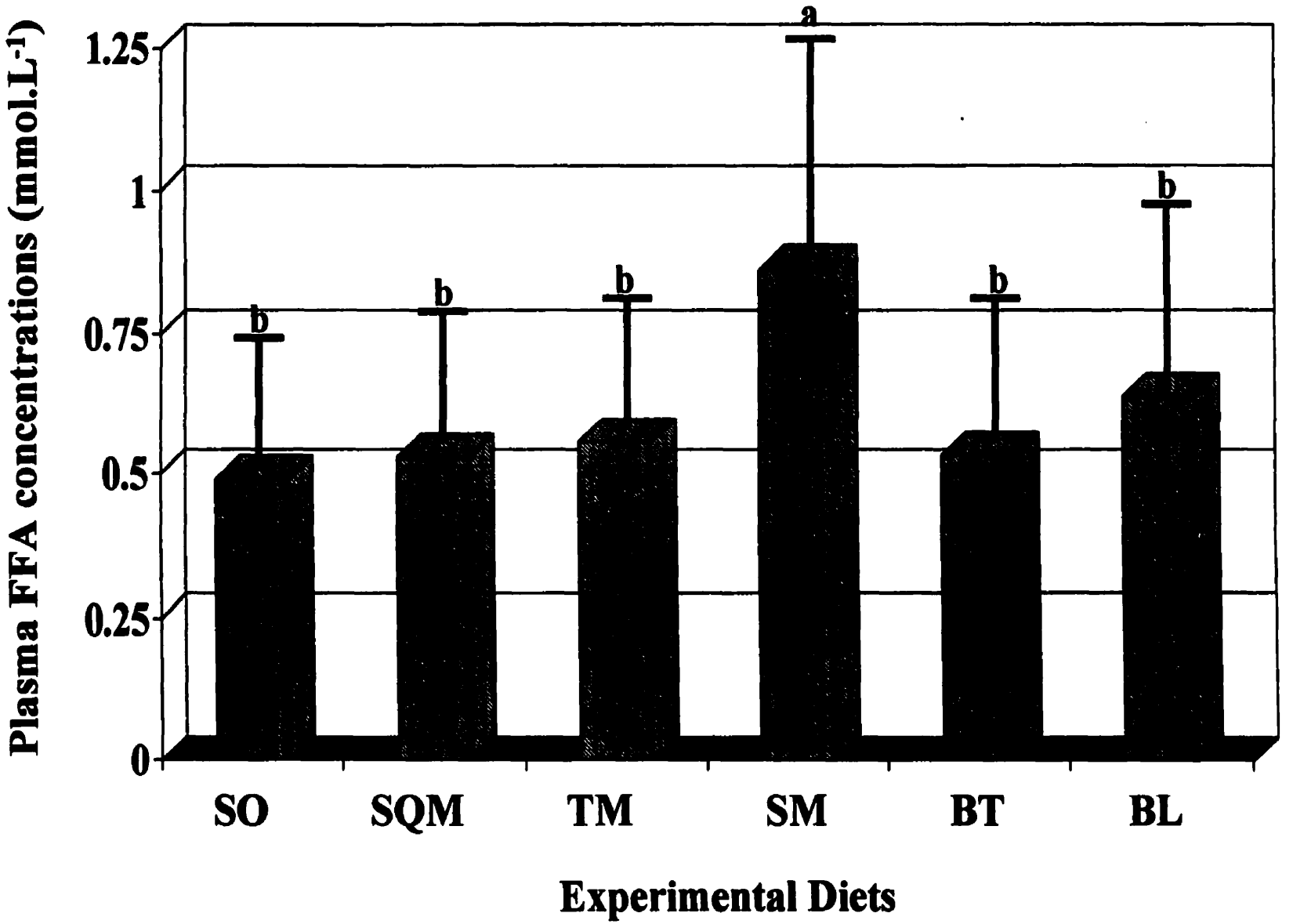
Figure 6-1: Plasma ASP concentrations ($nmol.L^{-1}$) at the end of each dietary phase. Data are presented as means ± SD, n =14. Diet abbreviations are as follows; SO: soybean oil, SQM: squeeze margarine, TM: tub margarine, SM: stick margarine, BT: butter, BL: baseline. Bars with different letters are significantly different ($p<0.05$).

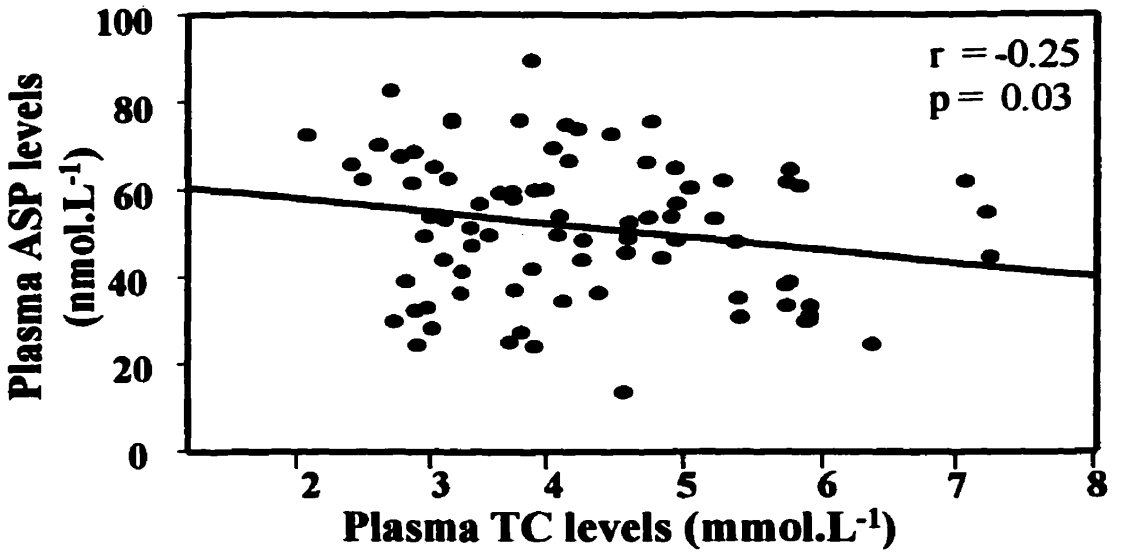
Figure 6-2: Plasma FFA concentrations ($mmol.L^{-1}$) at the end of each dietary phase. Data are presented as means ± SD, n =14. Diet abbreviations are as follows; SO: soybean oil, SQM: squeeze margarine, TM: tub margarine, SM: stick margarine, BT: butter, BL: baseline. Bars with different letters are significantly different ($p<0.05$).

Figure 6-3: Association between plasma ASP levels ($nmol.L^{-1}$) and (**A**) plasma total (TC), (**B**) low density lipoprotein cholesterol (LDL-C), and (**C**) plasma triacylglycerol (TG) levels in $mmol.L^{-1}$. Data are presented for 14 subjects after consumption of the six experimental diets.

Figure 6-4: Association between plasma FFA levels ($mmol.L^{-1}$) and (**A**) plasma triacylglycerol (TG), (**B**) very low density lipoprotein cholesterol (VLDL-C) and (**C**) plasma high density lipoprotein cholesterol (HDL-C) levels in $mmol.L^{-1}$. Data are presented for 14 subjects after consumption of the six experimental diets.


Plasma ASP concentrations (nmol.L-1)
80
70
60
50
40
30
20
10
0
ab
a
abc
c
bc
abc
SO
SQM
TM
SM
BT
BL
Experimental Diets


Plasma FFA concentrations (mmol.L-1)
1.25
1
0.75
0.5
0.25
0
a
b
b
b
b
b
SO
SQM
TM
SM
BT
BL
Experimental Diets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p = 0.03
Plasma ASP levels (nmol.L-1)
Plasma TC levels (mmol.L-1)
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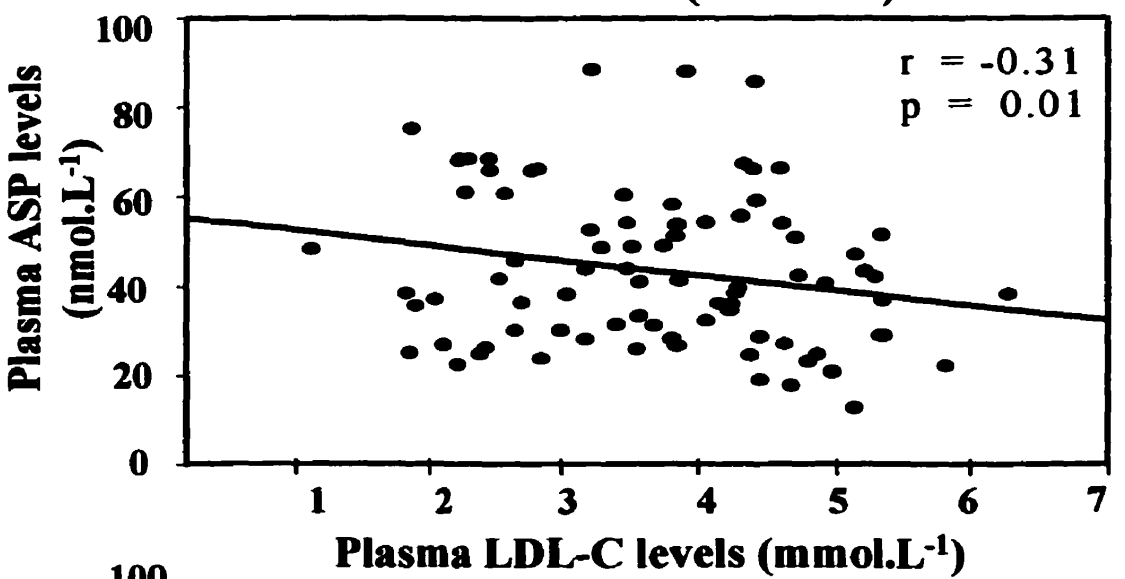
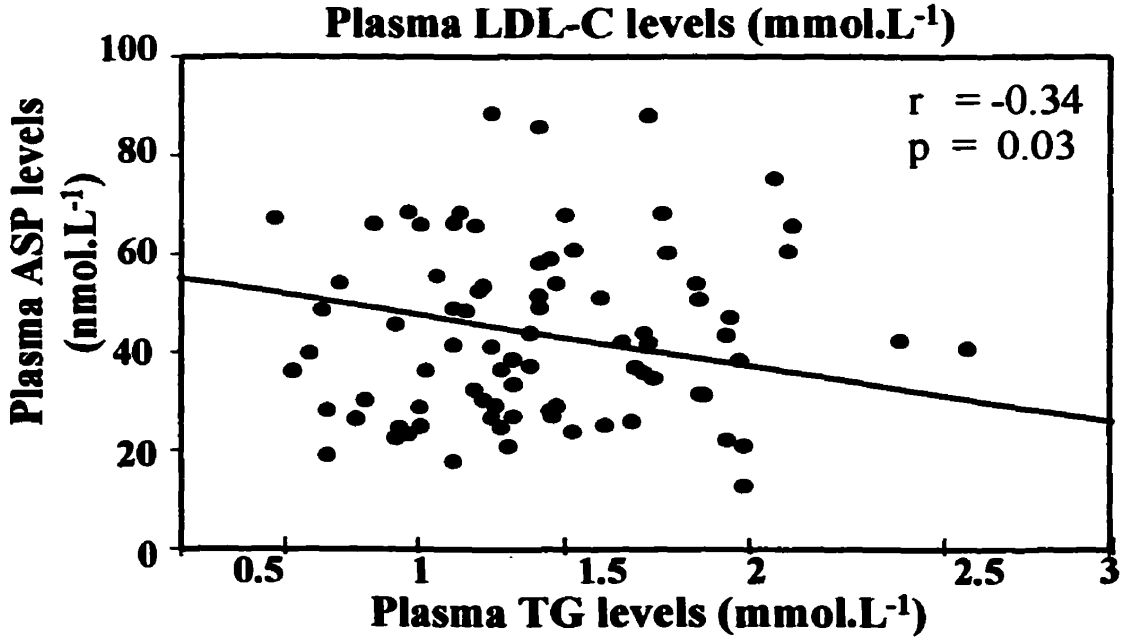
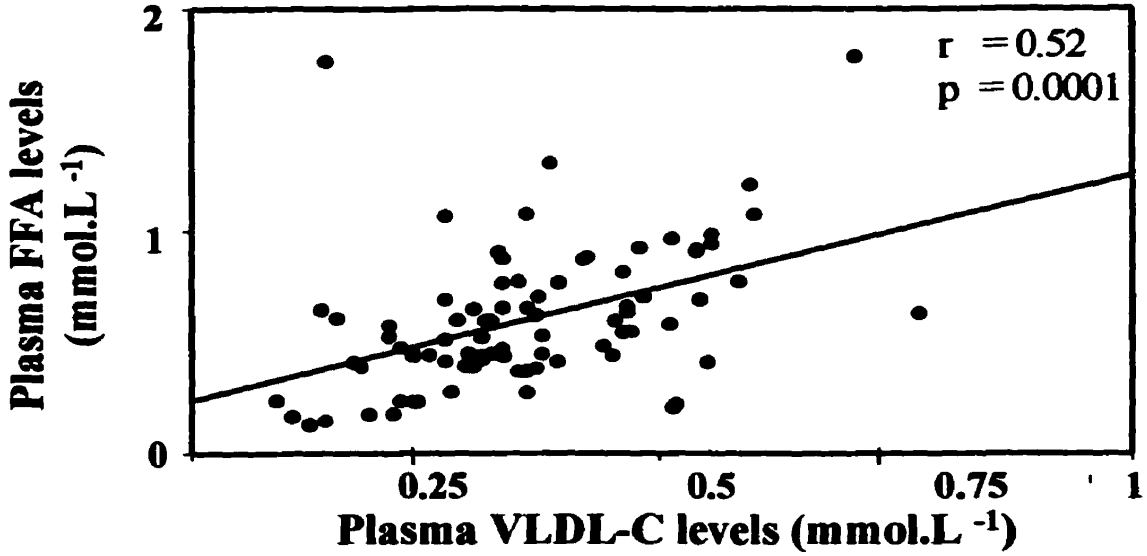
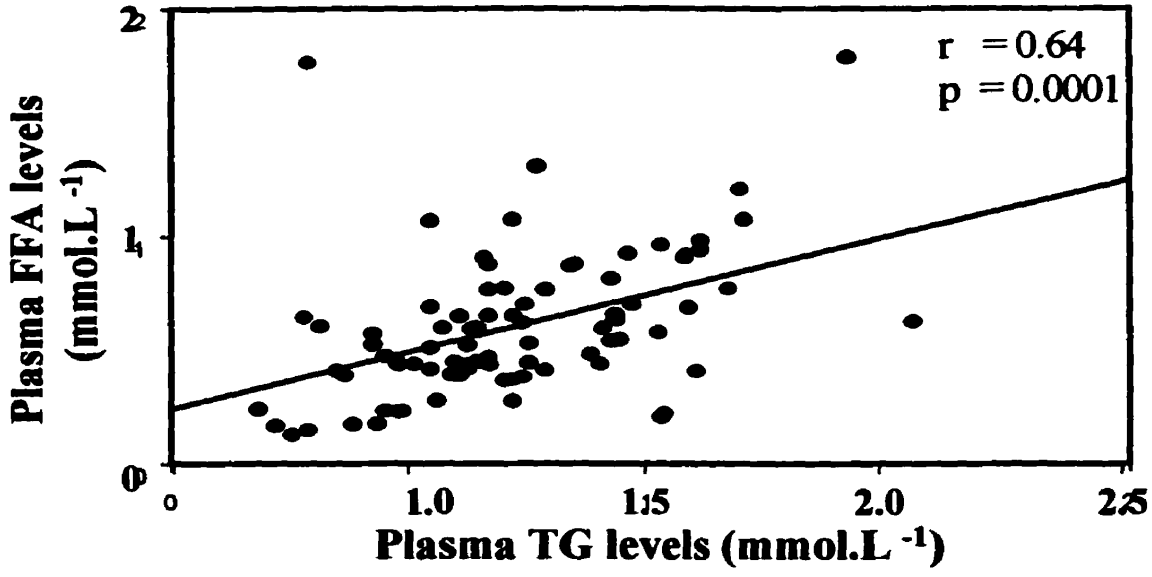
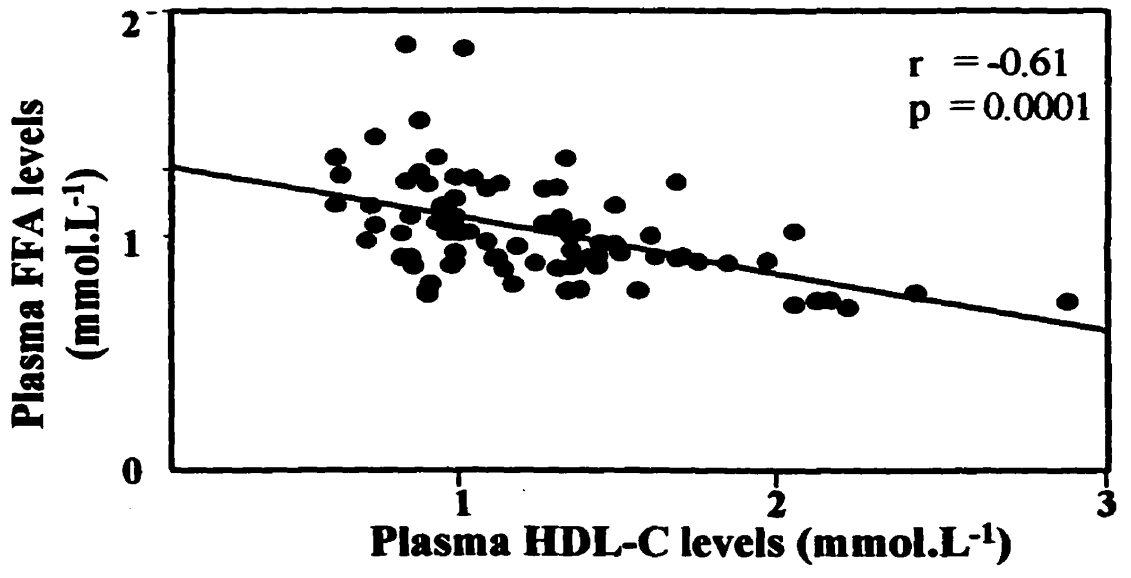
20
40
60
80
100
2
3
4
5
6
7
8


r = -0.31
p = 0.01
Plasma ASP levels (nmol.L-1)
Plasma LDL-C levels (mmol.L-1)
0
20
40
60
80
100
1
2
3
4
5
6
7


r = -0.34
p = 0.03
Plasma ASP levels (nmol.L-1)
Plasma TG levels (mmol.L-1)
0
20
40
60
80
100
0.5
1
1.5
2
2.5
3


r =0.52
p =0.0001
Plasma FFA levels (mmol.L-1)
2
1
0
0.25
0.5
0.75
1
Plasma VLDL-C levels (mmol.L-1)


r =0.64
p =0.0001
Plasma FFA levels (mmol.L-1)
2
1
0
0
1.0
1.5
2.0
2.5
Plasma TG levels (mmol.L-1)


r =-0.61
p =0.0001
Plasma FFA levels (mmol.L-1)
2
1
0
1
2
3
Plasma HDL-C levels (mmol.L-1)

CHAPTER 7

OVERALL SUMMARY AND CONCLUSIONS

The present study examined the effect of reduced fat diets enriched with either soybean oil (SO), low *trans* squeeze (SQM), medium *trans* tub (TM) or high *trans* stick (SM) margarines, or butter (BT) relative to a high fat baseline (BL) diet on cholesterol and triglyceride metabolism in moderately hypercholesterolemic, postmenopausal women. An understudied population group was assessed under carefully controlled metabolic conditions, using diets that conformed to current dietary guidelines for CVD risk reduction. Our results offer new evidence into the mechanisms responsible for the adverse plasma lipid and lipoprotein profile seen after consumption of different forms of hydrogenated fat diets.

However, prior to measuring alterations in cholesterol metabolism, it was deemed appropriate to validate the tool used to measure these changes. Thus, our first objective was to determine if the deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women. The results provided in Chapter 3 demonstrate that both methods compare well over the short-term for measurement of cholesterol synthesis. An added advantage of the DI method is that it gives a direct estimate of synthesis unlike cholesterol precursor levels which are relative indices of synthesis. other investigators have also demonstrated good agreement between the DI method and plasma mevalonic acid concentrations (1), sterol balance (2) and MIDA (3) techniques. Taken together, these results provide evidence that the DI method is reliable and suitable for relatively uninvasive, short-term detection of

cholesterol synthesis. Furthermore, this methodology can be used to study factors known to influence human cholesterol metabolism and consequently the risk of developing CVD.

Having demonstrated that our choice of methodology was technically sound, our next objective was to determine if hydrogenated fat consumption affected rates of endogenous cholesterol biosynthesis. A new equation, which takes into account uptake of D label from the FC pool, was developed to determine cholesterol esterification rates. Our final objective was to determine the effect of consumption of hydrogenated fats on TG metabolism by measuring changes in the concentrations of key components of the ASP pathway, namely plasma ASP, FFA, glucose and insulin. The overall changes observed in both TG and cholesterol metabolism, and the subsequent impact on lipoprotein profile, have been depicted in Figure 7-1. We speculate that incorporation of *trans* and SFA into the chylomicron membrane interferes with the normal binding of TTR to the chylomicron surface and thus affects ASP induction and FFA uptake. This hypothesis is supported by the lower plasma ASP and higher plasma FFA levels observed after consumption of the SM diet, as reported in Chapter 6. Consequently, the hydrolyzed FFA cannot be taken up by the adipose tissue and are diverted to the liver, where they are secreted as an increased number of hepatic B100 containing particles (4). This increase in circulating VLDL and LDL levels could explain the downregulation of endogenous cholesterol synthesis seen with consumption of the SM, BT and BL diets.

The lowering of cholesterol esterification rates could interfere with the normal maturation and transformation process of HDL particles in plasma, and thereby explain the lowered HDL levels, typically observed with high *trans* feeding. Furthermore, the reduction in

esterification rates could result in an expanded pool of hepatic unesterified cholesterol when hydrogenated and saturated fat diets are consumed, which is thought to contribute to the down regulation of the LDL receptor and subsequently increase circulating cholesterol levels (5).

Several studies (5,6) have also shown that SFA rich diets tend to decrease rates of cholesterol esterification. Although a trend toward lower AER and ER was observed in our study after consumption of the SFA rich BT and BL diets, differences did not attain statistical significance, possibly due to the high variability among subjects. However, if SFA diets lower esterification rates, then one would expect the lowering of HDL-C levels with SFA to parallel those seen with *trans* fatty acid feeding, but the reverse is usually observed. This paradox is not readily explained but is presumed to occur via alterations in the positional specificity of LCAT by different fatty acid species, and the subsequent substrate available for the CETP reaction (7), which has been discussed in detail in Chapter 5.

Taken together, our results demonstrate that the increases in circulating cholesterol levels, commonly observed with consumption of *trans* and SFA rich diets are due to alterations in endogenous synthesis of free cholesterol as well as the functioning of the ASP pathway. The decrease in HDL-C levels, which is a characteristic feature of high *trans* consumption are possibly related to reduction in cholesterol esterification rates. Thus intake of *trans* fatty acids at levels > 6.7% of energy from total fat (SM diet) results in higher risk ratios of TC/LDL-C and LDL-C/HDL-C, and consequently CVD risk when compared to SFA consumption. These findings support present dietary guidelines to substitute unhydrogenated

oil for hydrogenated or saturated fat in processed foods. Additionally, the recommendation to substitute softer for harder margarines and cooking fats seems justified.

7.1 Limitations and Future Work

The overall objective of the thesis was to evaluate the impact of hydrogenated fat consumption on *in vivo* lipid metabolism. Diets were formulated so as to provide 2/3rd of fat calories from the experimental fat or oil. Consequently, the fatty acid profile of the diets resembled that of the experimental fat or oil utilized, with varying *trans* and fatty acid profile. A limitation of this protocol is the difficulty in determining whether the cholesterol raising effects of hydrogenated fats are due to the *trans* fatty acid or lowered PUFA content of the experimental fat. However, recent well-controlled metabolic studies have demonstrated that *trans* fatty acids independently affect plasma TC and LDL-C concentrations relative to MUFA and PUFA (8-10), and at high levels lower HDL-C concentrations relative to SFA (9,11,12). Since consumption of the hydrogenated fat diets in our study produced similar changes in plasma lipid and lipoprotein profile as seen in studies evaluating the independent effect of *trans* fatty acids, we feel that these changes are more likely attributable to the *trans* fatty acid rather than to the lowered PUFA content of the margarine enriched diets. Additionally, in the present study, only fats derived from soybean oil were studied, to minimize dietary variables other than hydrogenation, and because soybean oil is one of the major oils that is hydrogenated. Hydrogenated fats made from other vegetable oils would be expected to have different fatty acid profiles, however, previous work has suggested that consumption of hydrogenated fats made from a wide range of oils has similar effects (13).

Another limitation of the present work is that relatively high levels of a single fat were used in each diet, in contrast to the more likely situation in real life in which multiple sources and forms of fat are freely exchanged in the diet. Our approach allowed us to assess different forms of commercially available fats, and we feel that our conclusions can be extrapolated to mixed products. The reason being that there is a wide range of absolute intake of fat and considerable variability in patterns of food intake among and within subgroups of the population. While precise data on the sources and quality of *trans* fatty acid intake levels are lacking, it appears that the range of intakes of *trans* fatty acids in our study spans that currently consumed in North America (14,15).

The sample population is also an important factor in the interpretation of the results. We used an understudied population, namely moderately hypercholesterolemic, postmenopausal women. However, study subjects were slightly overweight (BMI of 26.5 ± 2.4 $kg.m^{-2}$) and they may have responded differently to some of the parameters measured. For example, studies (16,17) have shown disparate responses in plasma ASP concentration between obese and normal subjects to oral fat loads. Therefore, results obtained in our study may be representative of hypercholesterolemic individuals, but care must be taken in extrapolation of results to other population groups.

Another concern is the possibility of a pre-treatment effect due to the variation in duration of washout periods between dietary phases. Several investigators (18,19) have demonstrated that plasma lipoprotein levels stabilize by week 4 following dietary manipulation. Consequently the use of data obtained at week 5 of each dietary phase obviates the need to

correct for dietary intake prior to starting a phase and for any residual effect from an earlier dietary treatment.

One of our arguments for the elevations in circulating cholesterol levels with hydrogenated fat diets, is the lack of induction of ASP and re-routing of FFA to the liver resulting in increased production of apo B100 rich lipoprotein particles. While this hypothesis is supported by the higher FFA concentrations with SM feeding and observed associations between FFA and plasma cholesterol and triglyceride levels, we do not have direct evidence to support this hypothesis. Future studies using labeled dietary fatty acids, and measuring the appearance of label in plasma apo B containing lipoprotein fractions, as well as in tissues such as adipose, liver, and muscle, obtained from ASP deficient mice fed different forms of hydrogenated fats, are warranted to substantiate the FFA flux theory.

Another area that requires further examination is the relative effects of individual *trans* isomers on CVD risk profile (20). Much of the data on the effect of hydrogenated fat and plasma lipid profile have been derived utilizing mixtures of isomers or specifically elaidic acid (*t*-C18:1n-9). Scant information exists on the effects of natural versus commercially produced *trans* fatty acids. Such studies comparing the effect of different *trans* fatty acid species on plasma lipid and lipoprotein concentrations are important because elaidic acid (*t*-C18:1n-9) is the major isomer found in commercially produced hydrogenated products, whereas in products resulting from biohydrogenation in the ruminant fat of animals nearly 50 to 60 % of the total *trans* C18:1 isomers are exclusively vaccenic acid (*t*-C18:1n-7). Thus, one of the major distinguishing features between products of biohydrogenation and commercial hydrogenation is the position of the double bond within the same fatty acid.

Another is the distribution of the *cis* C18:1 isomers. In hydrogenated fat only 60 % of the C18:1 isomers are *c*-C18:1n-9, whereas in animal fats such as butter, *c*-C18:1n-9 accounts for almost 90 % of the total. Willet et al (21) have shown that vaccenic acid (*t*-C18:1n-7) does not appear to be a risk factor for CVD. In addition, Van Greevenbroek et al (22) have demonstrated alterations in chylomicron TG composition with elaidic acid (*t*-C18:1n-9) but not vaccenic acid (*t*-C18:1n-7), which raises the question of whether all *trans* fatty acid isomers have deleterious effects on lipoprotein metabolism.

While these are important issues that remain to be resolved, results obtained from the present study, despite the limitations, have contributed to knowledge in the field of *trans* fatty acid research, specifically related to cholesterol and triglyceride metabolism. Future research can in part be directed by results obtained here, and the limitations of the current study can be addressed in the execution of upcoming studies. In addition, research examining the uptake of labeled dietary fatty acids into various tissue and lipoprotein fractions, as well the effect of specific *trans* fatty acid isomers on lipoprotein metabolism, may offer further insight into the body's handling of *trans* fatty acids from hydrogenated fats.

7.2 References

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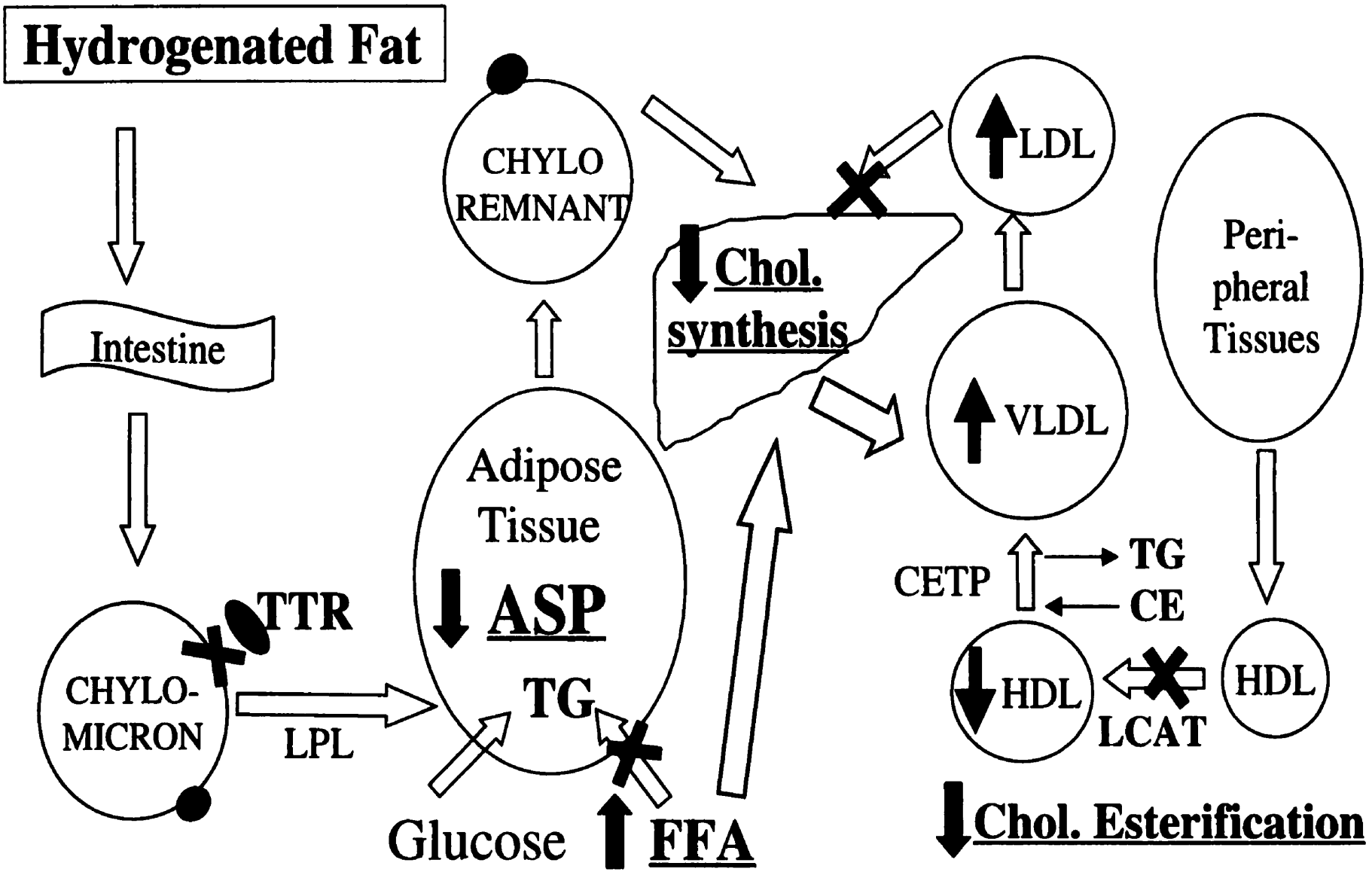
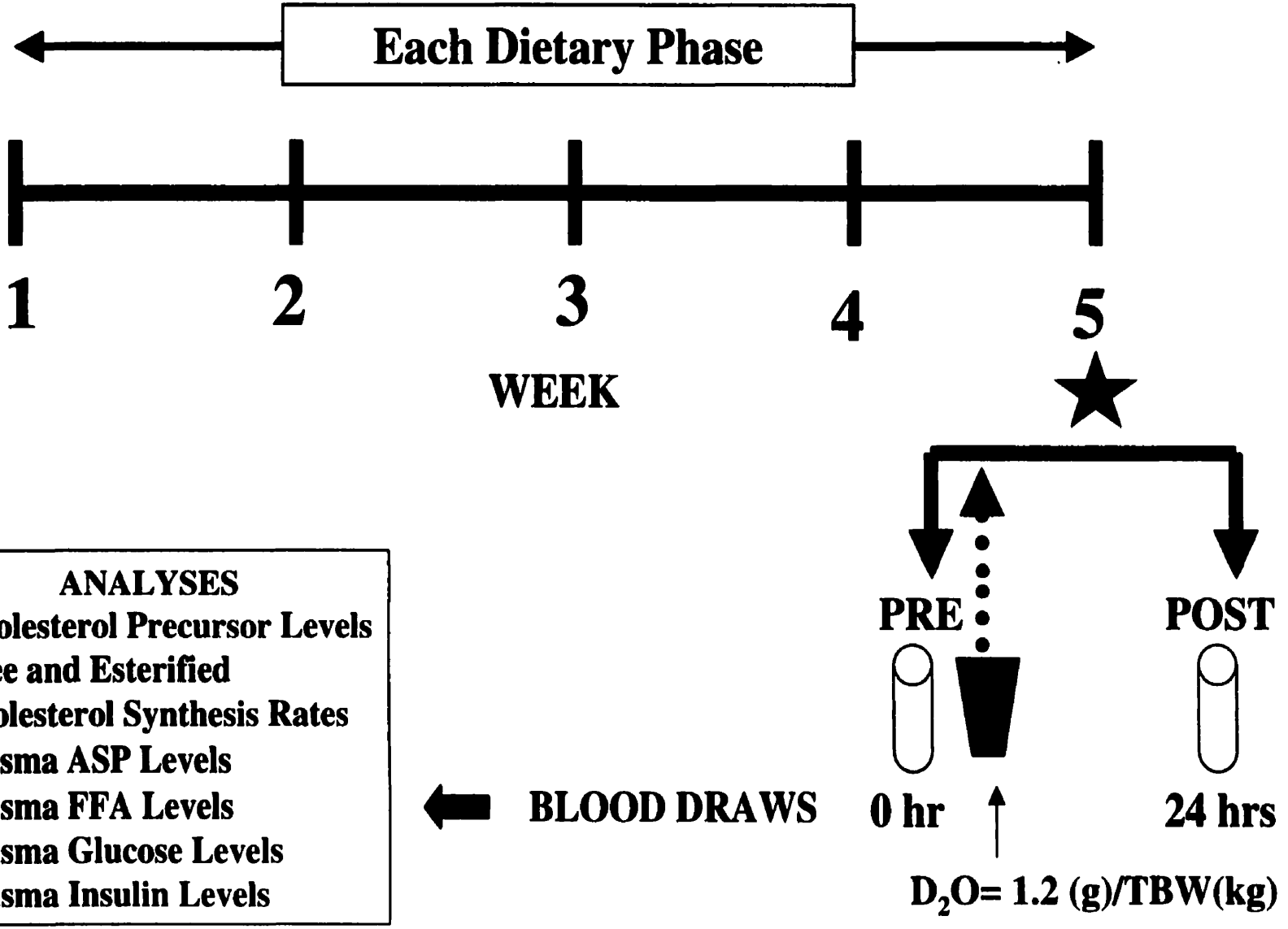
7.3 Figure Legend

Figure 7-1: Effect of *trans* fatty acids from hydrogenated fats on triglyceride and cholesterol metabolism, specifically plasma ASP and FFA levels, and endogenous synthesis rates of free and esterified cholesterol. Abbreviations are as follows: TTR: transthyretinin, LPL: lipoprotein lipase, FFA: free fatty acids, ASP: acylation stimulating protein, CETP: cholesteryl ester transfer protein, LCAT: lecithin cholesterol acyl transferase, and CE: cholesteryl ester.


Hydrogenated Fat
Intestine
CHYLO-
MICRON
TTR
LPL
CHYLO
REMNANT
Adipose
Tissue
ASP
TG
Glucose
FFA
Chol.
synthesis
LDL
VLDL
CETP
TG
CE
HDL
LCAT
HDL
Peri-
pheral
Tissues
Chol. Esterification

APPENDIX 1

Figure A1-1: Overall thesis experimental protocol



ANALYSES

- Cholesterol Precursor Levels
- Free and Esterified Cholesterol Synthesis Rates
- Plasma ASP Levels
- Plasma FFA Levels
- Plasma Glucose Levels
- Plasma Insulin Levels

APPENDIX II

Determination of caloric requirement for each subject

Caloric requirement was approximated by multiplying BEE by 1.5 for moderate activity

BEE was derived using the Harris-Benedict equation

BEE (females) = 655 + (9.6xW) + (1.7x H) - (4.7xA)

where: W = weight (kg)

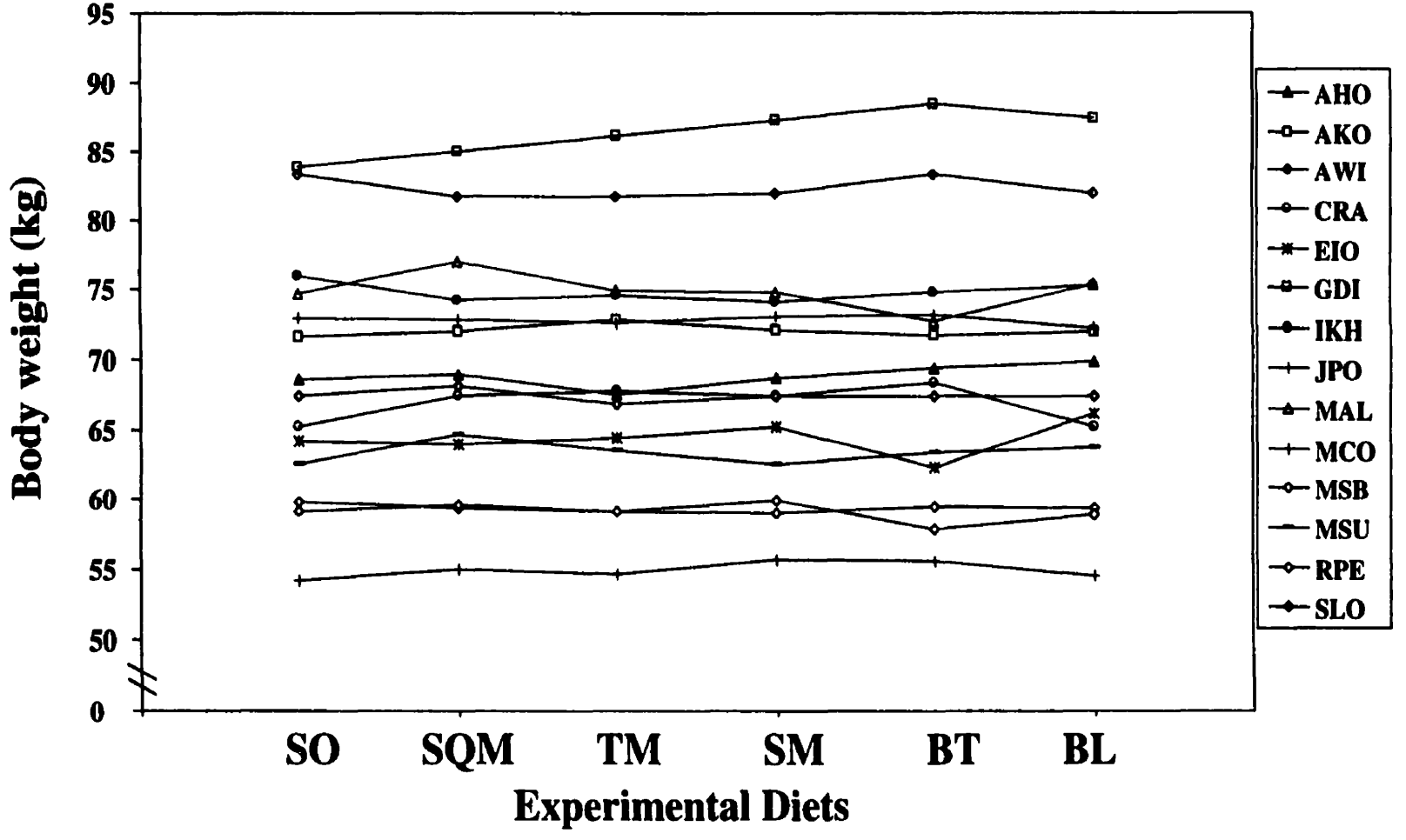
H = height (cm)

A = age (years)

APPENDIX III

Body weight of individual subjects at the end of each dietary phase

(n=14)



APPENDIX IV

Sample of the three day rotating menu

MEAL	DAY 1	DAY 2	DAY 3
Breakfast	*Cream of wheat + fat Maple syrup Raisins Heavy cream Apple sauce Whole wheat bread Grape jelly Skim milk Banana	Orange juice *Muffin + fat Strawberry jam Grapefruit Whole milk	Cranberry juice Cold cereal Heavy cream Raisins Skim milk
Lunch	*Poarched chicken + fat White rice, onions, peas Pita bread Orange	*Spaghetti + fat Tomato sauce Broccoli spears, Olive oil Lettuce salad Fat free Italian dressing Skim milk Apple	Tuna packed in water Fat free mayonnaise Whole wheat bread *White rice + fat Lettuec salad Olives Fat free french dressing * Cinnamon cookie + fat Canned peaches
Dinner	Tenderloin *Roast potatoes + fat Carrots *Dinner roll + fat, garlic Pumpkin muffin	Garlic chicken Corn Carrots *Biscuit + fat Sliced peaches	Turkey breast *Macaroni + fat Parmesan cheese Carrots Broccoli spears *Chocolate pudding + fat

*Denotes items containing the experimental fat. All foods containing the experimental fat (+ fat) were either prepared with the fat or had the fat incorporated into the food prior to serving.

APPENDIX V

Fatty acid composition of experimental oil and fats as determined by chemical analysis

VARIABLE	SOYBEAN OIL	SQUEEZE MARGARINE	TUB MARGARINE	STICK MARGARINE	BUTTER
			(percent)		
C4:0	n.d	n.d	n.d	n.d	4.06
C6:0	n.d	n.d	n.d	n.d	2.22
C8:0	n.d	n.d	n.d	n.d	1.16
C10:0	n.d	n.d	n.d	n.d	2.35
C11:0	n.d	n.d	n.d	n.d	0.20
C12:0	n.d	n.d	n.d	n.d	2.54
C14:0	n.d	n.d	n.d	n.d	9.38
C14:1	n.d	n.d	n.d	n.d	1.07
C15:0	n.d	n.d	n.d	n.d	1.22
C16:0	8.55	10.24	9.82	9.29	26.01
C16:1	0.12	0.09	0.09	0.12	1.83
C17:0	n.d	n.d	n.d	n.d	1.26
C18:0	3.56	5.02	6.96	6.80	11.25
C18:1 (total)	28.88	22.94	33.11	54.54	23.98
C18:1Δ6t	n.d	n.d	1.40	2.49	0.30
C18:1Δ9t	n.d	n.d	1.77	5.96	0.28
C18:1Δ10t	n.d	n.d	1.73	6.03	0.56
C18:1Δ11t	n.d	n.d	1.84	6.05	1.21
C18:1Δ12t	n.d	n.d	0.77	3.89	0.27
C18:2 (total)	46.87	52.91	41.46	23.99	3.20
C18:2Δ9Δtt	n.d	n.d	0.98	1.09	n.d
C18:2Δ9Δtc	0.15	0.33	0.5	0.26	n.d
C18:2Δ9Δ12 tc	0.11	0.29	0.41	0.30	n.d
Total trans	0.26	0.62	9.40	26.07	2.62
C18:3	9.00	6.77	5.38	2.49	0.47
C20:0	0.38	0.33	0.33	0.36	n.d
C20:1	0.50	0.32	0.33	0.53	n.d
C22:0	0.31	0.40	0.37	0.31	n.d
C24:0	0.07	0.04	0.12	0.12	n.d

APPENDIX VI

Differential Effects of Individual Trans Fatty Isomers on Lipoprotein Assembly and Metabolism

Nirupa R. Matthan and Peter J. H. Jones

Published in Nutrition Reviews 1999; 57:282-284

11.1 Abstract

Recent findings indicate that certain (t-C18:1Δ9) but not all (t-C16:1Δ9 and t-C18:1Δ11) *trans* fatty acid isomers influence intestinal lipoprotein secretion. A mechanism is suggested that appears sensitive to properties associated not only with geometrical configuration but also fatty acid chain length and double bond position. This mechanism could explain in part the conflicting results seen among studies investigating the effects of total dietary *trans* fatty acids on serum lipid and lipoprotein concentrations.

11.2 Brief Critical Review

The health consequences of *trans* fatty acid consumption have generated much debate and scrutiny in the media and scientific press. This controversy has been largely due to the conflicting results regarding effects or lack thereof of dietary *trans* fatty acids on plasma lipid and lipoprotein profile and subsequent risk of Coronary Heart Disease (CHD).[1] Evidence marshaled to implicate *trans* fatty acids in the etiology of CHD is derived from two sources, epidemiological and metabolic studies. In the case of epidemiological studies, the multiple problems of bias, misclassification, improper time sequence and confounding have made it difficult to establish causation.[2] However, the majority of metabolic data indicate that *trans* fatty acids as hydrogenated fat tend to have

adverse effects on plasma lipid levels relative to their *cis* or native unhydrogenated counterparts.[3-6] Dietary *trans* fatty acids generally increase levels of low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C) and triglycerides (TG), and decrease levels of high density lipoprotein cholesterol (HDL-C) Unfortunately, available studies have focussed primarily on surrogate risk factors for CHD with limited evidence available delineating the mechanism of action of *trans* fatty acids. Suggested mechanisms of *trans* compared with *cis* fatty acids include LDL receptor down-regulation, decreased lecithin cholesterol acyltransferase (LCAT) for which *trans* fatty acids are poor substrates, and increased cholesterol ester transfer protein (CETP) when compared with fatty acids having *cis* double bonds.[7,8] Much of these data have been derived from studies utilizing mixtures of isomers or specifically elaidic acid (t-C18:1Δ9). Scant information exists on effects on plasma lipid and lipoproteins of other positional *trans* isomers found in both products of commercial and biohydrogenation.

In this regard, the recent paper by van Greevenbroek *et al* indicating that the differential effects of *trans* fatty acids on intestinal lipoprotein secretion are influenced by fatty acid chain length and position of the double bond offers novel findings. [9] In humans, all *trans* fatty acids are diet- derived, [10] thus their incorporation into chylomicrons represents the first step in the cascade of metabolic events relating dietary *trans* fatty acid intake and subsequent lipoprotein profile. In the present study[9], the authors have primarily focussed on this aspect of *trans* fatty acid metabolism by utilizing two sets of dietary fatty acids C_{16} (C16:0, c-C16:1Δ9 and t-C16:1Δ9) and C_{18} (C18:0, c-C18:1Δ9, t-C18:1Δ9, c-C18:1Δ11 and t-C18:1Δ11). A human intestinal enterocyte model was developed using

polarized Caco-2 cells. This model enabled the investigators to determine whether the effects of *trans* fatty acids on lipoprotein secretion more closely resembled that of saturated or of *cis* unsaturated fatty acids. The system also allowed for evaluation of the effects of carbon chain length and positionality of the double bond within the fatty acid molecule on chylomicron production and composition.

The major finding was that, relative to the C18:0 saturated fatty acid control, the increase in triglyceride secretion by t-C18:1Δ9 was greater than seen with c-C18:1Δ9. However, increases in apo B-48 and apo B-100 secretion were similar between the *cis* and *trans* C18:1Δ9 fatty acids relative to control. Based on these results, the investigators propose that the TG- rich chylomicrons induced by t-C18:1Δ9 feeding are inefficiently hydrolyzed causing delayed postprandial clearance. Retarded TG-rich chylomicron clearance may contribute to the lower HDL-C and higher TG concentrations observed in some *trans* fatty acid feeding trials. The lack of induction of TG and apo B secretion by t-C16:1Δ9 and t-C18:1Δ11 seems to indicate an effect on some other aspect of the lipoprotein assembly line.

The finding of increased TG secretion and incorporation into chylomicrons by specific (t-C18:1Δ9) but not all (t-C16:1Δ9 and t-C18:1Δ11) *trans* fatty acid isomers is provocative, and offers a possible explanation for the variation in lipid and lipoprotein profiles seen across studies. However, the Caco-2 cell line has its specific limitations when compared to the human intestine. These include a long period of post-confluent differentiation, unlike the human enterocyte which is replaced every 36 hours, and also a lack of expression of the intestinal fatty acid binding protein. Thus, one must use caution

while extrapolating the results from an *in vitro* isolated model of the human intestinal cell to the *in vivo* situation of the human intestine.

In addition, the authors have not indicated whether concentrations of the specific fatty acids used are physiologically relevant to the postprandial environment in the human intestine. Given the evidence of a concentration dependant effect of *trans* fatty acids on plasma lipid profile,[11] applicability of the present results within the context of current intake levels remains unclear. However, Caco-2 cells are currently one of the best available *in vitro* intestinal cell models,[12] and despite the above mentioned drawbacks, provide a basis for conducting future animal/human studies examining the mechanism of action of *trans* isomers on chylomicron composition.

Another limitation of the present study[9] is the lack of direct comparison between t-C18:1Δ9 and t-C18:1Δ11 fatty acid species. While, the authors have extensively compared the effects of t-C16:1Δ9 and t-C18:1Δ9, emphasis on positional isomers of the same carbon length could have shed more insight into the differential effects of natural versus commercially produced *trans* fatty acids. Such a cross comparison is important because elaidic acid (t-C18:1Δ9) is the major isomer found in commercially produced hydrogenated products, whereas in products resulting from biohydrogenation in the ruminant fat of animals nearly 50 to 60 % of the total *trans* C18:1 isomers are exclusively vaccenic acid (t-C18:1Δ11). [7] Thus, one of the major distinguishing features between products of biohydrogenation and commercial hydrogenation is the position of the double bond within the same fatty acid. Another is the distribution of the *cis* C18:1 isomers. In hydrogenated fat only 60 % of the C18:1 isomers are c-C18:1Δ9, whereas in animal fats

such as butter, c-C18:1Δ9 accounts for almost 90 % of the total. The fact that oleic acid (c-C18:1Δ9) produced a higher secretion of TG than vaccenic acid (t-C18:1Δ11), and was as potent a stimulus as elaidic acid (t-C18:1Δ9) is contrary to what might be expected. The question is also raised as to whether a specific ratio for both *cis* and *trans* positional isomers in hydrogenated products needs to be achieved for optimal health benefits.

In conclusion, short term human experiments indicate that positional C18:1 isomers have differential effects on human serum lipid and tissue fatty acid composition.[13-15] The present study by van Greevenbroek and colleagues demonstrates a similar effect on TG secretion and incorporation at the level of the intestine.[9] Thus, the adverse effects of *trans* fatty acids on human plasma lipids may be confined to specific isomers and future studies delineating their effects could explain, if only in part, the so far elusive mechanism of action of *trans* fatty acids.

11.3 References

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