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THE INFLUENCE OF WEIGHT LOSS THROUGH ENERGY RESTRICTION ON CHOLESTEROL METABOLISM IN HUMANS

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Science

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In memory of my Nonnos and my Nonnas.
I will always remember and love you.

ABSTRACT

Our first objective was to compare deuterium incorporation and mass isotopomer distribution analysis in the measurement of *in vivo* cholesterol biosynthesis in humans. Twelve healthy subjects were recruited to participate in a 24 h stable isotope infusion study which mimicked temporal conditions typical of both deuterium incorporation and mass isotopomer distribution analysis techniques. There was a difference ($p<0.05$) observed between cholesterol fractional synthesis rates measured at 15 h using either deuterium incorporation (4.8±1.2 %/day) or mass isotopomer distribution analysis (6.3±1.8 %/day); however, there was no difference in the 24 h measurement (7.8±2.5 versus 6.9±2.2 %/day). A strong correlation was found between cholesterol synthesis measured using both techniques at 24 h ($r=0.84$, $p<0.01$). These data suggest that both deuterium incorporation and mass isotopomer distribution analysis accurately define cholesterol synthesis in humans when measured over a period of 24 h.

Our second objective was to examine the effects of weight loss through energy restriction upon human in vivo circulating cholesterol concentrations and synthesis using the deuterium incorporation methodology. Seven overweight subjects with a mean BMI of 30.6±1.6 $kg \cdot m^{-2}$ were recruited into a 2-phase prospective clinical trial including 3 mo on a weight stable American Heart Association Step I diet and 6 mo on an energy-reduced American Heart Association Step I diet. A mean weight loss of ($p<0.05$) 6.27±2.61 % initial body weight was associated with significant reductions ($p<0.05$) in cholesterol biosynthesis from 7.90±2.25 %/day to 2.73±0.62 %/day. No significant reductions were observed in either circulating total, HDL or LDL cholesterol concentrations; however, circulating triglyceride concentrations did decrease ($p<0.05$) from 1.61±0.28 $mmol \cdot L^{-1}$ to 1.27±0.39 $mmol \cdot L^{-1}$. These data suggest that deuterium incorporation and mass isotopomer distribution analysis are equally effective methods for measuring cholesterol biosynthesis in humans, and that energy restriction resulting in modest weight loss effectively reduces endogenous cholesterol synthesis thus decreasing the risk of CVD development in obese and overweight men.

RÉSUMÉ

Notre premier objectif était de comparer l'incorporation du deuterium et MIDA pour mesurer la synthèse *in vivo* du cholestérol. Douze bénévoles ont été recruités pour participer dans une étude d'infusion d'isotope stable de 24 h qui imitait les conditions temporelles des techniques d'incorporation du deuterium et MIDA. Il y avait une différence ($p<0.05$) entre les taux de synthèse du cholestérol mesurés à 15 h avec l'incorporation du deuterium (4.8±1.2 %/jour) et le MIDA (6.3±1.8 %/jour); tandis qu'il n'y avait pas de différence à 24 h (7.8±2.5 versus 6.9±2.2 %/jour). Il existe une forte corrélation entre les taux de synthèse mesurés avec les deux méthodes à 24 h ($r=0.84$, $p<0.01$). Ces données suggèrent que l'incorporation du deuterium et le MIDA mesurent la synthèse du cholestérol de façon précise dans les humains sur des intervalles de
24 h.

Notre deuxième objectif était d'examiner les effets de perte de poids par restriction d'énergie sur les niveaux de cholestérol sanguin ainsi que sur la synthèse du cholestérol en utilisant l'incorporation du deuterium. Sept bénévoles obèses ont été recruité pour participer dans une étude en deux parties incluant 3 mois d'un régime American Heart Association Step I à poids stable et 6 mois d'un régime American Heart Association Step I réduite en énergie. Une perte significative de 6.27±2.61 % du poids initial était associée avec des réductions dans le taux de synthèse du cholestérol de 7.90±2.25 %/jour à 2.73±0.62 %/jour. Il n'y a pas eu de réductions significatives dans les niveaux de cholestérol total, LDL, ni HDL; pourtant, les niveaux de triglycérides ont baissé de 1.61±0.28 $mmol \cdot L^{-1}$ à 1.27±0.39 $mmol \cdot L^{-1}$. Ces résultats indiquent que les techniques d'incorporaton du deuterium et MIDA sont des techniques également efficaces pour mesurer la synthèse du cholestérol dans les humains, et qu'une modeste réduction dans l'apport en énergie qui mène à une perte de poids réduit efficacement la synthèse du cholestérol et réduit les risques de maladies cardiovasculaires dans les personnes obèses.

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I dedicate this thesis to my parents, without whom my dreams would never come true, my achievements would not be worthwhile, and my life would not be complete. Their sacrifices made this possible.

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I would also like to acknowledge and thank my international collaborators at the Medlantic Research Institute, Washington, D.C. and Department of Medicine, University of Maryland, Baltimore, MD who made this study possible. The staff at the University of Maryland provided the subjects, and managed the clinical trial from which the data in Manuscript 3 were generated. Dr. Leslie I. Katzel, and Dr. Judy S. Hannah also provided feedback on the interpretation of the results.

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When I first started my degree, I was under the supervision of one man, Dr. Peter Jones. Working with him has been an experience comparable to a roller coaster ride in an amusement park. Dr. Jones did not open any doors for me, he just pointed the way and forced me to open them myself. Half way through my degree, I acquired a co-supervisor. Dr. Linda Wykes had been a mentor to me before she took on that role; yet, that did not stop her from offering me more insight, more knowledge, and more opportunities.

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CHAPTER 1

LITERATURE REVIEW

1.1 OBESITY AND CARDIOVASCULAR DISEASE

1.1.1 Epidemiological Evidence

Obesity is a chronic widespread disease of increasing prevalence for men, women and children in western societies. It is widely accepted that increasing weight is associated with health risks. Several population-based studies have established strong relationships between obesity and several cardiovascular disease (CVD) risk factors, including hypertension, hyperlipidemia, glucose intolerance, hyperinsulinemia and Type II diabetes, and it is presumed that obesity actually predisposes to these risk factors through a breakdown of normal metabolic processes (Schwartz & Brunzell, 1997). Long-term longitudinal studies, however, indicate that obesity as such not only relates to but independently predicts coronary atherosclerosis (Robkin *et al.* 1977, Garrison & Castelli 1985, Mansen *et al.* 1995). In 1974, the Framingham study found that for a 10% reduction in weight in men, close to a 20% reduction in CVD incidence can be expected, whereas for each 10% increase in weight a 30% increase in CVD incidence can be expected (Ashley & Kannel, 1974). Findings such as these add strength to the claim that obesity is a life-threatening disease. But despite public warnings of the dangers of being overweight and obese, the disease is more prevalent today than it was almost 10 years ago (Health Canada, 1997) (Figure 1.1).

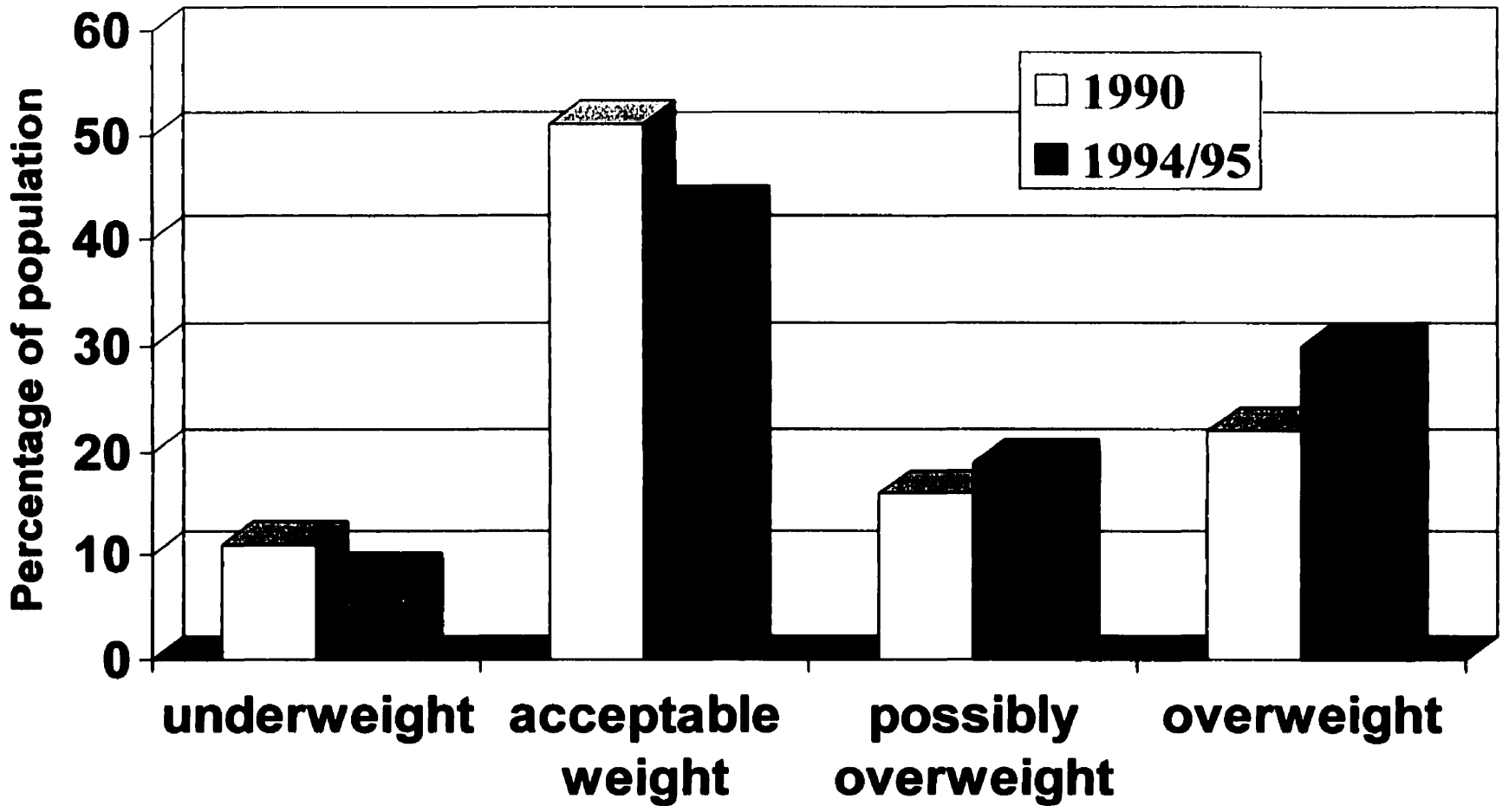


Figure 1.1. Data from Health Canada's Health Promotion Survey 1990: Technical report, which defines acceptable weight as a BMI of 20.0-25.0, possibly overweight as a BMI of 25.1-27.0 and overweight as a BMI >27.0.

According to current Health Canada statistics, 50% of Canadians aged 18 to 74 are overweight (Petrasovits, 1997) (Table 1.1). In the United States, obesity affects some 58 million adults and 25% of children and adolescents (D'Eramo-Melkus, 1997), and there is growing evidence that this epidemic has spread to the northern regions of Mexico and Europe (Seidell, 1995; Vartianinen *et al.* 1994; Sørensen et al. 1997). Genetic predisposition may offer a partial explanation for the high numbers of people affected, but environmental contributions seem to be a more likely cause. Furthermore, despite a reduction in the consumption of fat from 40% of total calories in the late 1960's to 34% in the early 1990's, the incidence of obesity among Americans has not decreased (Popkin *et al.* 1996). High energy intakes and reduced energy expenditure in the form of physical activity are the most likely explanations for the increase in obesity in the United States and elsewhere (Eckel, 1997).

The degree of CVD risk depends more on the site of excess fat deposition than on the level of adiposity per se (Schwartz & Brunzell, 1997). Body mass index (BMI) is generally used as a measure of adiposity (Kraemer *et al.* 1983). A BMI>27 $kg \cdot m^{-2}$ is indicative of obesity in both men and women. However, indices such as BMI provide only a rough indication of at-risk individuals because they only take into account total body fat and ignore fat distribution and body composition. Substantial evidence now indicates that an increased waist circumference, or waist-to-hip ratio, predicts comorbidities and mortality from

obesity better than BMI (Eckel, 1997). A waist -to-hip ratio greater than 1.0 is characteristic of obesity associated with hypertension, hyperlipidemia, and glucose intolerance. Obesity predisposes to these symptoms through a systemic insulin resistance syndrome. This syndrome has become known as Syndrome X (Schwartz & Brunzell, 1997) (Figure 1.2).

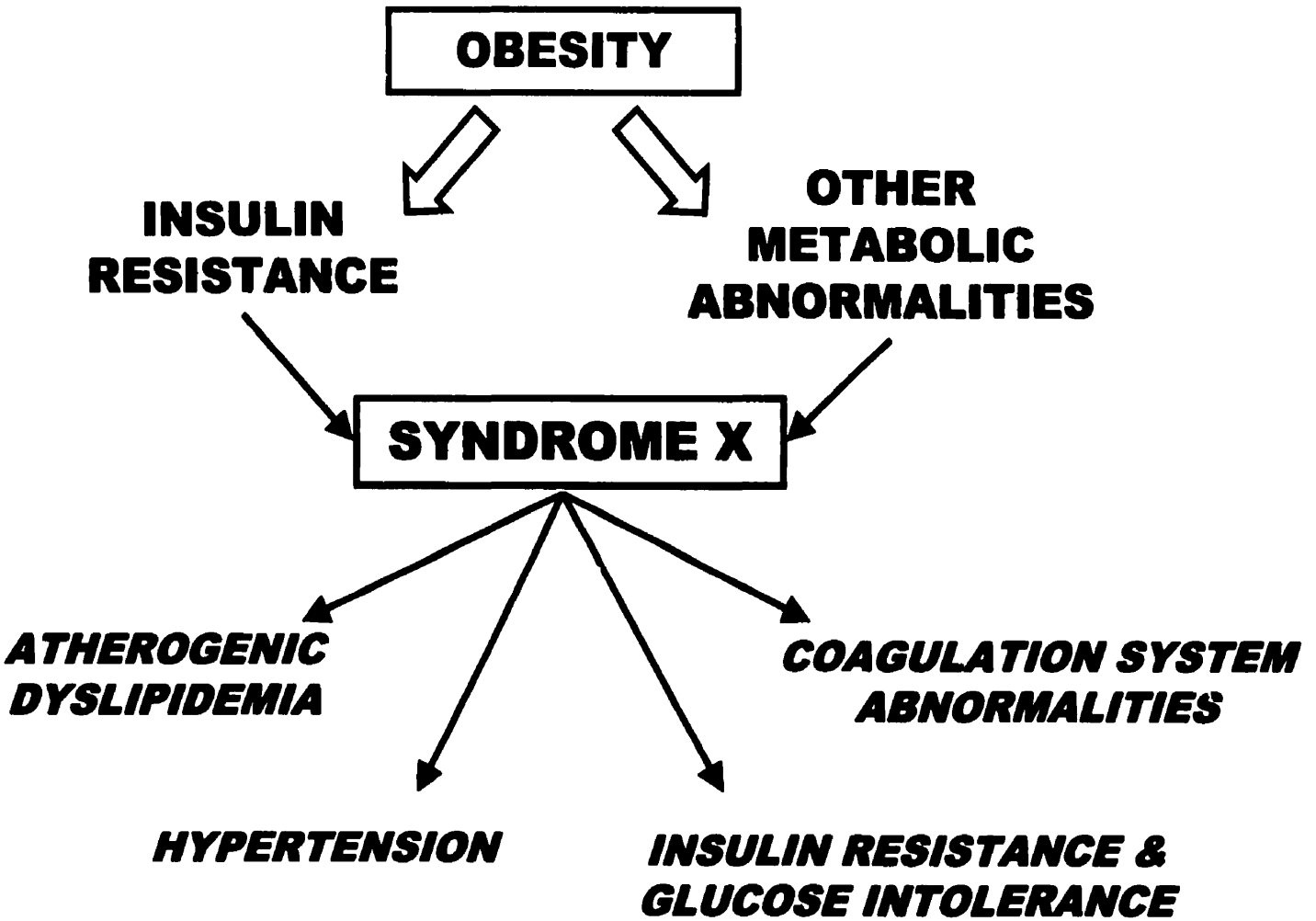


Figure 1.2. Link between obesity and cardiovascular disease risk factors

Table 1.1. Percentage of population aged 18-74 with selected CVD risk factors, by province, Canada, 1986-1992

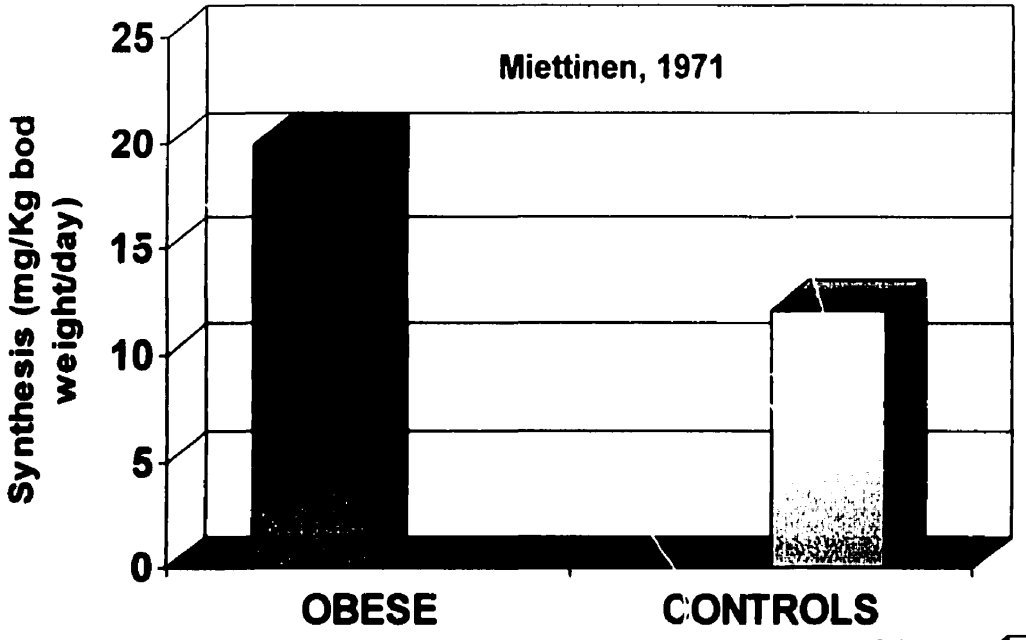
Risk Factors	Provinces[1] (%)										
	BC	AB	SK	MB	ON	PQ	NB	NS	PE	NF	Canada[2]
Smoking[3]	25	27	23	25	23	32	31	33	29	36	27
High blood pressure[4]	20	17	21	22	23	19	26	25	24	27	21
Elevated blood cholesterol[5]	46	36	44	44	42	49	47	45	44	42	44
Obesity[6]	**28**	**33**	**35**	**36**	**33**	**28**	**36**	**36**	**37**	**41**	**31**
Sedentary lifestyle[7]	29	37	30	45	39	37	45	42	44	48	38

1. British Columbia (BC), Alberta (AB), Saskatchean (SK), Manitoba (MB), Ontario (ON), Quebec (PQ), New Brunswick (NB), Nova Scotia (NS), Prince Edward Island (PE), Newfoundland (NF).
2. Excluding Yukon Territory and Northwest Territiories.
3. One or more cigarettes per day, every day.
4. Diastolic pressure ≥90 mm Hg and/or pharmacologic or non-pharmacologic treatment.
5. Total plasma cholesterol level ≥5.2 mmol/L.
6. Body mass index ≥27.
7. Respondents not physically active during leisure time at least once a week during the month preceding the survey.

Source: Canadians and Heart Health: Reducing the Risks; Health Canada 1995; Canadian Heart Health Surveys

1.1.2 Metabolic Changes in Obesity

Circulating cholesterol concentrations are determined by the size of the body pool of cholesterol, which in turn is determined to a large extent by endogenous cholesterol synthesis rates (Dietschy, 1984; Rudney & Sexton, 1986). Up to 2/3 of the body pool can actually be derived from endogenous synthesis (Rudney & Sexton, 1986). In 1971, Miettinen provided an explanation for the strong link between obesity and CVD. Using the sterol balance method, which makes the assumption that in the steady state cholesterol synthesis equals sterol balance, he determined that obese subjects synthesized an average of 20±2 mg cholesterol per kg body weight per day as compared to non-obese controls at 12 $mg \cdot kg^{-1} \cdot day^{-1}$ (Miettinen, 1971). Nestel *et al.* later confirmed the finding that obese individuals synthesized twice as much cholesterol as non-obese individuals (Nestel *et al.* 1973). By comparing 8 obese subjects of their own to 6 non-obese subjects used in a previous study by Connor *et al.* (1969) and 9 non-obese subjects used in a previous study by Grundy and Ahrens (1969), Nestel *et al.* found synthesis rates as high as 1754 $mg \cdot day^{-1}$ in their obese cohort versus rates of 915 and 865 $mg \cdot day^{-1}$ in the two non-obese cohorts (Figure 1.3). Recently, Stahlberg *et al.* (1997) compared operative liver biopsies obtained from 17 morbidly obese subjects to those obtained in 15 non-obese controls. They found that both activity and messenger RNA (mRNA) level of 3-hydroxy-3-methyl glutaryl Co-enzyme A (HMG CoA) reductase, and the activity of acyl CoA:cholesterol acyltransferase (ACAT) was significantly higher in the obese subjects. Stahlberg *et al.* (1997)



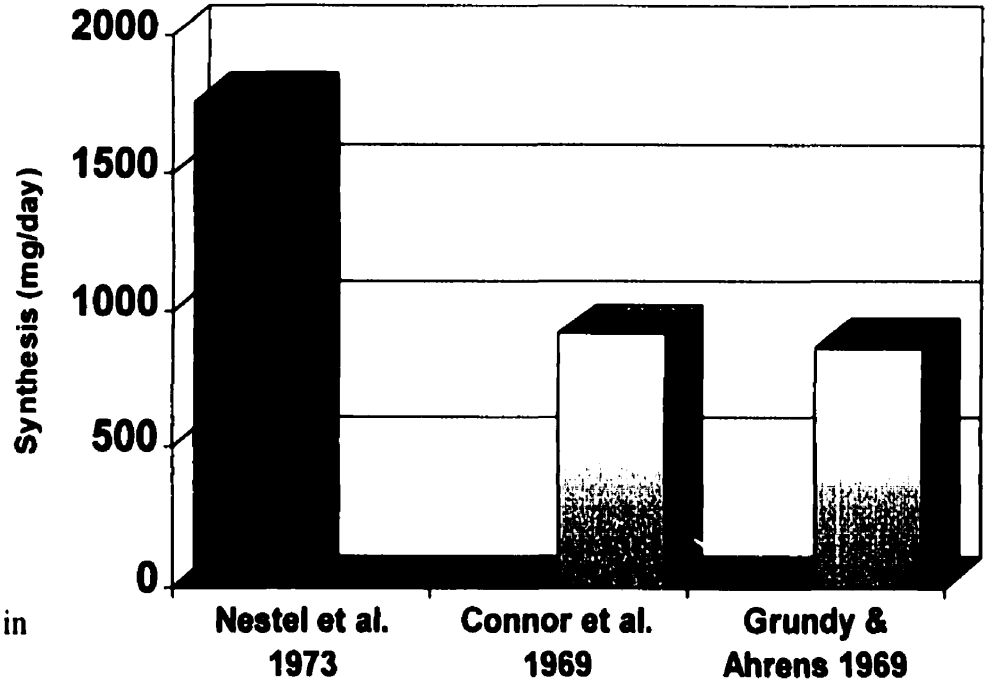


Figure 1.3. Enhanced cholesterol biosynthesis observed in obesity. This is one of several metabolic alterations which commonly occur in obese individuals.

concluded from their results that the hypersecretion of cholesterol which occurs in obesity and generally leads to elevated serum cholesterol concentrations is due to an increased synthesis of cholesterol.

Obesity is characterized by other metabolic and hormonal changes which include atherogenic dyslipidemia, hypertension, insulin resistance and glucose intolerance, and abnormalities in the coagulation system (McGarry, 1998). In fact, it is widely accepted that obesity predisposes to a condition known as Syndrome X, which itself leads to the development of these symptoms (McGarry, 1998; Grundy, 1998). Some scientists believe that insulin resistance forms the basis of Syndrome X (DeFronzo, 1988; Howard *et al.* 1996); however, insulin sensitivity may be but one of several abnormalities resulting from a generalized metabolic derangement induced by obesity (Grundy, 1998).

Obesity results from an imbalance between energy intake and energy expenditure. Under normal circumstances, adipose tissue triglycerides are broken down through lipolysis to provide nonesterified fatty acids (NEFA) as lipid fuel to fat-oxidizing tissues. The excess amounts of body fat that inevitably arise as a result of the energy imbalance in obesity may lead to aberrant metabolism of NEFA and may explain the constellation of symptoms seen in Syndrome X. High circulating concentrations of NEFA may shift energy utilization from carbohydrates to fat in skeletal muscle, which is the major site of NEFA

utilization (Randle *et al.* 1994). In doing so, skeletal muscle may develop a resistance to the action of insulin (Abate *et al.* 1995). If glucose uptake in muscle becomes impaired, the β cells of the pancreas may be stimulated to secrete more insulin. Furthermore, NEFA may act directly on the pancreatic β cells, inducing them to produce more insulin as well. If the overstimulation of these cells is prolonged, their function may become impaired, leading to less insulin secretion and eventually to type II diabetes (Zimmet *et al.* 1991). In the liver, high circulating concentrations of NEFA can lead to enhanced hepatic synthesis of triglycerides and cholesterol, and increased secretion of very low density lipoproteins (VLDL) (Grundy *et al.* 1979; Miettinen, 1971). The increased VLDL entering the bloodstream raises serum lipid concentrations to atherogenic levels (Grundy *et al.* 1979). Finally, high levels of insulin may act upon the autonomic nervous system to raise arteriolar tone, thereby increasing blood pressure (Grundy, 1998).

The extent to which adipose tissue contributes to circulating NEFA concentrations is dependent upon fat distribution. It is widely accepted that visceral obesity leads to a greater risk of developing Syndrome X (Jensen, 1998). But regardless of the cause and type of obesity, the evidence is substantial that reductions in weight do ameliorate the common complications of obesity.

1.1.3 Benefits of Moderate Weight Loss

It is extremely difficult to dispute that intentional weight loss among obese individuals is beneficial to their health. Most scientists will agree that reductions in initial weight of as little as 5-10% are sufficient to ameliorate common complications of obesity (Van Gaal *et al.* 1997). Although weight loss affects the metabolic and cardiovascular abnormalities associated with obesity differentially (Muscelli *et al.* 1997), it still provides protection against CVD. For example, Muscelli *et al.* (1997) found that a 15% weight loss leads to a 12% improvement in oral glucose tolerance, a 25% improvement in insulin sensitivity and significant reductions in systolic blood pressure. However, no dramatic changes occur with respect to resting energy expenditure, fasting and post-oral glucose tolerance test insulin delivery, or peripheral vascular resistance (Muscelli *et al.* 1997). Bosello *et al.* (1997) have also determined that some complications seen in obesity are reversible. In patients with type II diabetes, a 10% weight loss improved glycaemic control, insulin sensitivity and cardiovascular risk factors.

Several studies have shown that weight loss does in fact improve serum lipid profiles. A meta-analysis of 70 studies on the effects of weight reduction on blood lipid concentrations and lipoproteins published in 1992 (Dattilo & Kris-Etherton) concluded that weight reduction is associated with significant decreases in total cholesterol, LDL-cholesterol, VLDL-cholesterol, and triglyceride concentrations. The decreases in these parameters were positively correlated with

weight reduction. HDL-cholesterol concentrations, on the other hand, were inversely correlated with weight loss. Studies conducted in 1993 (Leenen *et al.*) and 1995 (Dengel et *al.*) reiterate these findings, therefore warranting clinical recommendations for weight loss in obese and overweight individuals.

According to Canada's Health Promotion Survey conducted in 1990, 31% of Canadians were trying to lose weight (Green *et al.* 1997). Interestingly, a significantly larger proportion of women (39%) compared to men (23%) were attempting to lose weight despite the fact that more men than women are overweight. In the Unites States, current estimates suggest that 40% of women and 25% of men are attempting to lose weight (Williamson, 1997). Despite all these attempts at weight loss, the prevalence of obesity in North America and abroad is still increasing. One might argue that it is not moderate weight loss per se that is favorable, but sustained moderate weight loss which has beneficial outcomes on morbidity and mortality from obesity-related causes (Rössner, 1997).

1.2 CHOLESTEROL

1.2.1 Basic Role and Function

Cholesterol is a 27-carbon alicyclic compound classified as a lipid. Although its solubility in water is considerably low, its solubility in blood is very high due to the presence of transport proteins, known as lipoproteins, with the ability to bind and solubilize large amounts of cholesterol. Cholesterol can reach

plasma concentrations of 3.88 - 5.12 mmol·L^{-1} in healthy individuals, 30% of which is found in its free form. Seventy percent is found esterified to a long-chain fatty acyl residue, usually linoleic acid, that further increases the hydrophobicity of cholesterol. The enzyme responsible for cholesterol esterification in plasma is lecithin:cholesterol acyltransferase (LCAT). Cholesterol-ester represents both a form of transport and storage of cholesterol in the body (Roberts et al. 1964).

Cholesterol, which can be derived from the diet or synthesized *de novo* in virtually all cells of the body, is a ubiquitous and essential component of mammalian cell membranes where its major role appears to be the maintenance of mechanical stability and the regulation of cell membrane fluidity (Myant, 1981; Bloch, 1983). The free form of cholesterol is especially abundant in myelinated structures of the brain and central nervous system where it is involved in the formation of the myelin sheaths that insulate nerve fibers. It is present in small amounts in the inner mitochondrial membrane.

Free cholesterol also serves as the immediate precursor of the bile acids. Because cholesterol is not metabolized to CO_2 and H_2O in humans, this represents the only route of excretion of cholesterol from the body. Various steroid hormones including progesterone, vitamin D, the corticosteroids of the adrenal cortex, and the sex hormones are derived from the esterified form of cholesterol.

1.2.2 Cholesterol Biosynthesis and Transport

Cholesterol production is conditioned by nutritional, endocrine and neurohormonal influences. Cholesterol enters the body pool from only two sources. 300-500 mg are absorbed from the diet daily, and 700-900 mg are synthesized *de novo* daily; therefore, endogenous biosynthesis contributes to greater than 2/3 of the total body pool (Dietschy 1984) (Figure 1.4). Jeske and Dietschy (1980) have shown in the rat that liver accounts for only 10-30% of total cholesterol synthesis at the lowest point in its diurnal cycle. More recently, Dietschy *et al.* (1993) have shown that human extra-hepatic synthesis accounts for over 80% of total cholesterol synthesis. Therefore, regulation of extra-hepatic cholesterol synthesis has a more profound influence on the size of the total body pool of cholesterol than does regulation of hepatic synthesis.

Cholesterol biosynthesis occurs in three stages and involves approximately 26 steps. The first stage involves the formation of 3-hydroxy-3-methyl glutaryl CoA (HMG-CoA) from acetyl CoA. Acetyl CoA can be obtained from several sources, including β-oxidized long-chain fatty acids and oxidized ketogenic amino acids. The second stage involves the conversion of HMG-CoA to squalene. This stage includes the rate-limiting-step in the formation of cholesterol which is catalyzed by the regulatory enzyme HMG-CoA reductase. This enzyme catalyzes the reduction of HMG CoA to mevalonic acid. In the final stage, cholesterol is formed from squalene through a cyclization reaction in the endoplasmic

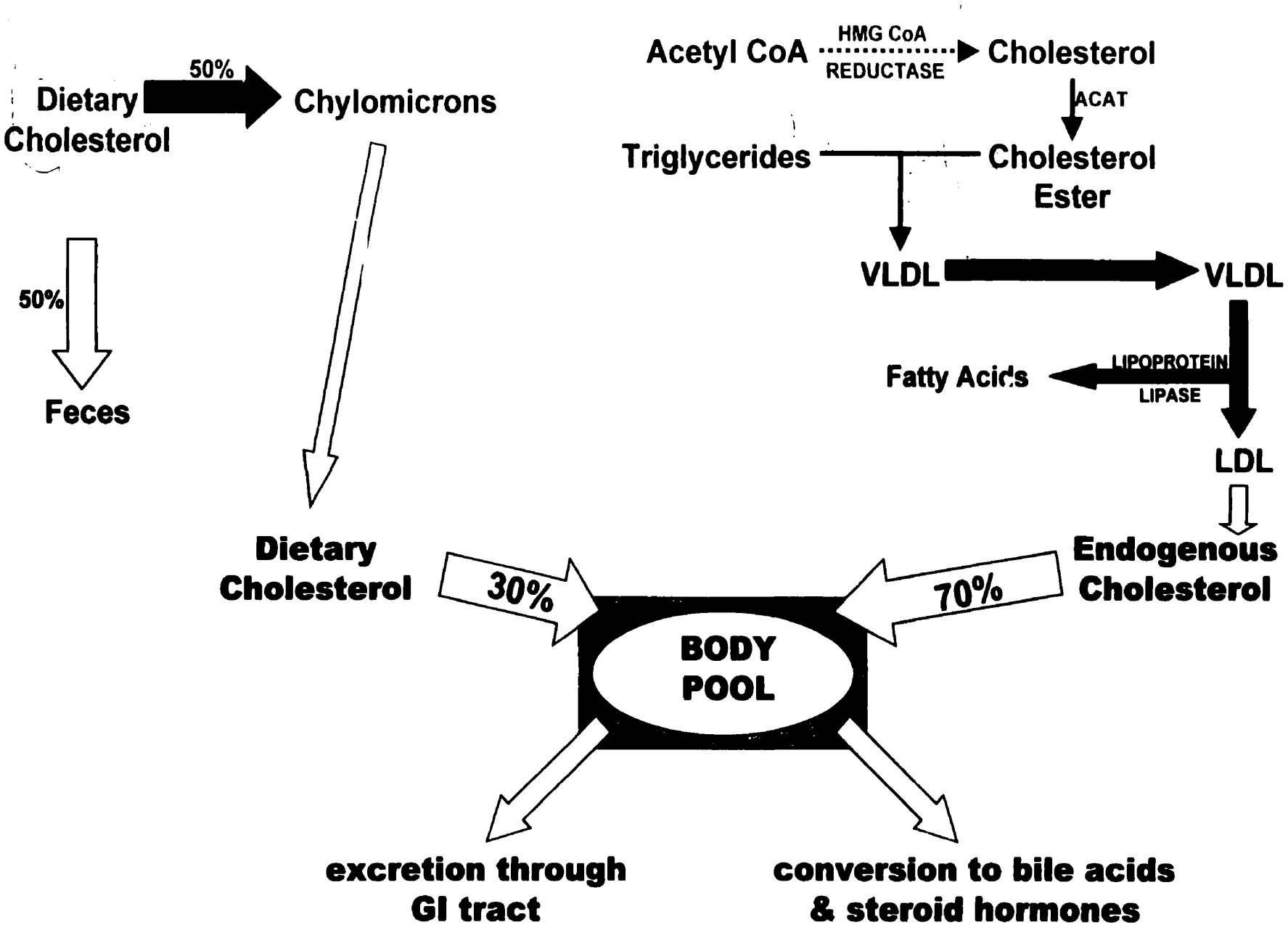


Figure 1.4. Schematic representation of endogenous cholesterol biosynthesis and transport. Also depicted are the relative contributions of dietary and endogenous cholesterol to the body pool pool of cholesterol

reticulum. Elevated concentrations of circulating cholesterol will decrease the rate of synthesis primarily through negative feedback regulation of hepatic HMG-CoA reductase (Dietschy 1984). Although the suppression of this enzyme's activity occurs in other tissues, it occurs to a grea'er extent in the liver (Dietschy 1984).

Once human cells have synthesized cholesterol, the isoprenoid begins its journey throughout the body. Cholesterol synthesized in extrahepatic tissues such as the small intestine is packaged into chylomicrons and sent to the liver. In the liver endogenous cholesterol can have 3 fates: 1) it will be secreted into the plasma as part of very low density lipoproteins (VLDL) and circulated to tissues in the body; 2) it will be converted to bile acids; and 3) it will be secreted in the bile and into the intestine. Fifty percent of cholesterol secreted into the intestine through bile will be reabsorbed and returned to the liver where it will regulate the activity of HMG-CoA reductase, the remainder is excreted into stools as fecal neutral steroids. This enterohepatic circulation represents the only route of elimination of cholesterol from the body.

In order to be secreted into the plasma, newly synthesized cholesterol is packaged in its esterified form along with triglycerides into VLDL in the liver. These particles are secreted into the plasma whereupon they encounter lipoprotein lipase, an enzyme made by adipose tissue and muscle but localized to the endothelial cell surface. This enzyme hydrolyzes triglycerides in order to release

free fatty acids into the bloodstream. In the process, it transforms the triglyceride-rich VLDL particle to a cholesterol-rich low density lipoprotein (LDL) particle. Approximately 75% of LDL particles return to the liver where they are taken up by the LDL-receptor mechanism and regulate hepatic cholesterol synthesis through modulation of HMG-CoA reductase activity. Twenty-five percent of LDL particles, however, are delivered to the peripheral tissues where they will be endocytosed through the activation of extra-hepatic cell-surface LDL-receptors. Once in the cell, the protein component of LDL is hydrolyzed to amino acids, and the cholesterol esters are hydrolyzed to free cholesterol by a lysosomal acid lipase. The released unesterified cholesterol will either be incorporated into the cell membrane, or re-esterified by acyl-CoA:cholesterol acyltransferase (ACAT) and stored for future use.

The cholesterol content of cells with an active LDL uptake is regulated in 2 ways. First, like the liver itself, free cholesterol suppresses the activity of extrahepatic HMG-CoA reductase. Second, LDL-receptor expression is subject to feedback inhibition. When the cellular level of cholesterol is too high, it will prevent the synthesis of new LDL-receptors and the recycling of older ones. This suppression of receptor expression effectively blocks the uptake of LDL from the circulation. When this block occurs, LDL particles will accumulate in the plasma and will eventually infiltrate the endothelial cell layer of arterial walls. This eventually leads to the formation of plaques and lesions in the arteries,

constituting an initial step towards the development of CVD as reviewed by Selwyn *et al.* (1997).

Animal studies have demonstrated a clear difference between regulation of hepatic compared to extrahepatic cholesterol synthesis. Cholesterol synthesis in hepatic tissue manifests a diurnal rhythm, is enhanced by stress or by interruption of the enterohepatic circulation, and is suppressed by fasting or caloric restriction, and cholesterol feeding (Jeske & Dietschy, 1980). Furthermore, there exists a negative correlation between sterol synthesis and plasma levels of lipoproteins, particularly LDL and chylomicrons (Nervi & Dietschy, 1975; Andersen *et al.* 1979). On the other hand, cholesterol synthesis in extrahepatic tissues, which does not manifest diurnal rhythmicity, is neither enhanced by stress, cholesterol feeding, plasma lipoprotein concentrations, or disruption of the enterohepatic circulation (Andersen & Dietschy, 1977). Therefore, the only regulatory mechanism in cholesterol synthesis common to all animal tissues is fasting or energy restriction.

Many intrinsic and extrinsic factors are capable of regulating cholesterol biosynthesis. Dietary factors alone represent a complex metabolic portrait that relies on fat type, cholesterol intake and plant sterol intake among others for regulating endogenous cholesterol synthesis in humans. But the factor which may have the most profound impact on cholesterogenesis is energy restriction.

1.2.3 Effect of Fasting and Energy Restriction on Cholesterol Biosynthesis

No studies to date have examined the effects of long term weight loss in animals on cholesterol synthesis; however, several studies have been conducted to assess the effects of short term energy restriction of varying degrees and complete fasting on cholesterol synthesis. Shapiro and Rodwell (1972), in an experiment designed to assess whether fasting could eliminate the cyclic variations observed in HMG-CoA reductase activity in female Wistar rats, discovered that although the cyclic variations in enzyme activity were unaltered, the enzyme's activity dropped 20-25 fold from initial levels following a 36-hour fast. Jeske and Dietschy (1980) whose subsequent findings support those of Shapiro and Rodwell, subjected female Sprague-Dawley-derived rats to fasting over a 48-hour period and observed a decrease in total body cholesterol synthesis to 27% of the initial control values by using $^{3}H_{2}O$ incorporation methodology. These animal studies led the way to similar studies in humans.

Kudchodkar *et al.* (1977) demonstrated a similar phenomenon in 5 human male subjects with varying degrees of obesity. They observed a decrease in synthesis using sterol balance from 1469±441 $mg \cdot day^{-1}$ to 1212±349 $mg \cdot day^{-1}$ following 9-12 days of energy restriction. Kudchodkar *et al.* (1977) credited the decrease in cholesterol synthesis to the metabolic state associated with energy restriction rather than to a reduction in body mass because the changes observed

in synthesis occurred prior to any significant changes in body weight. A decrease in plasma cholesterol concentrations of 11±9 % was also observed. Bennion and Grundy (1975) and Jones *et al.* (1988) found a similar influence of energy restriction upon human *in vivo* cholesterogenesis. Bennion and Grundy showed enhanced excretion of cholesterol in bile acids during periods of energy restriction in 10 obese subjects. Jones *et al.* (1988) demonstrated for the first time using deuterium incorporation methodology that cholesterol synthesis declines by approximately 40% following a 12 h fast. Based on these findings of Bennion and Grundy (1975), Vaswani (1983) postulated that HMG-CoA reductase is acutely inhibited following energy restriction, thereby reducing cholesterol synthesis rates, but that once the body has adapted to the new metabolic demands imposed upon it, HMG-CoA reductase activity returns to initial levels. To date, no one has tested this hypothesis.

1.2.4 Measuring Cholesterol Synthesis

The history of the use of stable isotopes in metabolic research parallels the development of the field of mass spectrometry. Prior to the use of stable isotopes, the measurement of endogenous cholesterol synthesis relied on indirect methods or methods that required extended measurement periods (Jones 1990). The sterol balance method, cholesterol kinetics studies with [^{14}C]-cholesterol, and plasma cholesterol precursor studies are of limited value in the study of cholesterol synthesis over short periods. Furthermore, these methods are either accurate but

require lengthy measurement periods, are immediate but overly invasive, or fail to measure cholesterol synthesis directly (Jones 1993). Stable isotope techniques such as deuterium incorporation and mass isotopomer distribution analysis correct these shortfalls by providing relatively quick, non-invasive methods of measuring cholesterogenesis directly and accurately. However, both stable isotope methods have their own procedural and theoretical drawbacks (Table 1.2).

The deuterium uptake method is based on the assumption that the incorporation of deuterium into cholesterol represents *de novo* synthesis of cholesterol. The deuterated tracer water equilibrates between the precise intracellular site of synthesis and the extracellular physiological fluid, such as plasma, once the tracer is administered. Therefore, a primary advantage of this technique is that the tracer which is incorporated into newly synthesized cholesterol originates from a pool of known enrichment.

The use of deuterium as a tracer to measure lipogenesis was pioneered by Schoenheimer in 1935 (Schoenheimer & Rittenberg 1935). The simplicity of the method is what makes it so attractive for use in human subjects. The tracer itself is inexpensive and safe to use, and the data analysis is relatively straightforward and does not involve the use of complex mathematics. The major procedural drawback lies in sample preparation. The isolation, combustion, reduction and isotopic assay of erythrocyte or plasma free cholesterol is a lengthy, multi-staged

process that easily lends itself to experimental error. Various improvements have been made to the methodology, including the use of high performance liquid chromatography (HPLC) (Wong *et al.* 1991) as a method for isolating cholesterol from lipid sources. Although improvements like this one increase the precision and accuracy of the final results, they do not eliminate the repeated sample manipulations inherent in multi-staged analysis.

Theoretically, the major concern with deuterium incorporation lies in establishing the maximum number of deuteriums per molecule of newly formed cholesterol (D_{max}). Acetyl CoA (n=24), H_2O (n=7) and NADPH (n=15) collectively contribute the 46 hydrogen atoms required for *de novo* cholesterol synthesis. It is generally accepted that in the presence of deuterium, only H_2O and NADPH become labeled over the short term. Therefore, D_{max} is considered to be relatively stable at 22 over periods of up to 48h (Figure 1.5).

The deuterium uptake method for measuring *in vivo* cholesterogenesis has recently been validated against the classic sterol balance method by Jones *et al.* (1998). Cholesterol synthesis was measured simultaneously by deuterium incorporation and sterol balance in 13 subjects consuming controlled weight maintenance diets. Jones *et al.* (1998) found a very high degree of correlation ($r=0.745$, $p<0.01$) between net cholesterol biosynthesis measured using both techniques, and they concluded that the deuterium uptake method was a reliable

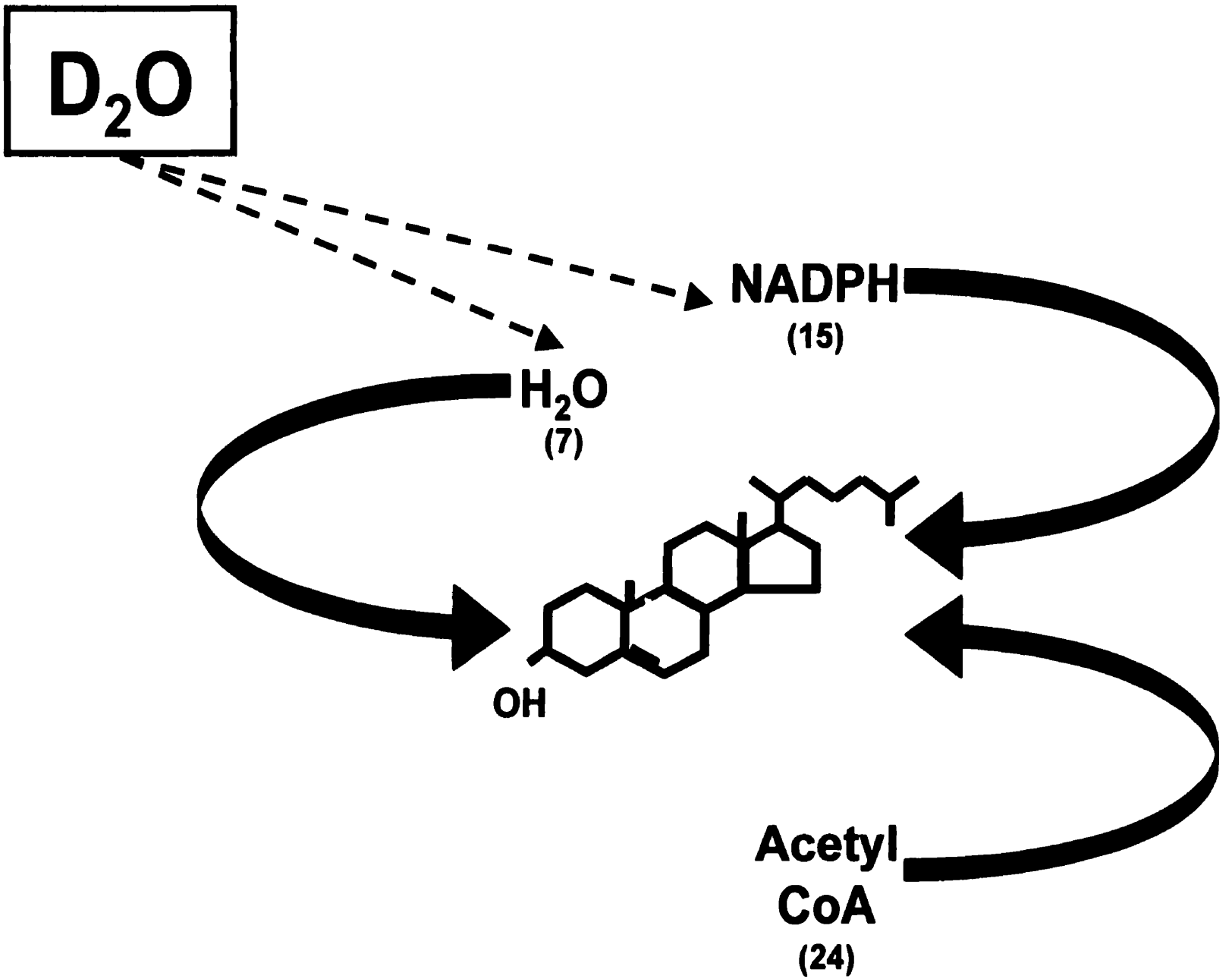


Figure 1.5. Origin of hydrogen species used in cholesterol biosynthesis and site of deuterium labeling within first 48 hours of D_2O dosing. Numbers in brackets represent number of hydrogen atoms contributed by each source. D_{max}, the maximum number of deuteriums per molecule of cholesterol, is 22 over periods up to 48h.

means by which to determine *in vivo* cholesterogenesis. Given the simplicity and non-restrictiveness of the deuterium uptake technique, it may serve as a more easily applicable method for the determination of synthesis compared with the labor-intensive and more inconvenient sterol balance.

MIDA is a relatively new technique developed by Neese *et al.* (1993) to study the synthesis of polymers from repeating monomeric subunits. Unlike deuterium incorporation, the isotope used to assess cholesterol synthesis in MIDA, Na[1-^{13}C]acetate, is expensive and must be infused through an indwelling catheter with serial blood sampling through another catheter. Furthermore, data analysis requires complex mathematics and modeling. However, sample preparation is very rapid and straightforward.

In theory, if one wishes to measure cholesterol biosynthesis, one needs simply to introduce a labeled substrate into a specific precursor pool of acetyl CoA, and then quantify the incorporation of labeled acetate into *de novo* cholesterol over a given period of time using a precursor:product relationship. However, because synthesis occurs at the cellular level it becomes extremely difficult to accurately determine the precursor pool enrichment due to subcellular compartmentalization, local microenvironments, and metabolite channeling (Hellerstein 1995). Whereas deuterium uptake uses a precursor pool which is not subject to subcellular compartmentalization, local microenvironments or

channeling, a key operational point about MIDA is that *de novo* cholesterol is analyzed to determine the isotopic enrichment of both the product and the precursor, thereby eliminating the need for sampling the precursor pool altogether (Hellerstein 1995).

In terms of theoretical considerations, the principal advantage of MIDA is that the enrichment of the precursor pool (acetyl CoA) at the site of synthesis is known because it is calculated from the mass isotopomer distribution (MID) of the newly synthesized cholesterol. However, two conditions must be met for this to hold true: 1) the precursor pool must be homogeneous and its enrichment constant; 2) the fractional synthesis rate of the product must be constant. These conditions may limit the applicability of the MIDA technique to the study of few metabolites. To date, MIDA has been applied to the study of fatty acids, cholesterol, glycogen and deoxyribonucleic acids (DNA).

Although the two methods have never been compared directly, MIDA generally provides synthesis values similar to deuterium uptake. Whereas deuterium uptake estimates average cholesterol synthesis at 750±50 $mg \cdot day^{-1}$ (Jones 1997), MIDA estimates 717±40 $mg \cdot day^{-1}$ (Hellerstein & Neese, 1992) (Table 1.3). Furthermore, the temporal conditions under which each methodology is applied are different. Whereas deuterium uptake measures synthesis over periods of 24 hours, MIDA estimates synthesis over periods of 15 hours.

Cholesterol synthesis is highly dependent on diet and physiological state; therefore, a direct comparison of the two methods is necessary in order to determine whether they both accurately define *de novo* cholesterogenesis.

Table 1.2 Comparison of two stable isotopic methods of determining human cholesterol synthesis

	Deuterium Incorporation	**MIDA**
Tracer	D_2O - inexpensive	^{13}C-acetate - expensive
Tracer dose	low	high
Infusion protocol	oral with supplemental drinking water over 48h	15h intravenous infusion
Sampling protocol	3 blood samples	serial blood samples requiring indwelling catheter
Sample preparation	complex multi-step extraction, separation, combustion and reduction	routine cholesterol derivitization (pentofluorobenzoyl chloride)
Mass spectrometry	IRMS - high sensitivity, low enrichments	GCMS - low sensitivity requires high enrichments
Data analysis	relatively straightforward	complicated
Precursor pool	NADPH, total body water	acetyl CoA from MID of product
Theoretical Issues	establishing D_{max} (constant labeling of NADPH in different states)	constant precursor pool labeling and FSR

Table 1.3 Comparison of typical cholesterol synthesis values estimated using deuterium incorporation and MIDA

Subjects	Deuterium Incorporation	MIDA
Men	750±50 mg/day[1] 11±2 mg/kg/day	717±40 mg/day[2] 9±2 mg/kg/day
Women	n/a	568±55 mg/day[3] 10±1 mg/kg/day

1. Hellerstein & Neese, 1992
2. Jones, 1997
3. Neese *et al.* 1993

1.3 SUMMARY AND RATIONALE

Obesity results from the accumulation of excess body fat due to an imbalance between energy intake and expenditure. Exactly why this process is facilitated in certain individuals is unknown, but many experts agree that it is most likely due to interactions between environmental, behavioral and biological factors. The risk of developing CVD increases with obesity (Ashley & Kannel, 1974), but despite public warnings of the dangers of being overweight or obese from health agencies the world over, the prevalence of this disease is continuously increasing.

It is currently believed that visceral obesity leads to a metabolic disorder known as Syndrome X, which predisposes to several cardiovascular risk factors through a breakdown in normal insulin action. Among the metabolic changes that occur in obesity is an enhanced synthesis of cholesterol. Endogenous cholesterol synthesis contributes to 2/3 of the body pool of cholesterol in man (Rudney & Sexton, 1986); hence, its regulation may be a very significant factor in the regulation of the body pool which ultimately determines circulating cholesterol concentrations. Although it has been established that weight loss leads to a significant decrease in circulating cholesterol concentrations, the effects of weight loss through energy restriction on endogenous cholesterol synthesis have not yet been investigated. The biochemical mechanisms which preside over *in vivo* cholesterol biosynthesis in humans are well understood, but investigation into

control mechanisms have only just begun. If a natural means by which humans could control their own rates of cholesterol production could be elucidated, this would have a great impact on the regulation of serum cholesterol concentrations, and thus lower CVD risk.

In order to properly assess *in vivo* cholesterogenesis, one requires a tool which is both accurate and practical for use in free-living subjects. Current stable isotope and mass-spectrometric technology have lead to the development of two such methodologies for assessing human *in vivo* cholesterogenesis. Although clinical studies employing the use of either deuterium incorporation into plasma free cholesterol or Na[1-^{13}C]acetate MIDA have shown that both methods have advantages relative to one another, a direct comparison of the two has not yet been made. Such a comparison is necessary if one wishes to accurately define the principles which regulate and modulate human cholesterol biosynthesis.

STATEMENT OF PURPOSE

A) The following objective is the focus of Manuscript 1, Thesis Chapter 2:

(1) To determine the difference between deuterium incorporation into plasma free cholesterol and mass isotopomer distribution analysis of Na[1-^{13}C]acetate in assessing rates of *in vivo* cholesterogenesis in humans.

The null hypothesis tested was that rates of cholesterogenesis measured using deuterium incorporation into plasma free cholesterol would not differ from rates measured using mass isotopomer distribution analysis of Na[1-^{13}C]acetate.

B) The following objective is the focus of Manuscript 2, Thesis Chapter 3:

(1) To examine the effects of weight loss as achieved through energy restriction upon *in vivo* circulating cholesterol concentrations and synthesis in older men, determined by deuterium incorporation into erythrocyte membrane free cholesterol.

The null hypothesis tested was that weight loss would not result in a change in circulating cholesterol concentrations or in rates of cholesterogenesis in older men.

Chapter 2. Manuscript 1.

Comparison of Deuterium Incorporation and Mass Isotopomer Distribution Analysis for Measurement of Human Cholesterol Biosynthesis

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2.1 ABSTRACT

To compare endogenous cholesterol biosynthesis measured using deuterium incorporation and mass isotopomer distribution analysis, cholesterol fractional and absolute synthetic rates were measured simultaneously with both techniques under identical physiological conditions.12 subjects (22 to 39 yrs) underwent a dual stable isotope protocol. Measurement of cholesterol biosynthesis by deuterium incorporation was achieved by accretion of deuterium into red cell free cholesterol after administration of a bolus oral dose of deuterium oxide. Measurement by mass isotopomer distribution analysis was achieved by delivering a constant infusion of Na[1-^{13}C]acetate. Synthesis was determined over 15 and 24 h with a 7 h feeding period for both methodologies in order to respect the temporal constraints of both techniques. No difference ($p>0.05$) was observed between fractional synthesis rates measured by deuterium incorporation or mass isotopomer distribution analysis at 24. Both techniques showed lower ($p<0.05$) fractional synthesis rates at 15 h as compared to 24 h. At 24 h the correlation of subjects' cholesterol fractional synthesis between both techniques ($r=0.84$, $p=0.0007$) was much stronger than at 15 h ($r=0.09$, $p=0.96$). Similarly, absolute synthesis rates correlated more strongly at 24 h ($r=0.81$, $p=0.0015$) than at 15 h ($r=-0.02$, $p=0.92$). These data suggest that deuterium incorporation and mass isotopomer distribution analysis yield comparable rates of cholesterogenesis in humans when measurements are made over 24 h.

2.2 INTRODUCTION

Elevated circulating cholesterol concentrations are associated with undesirable plasma lipid responses that increase the risk of developing cardiovascular disease (CVD) (Gotto *et al.* 1990, Ulbricht & Southgate 1991). Although up to 2/3 of the body pool of cholesterol originates from endogenous biosynthesis (Rudney & Sexton 1986), cholesterol synthesis has been largely overlooked as a potential factor in CVD development. Furthermore, there is increasing evidence that the suppression of cholesterogenesis is beneficial in the reduction of CVD risk independent of the lipid lowering effect (Corsini *et al.* 1996, Hughes 1996).

Prior to the use of stable isotopes, the measurement of endogenous cholesterol synthesis required indirect methods or methods that required extended measurement periods (Jones 1990). The sterol balance method, cholesterol kinetics studies with [^{14}C]-cholesterol, and plasma cholesterol precursor studies are of limited value in the study of cholesterol synthesis because these methods are either accurate but require lengthy measurement periods, are immediate but overly invasive, or fail to measure cholesterol synthesis directly (Jones 1993). Stable isotope methods currently in use such as deuterium incorporation (DI) and mass isotopomer distribution analysis (MIDA) meet the demand for immediate, accurate and non-invasive measurement of cholesterol biosynthesis. Both methods share a common basis of measuring fractional synthesis rate (FSR) as the rate of

appearance of product from a precursor pool at steady state enrichment. However, both stable isotope techniques rely on different assumptions and thus have their own procedural and theoretical advantages and drawbacks (Table 2.1). The fundamental difference in the two techniques lies in their approach to measuring precursor pool enrichment.

The DI methodology 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 (Jones 1990). The MIDA technique determines synthesis of the polymer cholesterol from repeating subunits of acetyl CoA during a constant infusion of ^{13}C-labeled acetate. The pattern of excess mass isotopomer frequencies of cholesterol is determined only by precursor pool enrichment. Therefore, MIDA eliminates the need to measure precursor pool acetyl CoA enrichment and allows determination of cholesterol synthesis through gas chromatography mass spectrometry (GCMS) analysis of cholesterol alone and the subsequent application of mathematical modeling (Hellerstein 1995).

DI shows good agreement with plasma mevalonic acid concentrations (Jones *et al.* 1992) as an index of short term cholesterogenesis and with the established sterol balance technique in measuring cholesterogenesis over the long term (Jones *et al.* 1998). In general, although DI and MIDA provide similar appraisals of cholesterogenesis (Jones 1997, Hellerstein & Neese 1992)) (Table

2.2), they have never been compared directly. Because cholesterol synthesis is highly dependent on diet and physiological state (Jones *et al.* 1998, Jones *et al.* 1993, Faix *et al.* 1993, Neese *et al.* 1993) and each approach relies on different assumptions and constraints, direct intercomparison of the two techniques is warranted . Our objective was to compare endogenous cholesterol biosynthesis using DI and MIDA methodologies simultaneously in human subjects under comparable temporal conditions.

Table 2.1. Comparison of two stable isotopic methods of determining human cholesterol synthesis

	Deuterium Incorporation	**Mass Isotopomer Distribution Analysis**
Tracer	D_2O - inexpensive	^{13}C-acetate - expensive
Tracer dose	low	high
Study conditions	free-living subjects	in-patients
Infusion protocol	oral bolus with supplemental drinking water over 24h	15h intravenous infusion
Sampling protocol	2 blood samples	serial blood samples requiring indwelling catheter
Sample preparation	complex multi-step extraction, separation, combustion and reduction	routine cholesterol derivatization (pentofluorobenzoyl chloride)
Mass spectrometry	IRMS - high sensitivity, low enrichments	GCMS - low sensitivity requires high enrichments
Data analysis	simple mathematical relations	complex mathematical modeling
Precursor pool	NADPH, total body water	acetyl CoA
Determination of precursor enrichment	plasma water	MID of product
Theoretical Issues	establishing D_{max} (constant labeling of NADPH[§] in different states)	constant precursor pool labeling and FSR

2.3 METHODS

Study design

Twelve subjects underwent a stable isotope infusion protocol designed to measure cholesterol biosynthesis using DI and MIDA simultaneously. Because DI typically measures cholesterol synthesis at 24 h, and MIDA measures cholesterol synthesis at 15 h, cholesterol synthesis was determined over 15 and 24 h with a 7 h feeding period for both methodologies in order to respect the temporal constraints of both techniques. The study received approval from the Ethics Review Board of the Faculty of Agricultural and Environmental Sciences at McGill University. The aims and procedures of the study as well as potential risks were explained to each subject before obtaining written consent.

Subjects and diets

Subjects (4 M, 8 F) were judged to be healthy by medical history and physical exam, as well as by a laboratory profile which determined fasting circulating lipid concentrations (Table 2.2). Subjects consumed their regular meal at 5:00 PM on day 1 of the study, prior to arriving at the Mary Emily Clinical Nutrition Research Unit on the Macdonald Campus of McGill University. Subjects consumed liquid mixed meals (Ensure) based on 130% of their resting energy expenditure as determined by the Mifflin predictive equation (Mifflin *et al.* 1990) in equal hourly doses (4.3±0.4 $kcal \cdot kg^{-1} \cdot h^{-1}$) between 6:00 AM and 1:00 PM on day 2. The liquid meal contained 30% calories as fat, 45% as glucose, 8% as

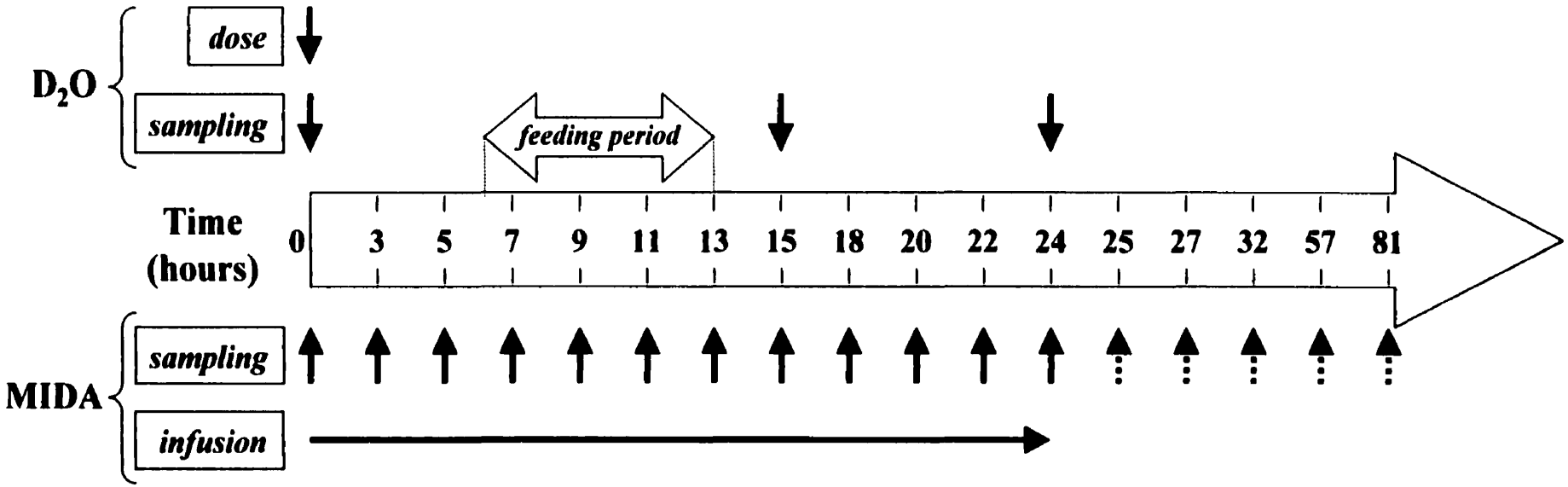

Figure 2.1. Experimental protocol.

fructose, and 17% as protein.

Infusions

At 11:00 PM on day 1 (t=0), a blood sample was drawn to determine baseline water and cholesterol enrichment. Subjects then received 0.7 $g \cdot kg^{-1}$ body water D_2O (Cambridge Isotope Labs, Cambridge, MA) as a bolus oral dose. Na[1-^{13}C]-acetate (Cambridge Isotope Labs, Cambridge, MA; 99% enriched) was infused at 90 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ using a volumetric infusion pump (IMED Corporation, San Diego, CA). Top-up doses of D_2O were given regularly throughout the 24 h protocol to ensure constant body water deuterium enrichment throughout the study (6, 7). At 11:00 PM on day 2, infusion of the labeled acetate was stopped and subjects remained in the Mary Emily Clinical Nutrition Research Unit until 7:00 AM the following morning.

Sampling

The blood sampling catheter was inserted in a superficial vein on the back of the non-dominant hand. Blood samples were drawn into EDTA tubes and immediately centrifuged for 10 min. Red blood cells were stored at -80^{0}C for analysis of cholesterol fractional synthesis by MIDA and plasma samples were stored separately at -20^{0}C for analysis of cholesterol fractional synthesis by DI. 8 mL blood samples for determination of cholesterol FSR by DI were taken at 15 and 24 h. 2 mL blood samples for determination of cholesterol FSR by MIDA

were taken at 3, 5, 7, 9, 11, 13, 15, 16, 20, 22 and 24 h. Additional samples were taken after cessation of label administration at 1, 3, 8, 33, and 57 h post-infusion in order to measure the fractional degradation of cholesterol.

Plasma lipid analysis

Serum cholesterol and lipid concentrations were performed prior to the study using enzymatic techniques validated by the National Institutes of Health Lipid Research Clinics (Friedewald *et al.* 1972). HDL-cholesterol concentrations were determined after treatment of plasma with dextran sulfate and Mg^{++} by enzymatic assay (Warnick *et al.* 1982). Serum LDL-cholesterol concentrations were calculated from serum total and HDL cholesterol concentrations using the equation formulated by Friedewald *et al.*

Cholesterol biosynthesis measurement using the deuterium incorporation methodology

All samples were analyzed in duplicate. Cholesterol FSR was determined as the rate of incorporation of deuterium into erythrocyte membrane free cholesterol (Jones *et al.* 1993). FSR obtained from erythrocyte membrane cholesterol corresponds to FSR obtained from plasma free cholesterol using the DI method (Jones *et al.* 1993, London & Schwarz 1953). Plasma lipids were extracted using a modified Folch extraction procedure (Folch *et al.* 1953) and dried under nitrogen. Free cholesterol was isolated from the extracted lipids by

thin-layer-chromatography (TLC) on silica gel against a free cholesterol standard. Free cholesterol bands were scraped from the TLC plates and the cholesterol was eluted from silica with hexane:chloroform:diethyl ether solution (5:2:1 vol:vol:vol). The free cholesterol was transferred to Pyrex™ combustion tubes containing CuO and silver wire. Tubes were subsequently flame-sealed under vacuum and cholesterol was combusted to CO_2 and H_2O at 520^{0}C for 4 h. Water resulting from the combustion was cryogenically separated from CO_2 by distillation into Pyrex™ tubes containing 50 mg zinc under vacuum. Tubes were flame-sealed under vacuum and the water was reduced at 520^{0}C for 30 minutes in order to obtain H_2/D_2 gas. Deuterium enrichment of the resultant gas was measured on a dual inlet isotope ratio mass spectrometer (IRMS) (VG Isogas 903D, Cheshire, England). Pilot studies by our group determined that the deuterium label was undetectable by GCMS.

Plasma-water enrichment was measured after dilution of 0, 15 and 24 h plasma samples with water of known isotopic enrichment to bring the enrichment into the working range of the International Atomic Energy Agency mass spectrometer calibration standards.

FSR represents that fraction of the free portion of the rapidly turning over central cholesterol pool that is synthesized per day as per the formula (Jones *et al.* 1993):

$$FSR(\%\cdot day^{-1})=(\delta_{cholesterol}/\delta_{plasma}) \times 0.478 \times 24 \times time^{-1} \times 100 \qquad \text{Eqn (1)}$$

where δ refers to deuterium enrichment of free cholesterol or water above baseline at either 15 or 24 h. The factor 0.478 represents D_{max}, the maximum proportion of hydrogen atoms that become enriched by deuterium per molecule of cholesterol over periods up to 48 h (Jones *et al.* 1993).

Absolute synthesis rate (ASR) was calculated as per the formula (Goodman *et al.* 1980):

$$ASR\ (mg\cdot kg^{-1}\cdot day^{-1})=(FSR \times (M_1/2))/body\ weight \qquad \text{Eqn (2)}$$

M_1 = 0.287 x body weight (kg) + 0.0358 x total cholesterol concentration ($mg\cdot dL^{-1}$) - 2.40 x TGGP. Where M_1 represents the size of the total cholesterol pool (mg), $M_1/2$ represents the size of the rapidly exchanging free cholesterol pool and TGGP is a variable equal to 1, 2, or 3 depending on serum triglyceride concentration (<200, 200-300, or >300 $mg\cdot dL^{-1}$) .

Cholesterol biosynthesis measurement using mass isotopomer distribution analysis

Cholesterol synthesis rates by MIDA were determined over 15 and 24 hours. Blood samples corresponding to time points 0, 3, 5, 7, 9, 11, 13, 15, 18, 20, 22, and 24 h after the start of the infusion, and 1, 3, 8, 33, and 57 h post-infusion were derivatized for GCMS analysis.

Plasma free cholesterol was extracted using a 95% ethanol:acetone (1:1 vol:vol) solution, dried under nitrogen, and derivatized to its pentofluorobenzoyl (PFB) derivative by incubating with pentofluorobenzoyl chloride (C_7ClF_5O) and pyridine according to Ostlund *et al.* (1996). The resulting PFB-cholesterol derivative was extracted with petroleum ether, dried under nitrogen, and resuspended in toluene. Triplicate split injections were made on a Hewlett Packard GC (model 5890) with a DB-5 capillary column of 0.25 mm i.d., 0.33 μ film thickness, and 10 m in length (J&W Scientific, Folsom, CA). A temperature program of 250°C for 1 min and then a $10^0 \cdot min^{-1}$ rise to 280^0 was used. Column effluent was admitted to a Hewlett Packard 5988A mass spectrometer operating in negative chemical ion mode with methane reagent gas (1 torr). Ions from *m/z* 580 corresponding to PFB-cholesterol $[M+0]^-$ to *m/z* 584 corresponding to PFB-cholesterol $[M+4]^-$ were monitored. The mole fractions (MF) 581/(580+581+582+583+584), 582/(580+581+582+583+584), 583/(580+581+582+583+584) and 584/(580+581+582+583+584) in samples

following infusion of the tracer were corrected by subtracting the same ratios at baseline in order to obtain mole excesses (EM_{581} to EM_{584}), as demonstrated by Hellerstein & Neese (1992).

MIDA FSR was calculated according to the model of Hellerstein & Neese (1992). Briefly, baseline isotopomer frequencies of PFB-cholesterol $[M + \chi]^-$ were calculated using natural abundances of constituent elements and binomial or polynomial expansion. Using the tracer [1-^{13}C]acetate, C_{max}, the maximum number of ^{13}C atoms that could be incorporated into cholesterol, is 12. The effect of varying excess enrichment of this precursor pool (p) was calculated also using ISOCOM (a generous gift of David Hachey). As demonstrated by Hellerstein & Neese (1992), using ratios among molar excesses in this model eliminates the need to correct for natural abundance. Ratios among molar excesses of cholesterol are determined uniquely by precursor enrichment (p) therefore the subunit precursor enrichment can be calculated from the ratios among excess enrichments (R) without the need to measure the actual acetate precursor units. This was done by plotting R of each mass isotopomer of theoretically generated PFB-cholesterol as a function of p (Figure 2.2). R_1 ($EM_{581}/\sum EM_{581\text{-}584}$) and R_3 ($EM_{583}/\sum EM_{581\text{-}584}$) were chosen to generate equations for p because both functions were linear and were not flat graphically (Hellerstein & Neese 1992). This relation between p and R minimizes the impact of analytical error and of multiple precursor pools if these are present (Hellerstein & Neese 1992). Estimates of p obtained with R_1 were

highly correlated (r=0.70, p=0.0001) to those obtained with R_3, therefore an average of R_1 and R_3 was used. Using these equations, experimentally determined R's were used to determine the enrichment of the precursor pool enrichment (p).

Due to the contribution of dietary cholesterol to the body pool, fractional synthesis never reaches 100%. The asymptote (A*), which is the frequency of each isotopomer in newly synthesized cholesterol for a given p, was calculated from molar excesses of theoretically generated PFB-cholesterol (Fig. 2.3). Experimentally, the asymptote of the m+1 isotopomer was used to calculate A* from p which was determined by equations in Fig. 2.2. This function (E_1A^*) is relatively flat over a wide range of p values, therefore if one is concerned that precursor enrichment is inconstant then selecting such a function between A* and p will minimize the effect of varying p on A* (Hellerstein & Neese 1992). The fraction of cholesterol molecules that were newly synthesized (f, in %) was calculated as the ratio of molar excess of the m+1 cholesterol species (EM_1) to E_1A^*. Fractional synthesis rates were thus obtained using the following equation:

$$FSR\ (\%\cdot day^{-1}) = f \times time^{-1} \times 24\ h \times (1/100) \qquad Eqn(3)$$

ASR was determined from molar excesses of PFB-cholesterol sampled 3, 8, 33, and 57 h post-infusion. Ratios among excesses (R) among higher mass isotopomers which were stable at those time points were used in order to minimize persistent label incorporation.(Hellerstein & Neese 1992). Per carbon enrichments were subsequently calculated from R and plotted against time on a semi-logarithmic scale in order to determine the rate constant of decay (k, i.e.: rate of change per carbon enrichment). The averaged decay constant obtained for the three higher mass isotopomers was then used to calculate ASR (Hellerstein & Neese 1992):

$$ASR\ (mg \cdot kg^{-1} \cdot day^{-1}) = [(k \times f \times pool\ size)/\ 1\text{-}e^{-kt}] \times 24\ h \times body\ weight^{-1} \times (1/100) \quad \text{Eqn (4)}$$

where f is the fraction of newly synthesized cholesterol at 15 and 24 h determined by MIDA in % and pool size is estimated at 130 $mg \cdot kg^{-1}$ body weight (Neese *et al.* 1993).

Statistical analyses

All data was tested for normality. Paired t-test was used to compare FSRs obtained with deuterium incorporation and MIDA using SAS statistical software (version 6.12, SAS Institute Inc., Cary, NC). Correlation statistics were used to measure the relation between DI-derived indices of synthesis and those from MIDA. Agreement between methods was also assessed using a graphical

approach developed by Bland and Altman (Bland & Altman 1986). Data is reported as means±SD. Results were considered significant at $p<0.05$.

2.4 RESULTS

Anthropometric data are summarized in Table 2.2. Deuterium enrichment of body water remained constant between 15 h (39518±301 $^{0}/_{00}$) and 24 h (3942±488 $^{0}/_{00}$) and is thus consistent with the procedural requirements of the DI technique (Jones *et al.* 1993). The MIDA curves used to calculate the isotope content of biosynthetic precursor subunits (p), and the asymptote (A*) representing the theoretical maximum isotopic content of new cholesterol molecules are depicted in Figures 2.2 and 2.3. These standard curves are consistent with those presented by Hellerstein & Neese (1992) for the trimethylsilyl (TMS) derivative of cholesterol.

At 15 h cholesterol FSR measured by DI (4.8±1.2 %·day^{-1}) was different (p=0.03) from FSR measured by MIDA (6.3±1.8 %·day^{-1}). However, there was no difference (p=0.06) at 24 h between FSR measured by DI (7.8±2.5 %·day^{-1}) or MIDA (6.9±2.2 %·day^{-1}). A difference between cholesterol FSR at 15 h and 24 h was detected with both methods (Table 2.3). Original methods were also compared. FSR measured by DI at 24 h was significantly different (p=0.009) from FSR measured by MIDA at 15 h.

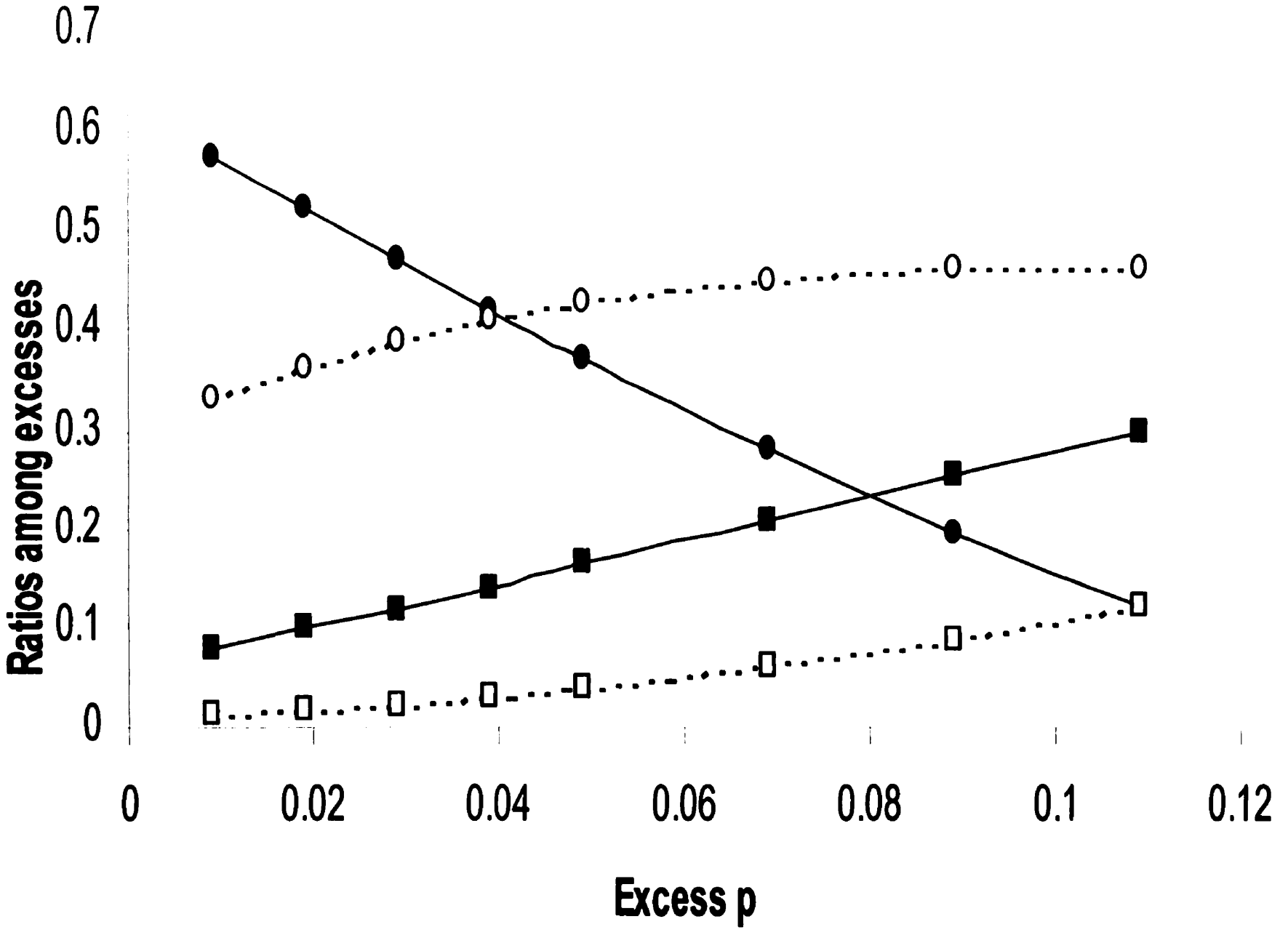

Figure 2.2. Ratios among excesses (R_x)versus excess isotopic enrichment of biosynthetic precursor subunit (p) calculated based on probability analysis of theoretical data (see text). $EM_1/\sum EM_{1-4}$ (R= -4.570p + 0.605) and $EM_3/\sum EM_{1-4}$ (R= 2.22p + 0.055) were used to calculate the actual enrichment of precursor subunits specific to the pentofluorobenzoyl (PFB) derivative of cholesterol. Also represented are $EM_2/\sum EM_{1-4}$ and $EM_4/\sum EM_{1-4}$.

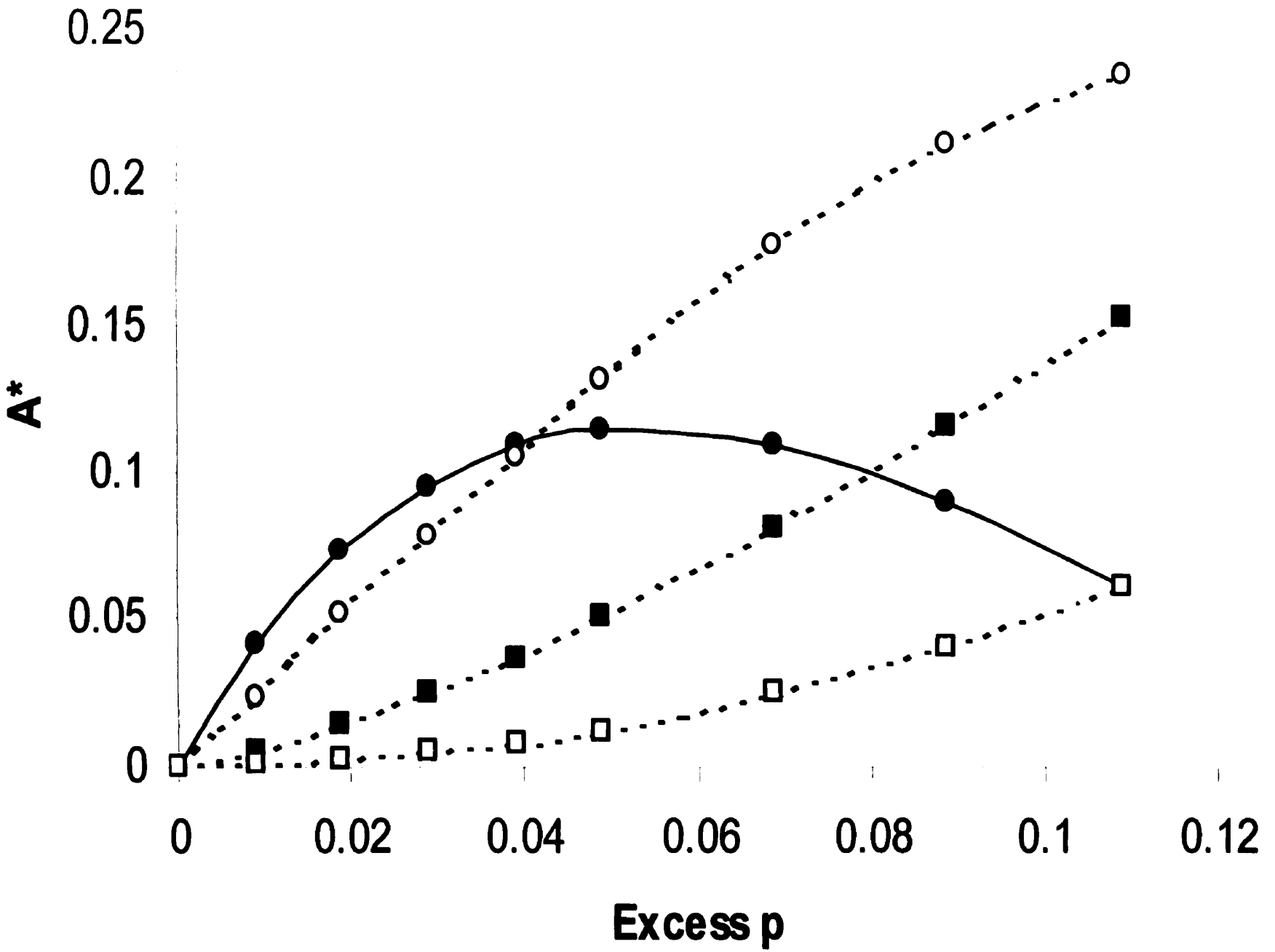

Figure 2.3. Asymptote (A*) versus p. The asymptote represents the theoretical maximum isotopic enrichment of new molecules expressed as molar excesses ($MF_x/\sum MF_{1-4}$). Calculation of the expected frequency of newly synthesized molecules of the PFB derivative of cholesterol was based on the excess mole fraction of the m+1 cholesterol species ($MF_1/\sum MF_{1-4}$) ($A^* = -29.97p^2 + 3.597p + 0.009$). Also represented are $MF_2/\sum MF_{1-4}$, $MF_3/\sum MF_{1-4}$ and $MF_4/\sum MF_{1-4}$.

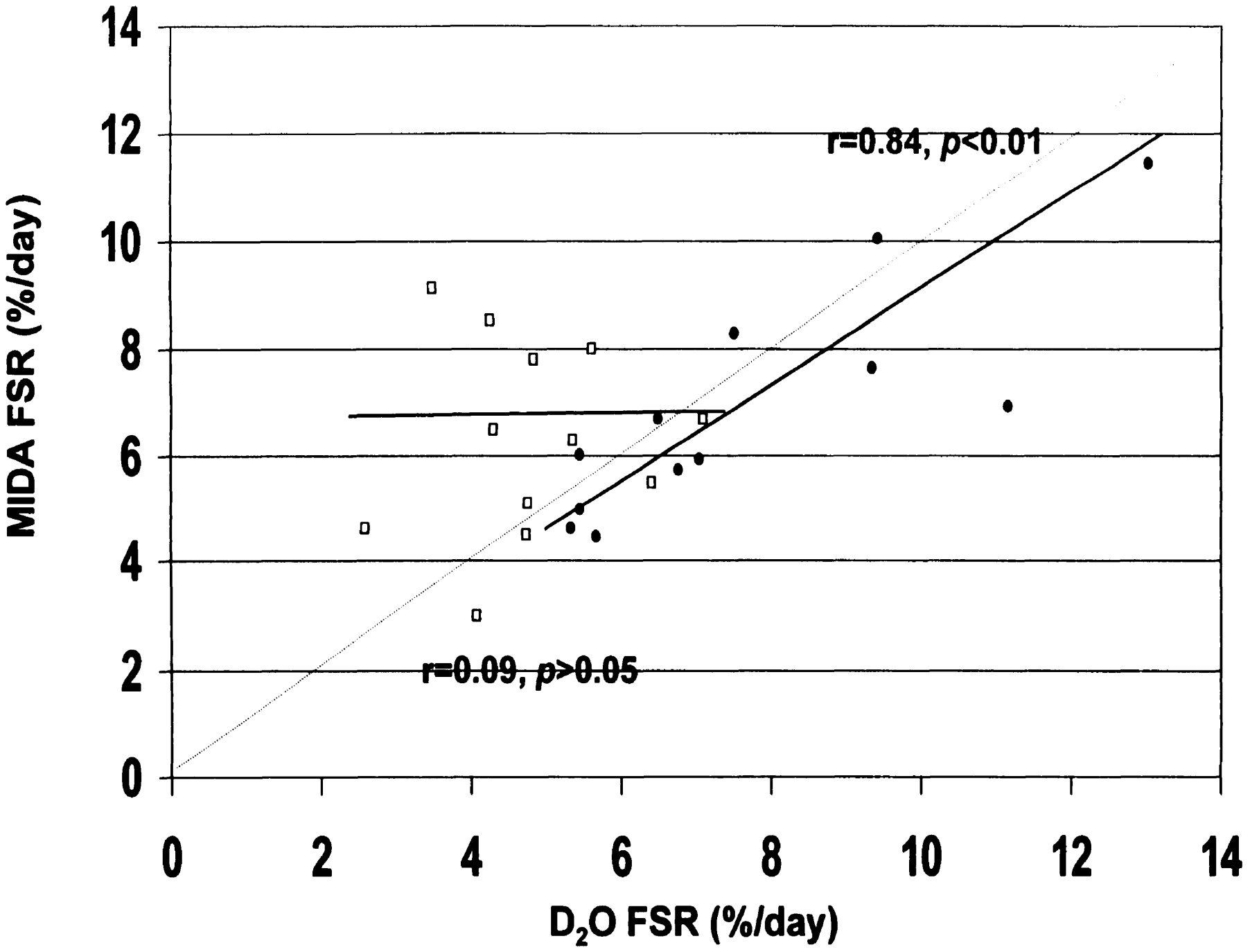

Figure 2.4. The correlation of subjects' cholesterol synthesis between deuterium incorporation and MIDA at 15 h (r=0.09, $p>0.05$) (□) and 24 h (r=0.84, $p<0.001$) (●).

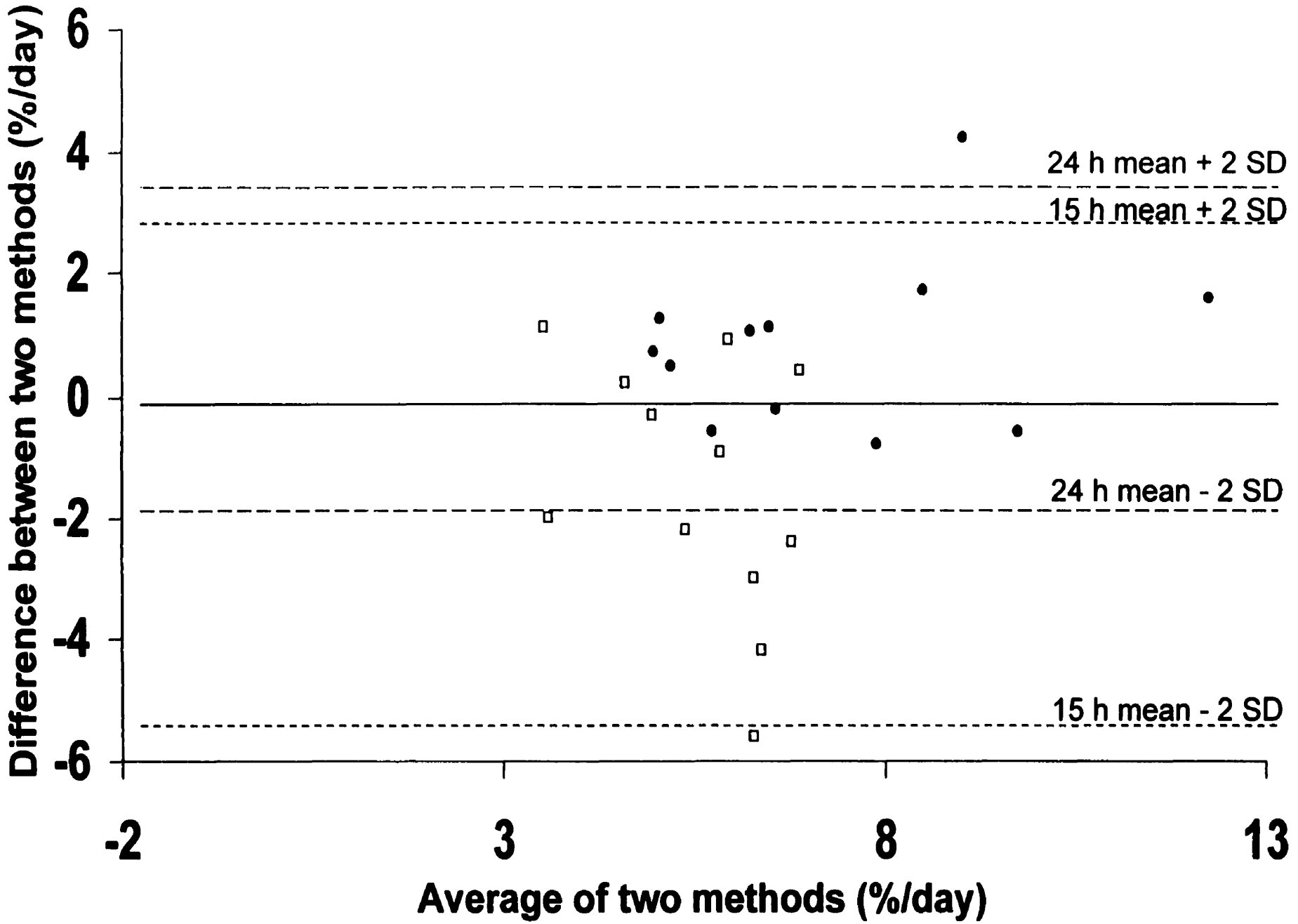

Figure 2.5. The differences between deuterium incorporation and MIDA as a function of the mean of the two measurements for each subjects' cholesterol synthesis data. Upper and lower 95% confidence limits of agreement (2SD) are indicated for both 15 h and 24 h measurements (15 h □, 24 h ●).

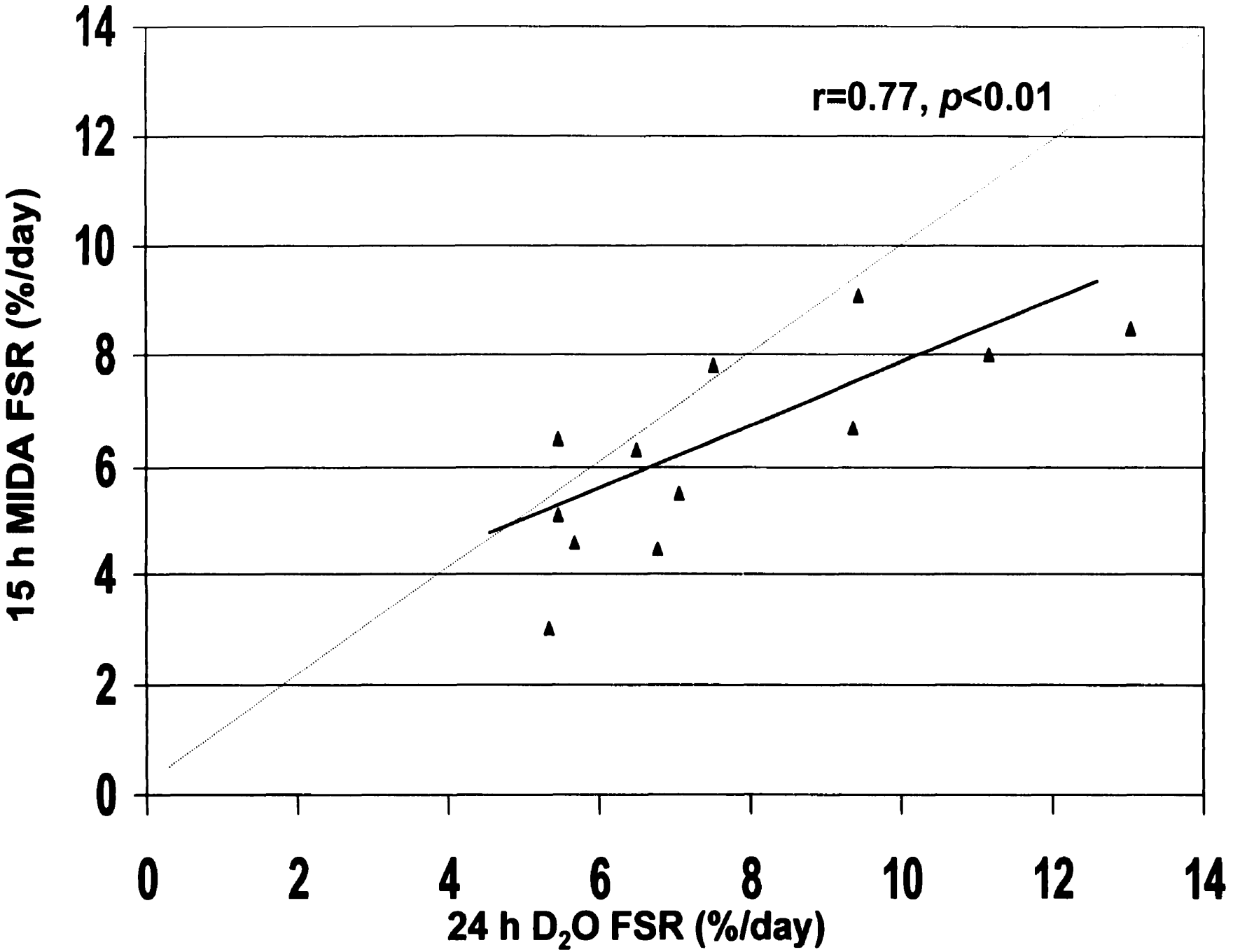

Figure 2.6. The correlation of subjects' cholesterol synthesis between deuterium incorporation at 24 h and MIDA at 15 h ($r=0.77$, $p<0.01$).

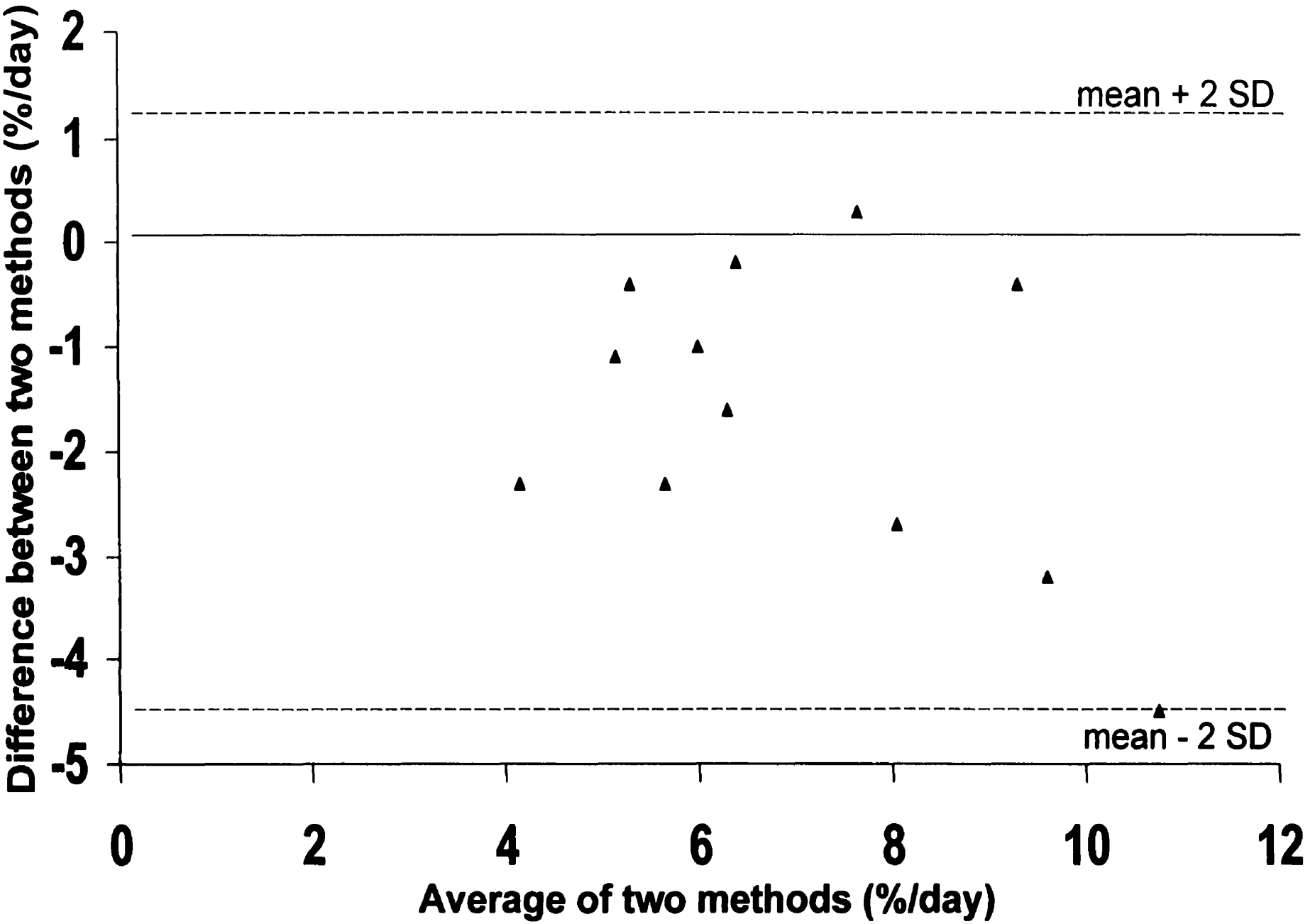

Figure 2.7. The difference between cholesterol synthesis measured by deuterium incorporation at 24 h and MIDA at 15 h as a function of the mean of the two measurements for each subject. Upper and lower 95% confidence limits of agreement (2SD) are indicated by the dashed lines.

There was no correlation (r=0.09, p=0.9) between FSR measured using DI and MIDA at 15 h, yet there was a very strong correlation (r=0.84, p=0.0015) between FSR measured at 24 h using both methods (Figure 2.4). Although FSR measured by DI at 24 h correlates with FSR measured by MIDA at 15 h (r=0.77, p=0.0081) (Figure 2.6), graphical analysis by the method of Bland & Altman (Figure 2.7) suggests that this association is random.

Total body pool of free cholesterol which were used in the calculation of ASR for the DI method (11.2±2.8 g) was different (p=0.015) from the estimate used for the MIDA technique (8.5±2.3 g). Nonetheless, ASR at 15 h measured by DI (8.4±2.5 $mg \cdot kg^{-1} \cdot d^{-1}$) was not different ($p$=0.32) from MIDA (9.5±2.9 $mg \cdot kg^{-1} \cdot d^{-1}$). There was a difference (p=0.17) between ASR measured at 24 h by DI (13.4±4.3 $mg \cdot kg^{-1} \cdot d^{-1}$) and MIDA (10.7±3.5 $mg \cdot kg^{-1} \cdot d^{-1}$). ASR measured by DI at 24 h was different (p=0.001) from ASR measured by MIDA at 15 h. No correlation between techniques was obtained for ASR measured at 15 h (r=-0.02, p=0.19); however, a strong correlation was obtained for ASR measured at 24 h (r=0.81, p=0.0001).

2.5 DISCUSSION

Despite the emergence of stable isotope techniques for the measurement of in vivo cholesterol biosynthesis in humans (Jones *et al.* 1993, Hellerstein 1995, Hellerstein & Neese 1992, Schoenheimer & Rittenberg 1935) their systematic

evaluation against one another has not been conducted. We compared cholesterol synthesis rates measured by DI and MIDA under temporal conditions typical of both methodologies. We present new evidence indicating that the DI method and the MIDA method yield values that correspond well in the measurement of human endogenous cholesterol biosynthesis. Estimates of absolute synthesis obtained by DI and MIDA are consistent with results presented elsewhere (11±2 $mg \cdot kg^{-1} \cdot d^{-1}$ by DI, 9±2 $mg \cdot kg^{-1} \cdot d^{-1}$ by MIDA) (Jones *et al.* 1998, Hellerstein & Neese 1992, Faix *et al.* 1993, Neese *et al.* 1993).

Our findings suggest that although there is no difference in FSR or ASR measured with either DI or MIDA at either 15 or 24 h, the two techniques yield values which correspond more strongly at 24 h. Furthermore, the greatest difference in cholesterol biosynthesis was observed between measurements made with MIDA at 15 h and those made by DI at 24 h which correspond to measurements for the both unmodified techniques. Interestingly, these two values are most often cited in the literature. It has been demonstrated that cholesterol synthesis exhibits a diurnal variation (Jones & Schoeller 1990) and that this 24 h variation is highly dependent on meal timing (Cella *et al.* 1995). Therefore, measuring synthesis over 24 h has the added benefit of accounting for changes in the diurnal rhythmicity of cholesterogenesis. Cholesterol synthesis was lower ($p<0.05$) at 15 h than at 24 h regardless of the methodology used, which is consistent with results presented elsewhere (Faix *et al.* 1993, Mazier & Jones

1997). However, evidence suggests that the highest rates of cholesterogenesis occur postprandially in the 24 h diurnal cycle (Jones *et al.* 1988, Jones et al. 1993). This corresponds to the period in our study where the highest fractional synthesis rates are observed.

It is particularly important to note that ASR did not differ between methods despite the significant difference in total body free cholesterol pool size estimates for either technique. This discrepancy in pool size calculation may be the result of differences in the approaches for estimating whole body cholesterol kinetics by DI and MIDA methodologies. Furthermore, in calculating ASR based on MIDA, decay constants are best calculated in the 48-72 h following a 12-18 h infusion (Neese *et al.* 1993). It is difficult to say whether extending the infusion period to 24 h and collecting samples for measurement of label decay at 8, 33, and 57 h post-infusion as was done in the current study could have impacted upon true decay rate calculation.

To measure cholesterol biosynthesis, a labeled substrate is introduced into a specific precursor pool of acetyl CoA, and the incorporation of labeled acetate into *de novo* cholesterol is then quantified (Hellerstein 1995). Cholesterol synthesis occurs at the cellular level; therefore, it becomes extremely difficult to determine accurately the precursor pool enrichment due to subcellular compartmentalization, local microenvironments, and metabolite channeling

(Hellerstein 1995). The key difference between DI and MIDA is in their approach to this problem. In the DI technique, deuterated tracer water equilibrates between the precise intracellular site of synthesis and the extracellular physiological fluid, thereby providing a homogeneous which is accessible for sampling. MIDA instead determines the isotopic content of both product and precursor based on the MID of the product thus eliminating the need for sampling the acetyl CoA precursor pool.

Theoretically, the major concern with DI lies in establishing D_{max}. D_{max} remains stable over periods as long as 48 h (Jones 1990). However, over the long term, D_{max} may be influenced by such factors as dietary fat type (Feingold & Grunfeld 1987) and ethanol ingestion (Selmer & Grunnet 1976). Diraison *et al.* (1996) administered deuterated water to rats and then determined that the maximum incorporation number of deuterium atoms in cholesterol from the MID of plasma cholesterol was 27. Lee *et al.* (1994) determined that D_{max} was as high as 30 after rats were exposed to deuterium in their drinking water for one-week. The general conclusion derived from these studies (Diraison *et al.* 1996, Lee *et al.* 1994) and others (Javitt & Javitt 1989) is that the contribution of deuterated acetyl CoA *in vivo* may be the source of the additional deuterium atoms when measurement periods exceed 48 h.

Although the principal advantage of MIDA is that the enrichment of the precursor pool at the precise site of synthesis is determined from the isotopic content of the product, this also constitutes one of the technique's theoretical complications because in order for the assumption to hold true, two conditions must be met. First, the precursor pool must be homogeneous and its enrichment constant: second, the fractional synthesis of the product must be constant. Recent reports have questioned these assumptions in the broader context of MID analysis of intermediary metabolism. Higher mass isotopomers can become more highly enriched in cultured cells than in vivo, thereby allowing more rigorous analysis of the MID pattern of cholesterol. Kelleher *et al.* (1994) measured cholesterol synthesis by isotopomer spectral analysis, a method based on similar principles to MIDA, using high concentrations of [1-^{13}C] acetate in cell culture. Their analysis showed the MID pattern to be inconsistent with an acetyl-CoA pool of constant enrichment or a constant synthesis rate. The MID pattern of glucose has been used by Landau *et al.* (1995) in perfused livers and Previs *et al.* (1995) in animals and humans. Both studies reported problems using MIDA with a heterogeneous precursor pool (triose phosphate). These conditions may limit the applicability of MIDA to only few polymers composed of repeating monomeric subunits. However, the impact of these factors can be minimized as discussed by Hellerstein & Neese (1992) and as was used in the present situation. When the possibility of multiple precursor pools arises, one can minimize the impact of these pools by selecting an as near to linear relation between p and R as possible

when inferring p from R. Furthermore, if one is concerned that precursor pool enrichment is not constant, selecting a relation between A* and p such that A* is relatively flat over a wide range of p values minimizes the effect of changing precursor pool enrichment.

Operationally, the DI technique requires a labor intensive, lengthy, multi-staged off-line preparation of cholesterol before it can be analyzed by IRMS. This may introduce some error. However, the methodology can be applied to free living subjects. Procedurally, MIDA has several drawbacks. An indwelling catheter is necessary for delivery of the isotope label, thus eliminating the possibility of using MIDA in free living subjects as is possible with the DI technique. Serial blood samples are necessary for GCMS analysis. This requires a second indwelling catheter for blood sampling making the technique more invasive than the DI method. Finally, data analysis is lengthy and requires elaborate mathematical processing.

Both deuterium incorporation (Jones *et al.* 1993, Jones *et al.* 1998, Jones *et al.* 1988, Jones *et al.* 1993, Mazier & Jones 1997, Cuchel *et al.* 1997) and MIDA (Faix *et al.* 1993, Neese *et al.* 1993, Empen *et al.* 1997, Bjorkhem *et al.* 1997, Bandsma *et al.* 1998) have has been applied to the study of cholesterol synthesis under various metabolic conditions (Table 2.4). DI has recently been evaluated against the cholesterol balance technique, which is considered the

standard of choice for measurement of synthesis over the long term (Jones *et al.* 1998). The results demonstrated that the DI method yields values that correspond well to those of sterol balance. In fact, Jones *et al.* obtained a strong correlation between synthesis measured by sterol balance and DI ($r=0.745$, $p<0.0001$). Comparison of the deuterium uptake method to plasma mevalonic acid levels as indices of short-term synthesis gives good agreement as well (Jones *et al.*1992).

In summary, present results represent the first direct comparison of DI and MIDA for measurement of human cholesterol synthesis. It is concluded that DI and MIDA provide similar estimates of fractional and absolute cholesterol synthesis in humans. Measurements of *de novo* cholesterogenesis are best made over a 24 h period to account for the inherent diurnal rhythmicity in cholesterol synthesis. The choice between DI and MIDA should be based on the specific study and available clinical and analytical facilities.

ACKNOWLEDGMENTS

The authors would like to thank Timothy M. Bayley, Catherine A. Vanstone and the staff at the Mary Emily Clinical Nutrition Research Unit for their assistance in conducting the clinical trial. Thanks are also extended to Dr. William Parsons for his assistance in the screening process. This work is supported by the Medical Research Council of Canada.

Table 2.2. Subject characteristics and plasma lipid concentrations

Subject	Gender	Age (years)	Weight (kg)	BMI ($kg \cdot m^{-2}$)	Total Cholesterol ($mmol \cdot L^{-1}$)	HDL-Cholesterol ($mmol \cdot L^{-1}$)	LDL-Cholesterol ($mmol \cdot L^{-1}$)	Triglycerides ($mmol \cdot L^{-1}$)
1	M	24	95.3	31.2	4.4	1.3	2.9	0.5
2	F	27	72.6	22.9	4.8	2.0	2.6	0.7
3	M	39	73.1	23.1	4.1	1.1	2.3	1.5
4	M	30	84.9	24.1	5.8	1.1	3.7	2.1
5	F	30	50.4	20.5	4.2	1.4	2.3	1.1
6	F	28	49.9	20.8	4.4	0.9	2.7	1.8
7	F	30	49.0	19.9	3.8	1.4	2.2	0.6
8	M	34	93.5	27.9	4.2	1.1	2.2	2.0
9	F	22	53.1	18.8	3.8	1.3	2.2	0.8
10	F	25	51.8	21.6	3.3	1.5	1.5	0.7
11	F	22	49.8	20.8	5.1	0.8	3.4	1.9
12	F	27	57.2	23.3	4.5	1.5	2.3	1.5
Mean (±SD)	**4 M 8 F**	**28 (±5)**	**65.1 (±17.2)**	**22.9 (±3.4)**	**4.4 (±0.7)**	**1.3 (±0.3)**	**2.5 (±0.6)**	**1.3 (±0.6)**

Table 2.3. Individual and mean fractional and absolute cholesterol synthesis rates obtained using deuterium incorporation and mass isotopomer distribution anaylsis

Subject	FSR ($\%\cdot d^{-1}$)				ASR ($mg\cdot kg^{-1}\cdot d^{-1}$)			
	15 h		24 h		15 h		24 h	
	DI	MIDA	DI	MIDA	DI	MIDA	DI	MIDA
1	4.1	3.0	5.3	4.6	6.7	6.4	8.7	7.4
2	4.3	6.5	5.5	5.0	7.4	9.3	9.4	7.5
3	2.6	4.6	5.7	4.4	4.3	6.7	9.4	7.1
4	5.4	6.3	6.5	6.7	9.5	9.0	11.5	10.0
5	5.6	8.0	11.2	6.9	10.0	11.8	19.8	10.9
6	4.8	5.1	5.5	6.0	8.6	7.1	9.8	8.7
7	3.5	9.1	9.5	10.0	6.1	15.8	16.4	16.3
8	4.3	8.5	13.0	11.5	6.9	12.4	21.1	17.9
9	4.7	4.5	6.8	5.7	8.1	6.4	11.6	8.8
10	4.8	7.8	7.5	8.3	8.0	11.5	12.4	12.8
11	7.1	6.7	9.4	7.7	13.5	9.9	17.8	12.2
12	6.4	5.5	7.1	5.9	11.4	7.9	12.5	9.1
Mean (±SD)	**4.8 (±1.2)**[‡]	**6.3 (±1.8)**[*‡¶]	**7.8 (±2.5)**	**6.9 (±2.2)**	**8.4 (±2.5)**[§]	**9.5 (±3.5)**[§|]	**13.4 (±6.2)**	**10.7 (±3.5)**

[*]Different from FSR by DI at 15 h ($p<0.05$). [‡] Different from FSR by DI at 24 h ($p<0.05$). [¶] Different from FSR by MIDA at 24 h ($p<0.05$). [§] Different from ASR by DI at 24 h ($p<0.05$). [|] Different from ASR by MIDA at 24 h ($p<0.05$).

Table 2.4. Applications of deuterium incorporation and mass isotopomer distribution analysis in the study of cholesterol biosynthesis *in vivo*.

	Subjects	Summary
Deuterium Incorporation		
Jones *et al.* 1988 (13)	normal males	Synthesis is sensitive to short term food restriction
Jones *et al.* 1993a (7)	normal males	Synthesis is sensitive to changes in meal frequency
Jones *et al.* 1993b (14)	normolipidemic adults	ApoE genotype does not influence cholesterogenesis
Cuchel *et al.* 1997 (37)	hyperlipidemic males	Declines in plasma lipid levels after Lovastatin treatment are attributable to reduction in synthesis
Mazier & Jones 1997 (28)	normal males	Synthesis is greater in fed than in fasted state; reduced synthesis is not responsible for effect of different fats on plasma cholesterol concentrations
Jones *et al.* 1998 (10)	normal adults	Synthesis rates measured by deuterium uptake correspond with those measured by sterol balance
Mass Isotopomer Distribution Analysis		
Faix *et al.* 1997 (15)	men, menstruating women	Synthesis is 1.5 to 3-fold higher at night and does not vary over the menstrual cycle
Neese *et al.* 1997 (16)	rats and humans	Absolute synthesis in women is 568±55 mg/day; women exhibit a diurnal variation
Empen *et al.* 1997 (38)	normal adults	Contribution of *de novo* cholesterol synthesis to bile exceeds that to plasma but is minor in humans
Bjorkhem *et al.* 1997 (39)	rats	Hydroxylation facilitates cholesterol transfer across the blood-brain barrier and may be critical for cholesterol homeostasis in the brain

BRIDGE

As stated in the introduction to Manuscript 1, regulation of cholesterol biosynthesis is an important factor in cardiovascular disease prevention. Recent findings have revealed that biosynthesis plays a dual role in the development of CVD. First, as demonstrated by the numerous studies conducted on the topic, cholesterol biosynthesis contributes to more than 2/3 of the body pool of cholesterol, which in turn contributes to circulating cholesterol concentrations. Therefore, controlling biosynthesis has a profound impact on circulating cholesterol concentrations. The development of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors remains the most potent weapon in the fight to lower circulating cholesterol concentrations of North Americans. These HMG CoA reductase inhibitors function on the premise that suppressing biosynthesis will lead to a decreased output of *de novo* cholesterol into the bloodstream. However, a lesser known effect of these drugs has lead to a new perspective on the role of *de novo* cholesterogenesis in CVD development.

As stated in the introduction, several recent studies have shown that isoprenoid metabolites that are synthesized as intermediates in the pathway of cholesterol synthesis play a role in the development of atherosclerotic lesions in arterial walls. These isoprenoid metabolites have been shown to enhance smooth muscle cell migration in the arterial wall, an early step in the development of

lesions. Suppression of cholesterol biosynthesis by administration of HMG CoA reductase inhibitors results in a decrease in the plasma concentrations of isoprenoid intermediates and therefore a reduction in the risk of developing atherosclerosis.

The objective of Manuscript 1 was to determine the difference between the use of deuterium incorporation and MIDA techniques in the measurement of *de novo* cholesterol synthesis in human subjects. We were able to demonstrate that both stable isotope techniques predict the same fractional synthesis rates under identical physiological conditions. Our findings strengthen the validity of both methodologies, and allow us to choose between two techniques to measure cholesterol biosynthesis in various metabolic states. Specifically, we will attempt to determine whether weight loss achieved through energy restriction can effectively reduce cholesterol biosynthesis and thus reduce the risk of developing cardiovascular disease. Although it is widely accepted that obesity leads to an increased production of cholesterol, it is yet to be determined whether weight loss can reduce *de novo* cholesterogenesis.

Having demonstrated that our choice of methodology is technically sound and valid will strengthen our elucidation of the biochemical principles which modulate cholesterol biosynthesis during weight loss and add credence to our findings.

Chapter 3. Manuscript 2.

Weight Loss Through Energy Restriction Suppresses Cholesterol Biosynthesis in Overweight, Mildly Hypercholestrolemic Men

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3.1 ABSTRACT

Objectives: Obesity constitutes a major risk factor for CVD. Obese individuals produce twice as much cholesterol as do lean individuals, yet the influence of weight loss on endogenous cholesterol biosynthesis has not been directly investigated to date. Therefore, our objective was to examine effects of weight loss as achieved through energy restriction upon human *in vivo* circulating cholesterol concentrations and synthesis.

Design and Subjects: Seven subjects (64 to 77 yrs) with a mean BMI of 30.6±1.6 $Kg \cdot m^{-2}$ were recruited into a 2-phase prospective clinical trial. In the first phase, subjects were placed on an American Heart Association (AHA) Step I diet for 3 mo with no change to their usual energy intake so as to maintain energy balance. Immediately following this weight-stable phase, subjects were placed on an AHA Step I diet with a targeted reduction in energy intake of approximately 1000 $kJ \cdot day^{-1}$ for 6 mo so as to achieve negative energy balance leading to weight loss. The incorporation rate of deuterium from body water into erythrocyte membrane free cholesterol over 24 h was utilized as an index of cholesterogenesis at the end of both phases.

Results: Subjects showed mean weight loss ($p<0.05$) from 91.3±12.5 kg to 85.6±12.3 kg (6.27±2.61% initial body weight). Cholesterol fractional synthetic rate (FSR) after weight loss (2.73±0.62 $\%\cdot d^{-1}$) was reduced ($p<0.05$) compared to pre-weight loss levels (7.90±2.25 $\%\cdot d^{-1}$). Absolute synthesis rate (ASR) also declined ($p<0.05$) between pre-weight loss

(2.35±1.15 $g \cdot d^{-1}$) and post-weight loss (0.81±0.44 $g \cdot d^{-1}$). The group showed a trend towards lower circulating lipid concentrations (4.85±0.78 $mmol \cdot L^{-1}$ to 4.47±0.96 $mmol \cdot L^{-1}$) while circulating triglyceride concentrations decreased ($p<0.05$) from 1.61±0.28 $mmol \cdot L^{-1}$ to 1.27±0.39 $mmol \cdot L^{-1}$, subsequent to weight loss.

Conclusions: These data suggest that energy restriction resulting in even modest weight loss effectively reduces endogenous cholesterol synthesis thus decreasing the risk of CVD development in obese men.

Sponsorship: This work is supported by the University of Maryland Claude Pepper Older Americans Independence Center NIH/NIA-P60-AG12583 and by the Medical Research Council of Canada.

Descriptors: weight loss; energy restriction; cholesterol; deuterium; humans

3.2 INTRODUCTION

Obesity represents a major risk factor for cardiovascular disease (CVD). In 1974, the Framingham study found that for a 10% reduction in weight in men, close to a 20% reduction in coronary incidence can be expected, whereas for each 10% increase a 30% increase in incidence is anticipated (Ashley & Kannel, 1974). Elevated circulating cholesterol concentrations are associated with undesirable plasma lipid responses that also elevate the risk of CVD (Gotto *et al*, 1990; Ulbricht & Southgate, 1991).

The body pool of cholesterol, which ultimately determines circulating cholesterol concentrations, receives up to 2/3 of its input from *de novo* synthesis (Rudney & Sexton, 1986). However, cholesterogenesis has been largely overlooked as a potential factor in CVD development. The few studies focusing on cholesterol metabolism in obesity have consistently observed cholesterol synthesis rates in obese subjects to be greater than those in non-obese subjects (Miettinen, 1971; Nestel *et al*, 1973, Ståhlberg *et al*, 1997). Furthermore, others have observed that energy restriction of varying degrees is capable of decreasing human endogenous cholesterol synthesis (Bennion & Grundy, 1975; Jones *et al*, 1988; Kudchodkar *et al*, 1977). Kudchodkar *et al*. (1977) credited the decrease in cholesterol synthesis observed in their subjects to the lower metabolic state associated with energy restriction rather than to body weight reduction because the changes observed in synthesis occurred prior to any significant shift in body

weight. In fact, none of these studies were of sufficient length to induce weight loss in their subjects. To date, no study has specifically monitored intraindividually the effects of long term weight loss on whole-body endogenous cholesterol synthesis in humans.

Therefore, we conducted a prospective controlled study evaluating the effects of weight loss, as achieved through energy restriction, upon *in vivo* circulating cholesterol concentrations and synthesis in a cohort of middle-aged males exhibiting mildly elevated serum cholesterol concentrations. We hypothesized that circulating lipoprotein cholesterol concentrations and synthesis rates would decrease following 6 mo of mild weight loss.

3.3 METHODS

Study Population and Protocol

Seven moderately obese men (76.4 kg to 104.8 kg) aged 64 to 77 years were recruited from the Baltimore, MD area to participate in the study. All subjects were screened for mildly elevated LDL-cholesterol (LDL-C) concentrations (3.36 - 4.91 $mmol \cdot L^{-1}$). Subjects were asked to complete a general health questionnaire prior to the start of the study, and were excluded from participation if they were smokers, exercising more than three times per week, consuming more than 3 alcoholic drinks per day or had undergone any oral hypolipidemic therapy within the 3 mo prior to the start of the study. None of the

subjects reported a history of CVD. The study was approved by the Institutional Review Board of the University of Maryland at Baltimore. All subjects gave written informed consent prior to the start of the study, and all procedures conducted were in accordance with research guidelines of the Institutional Review Board of the University of Maryland at Baltimore.

Subjects were enrolled in a 9 mo, 2-phase sequential study evaluating the effects of weight reduction on CVD risk factors. In phase 1, which lasted 3 mo, subjects were placed on an American Heart Association (AHA) step1 diet under the guidance and supervision of a registered dietitian with no change to their regular energy intake which was assessed using 3-day food records. During this period, in which subjects maintained energy balance, subjects were asked not to exercise more than three times per week and for no more than 30 minutes per occasion. Assignments such as activity logs and 3-day food records were collected on a weekly basis to monitor compliance to the protocol. Weights were measured on a weekly basis in order to ensure that subjects remained weight stable throughout the first phase.

Immediately following phase 1, subjects embarked on a 6 mo energy-restricted AHA Step 1 diet (phase 2). Under the supervision of a registered dietitian, subjects were instructed to reduce their energy intake by approximately 1000 $kJ \cdot day^{-1}$ in order to achieve a total weight loss goal of 8 kg over the 6-month

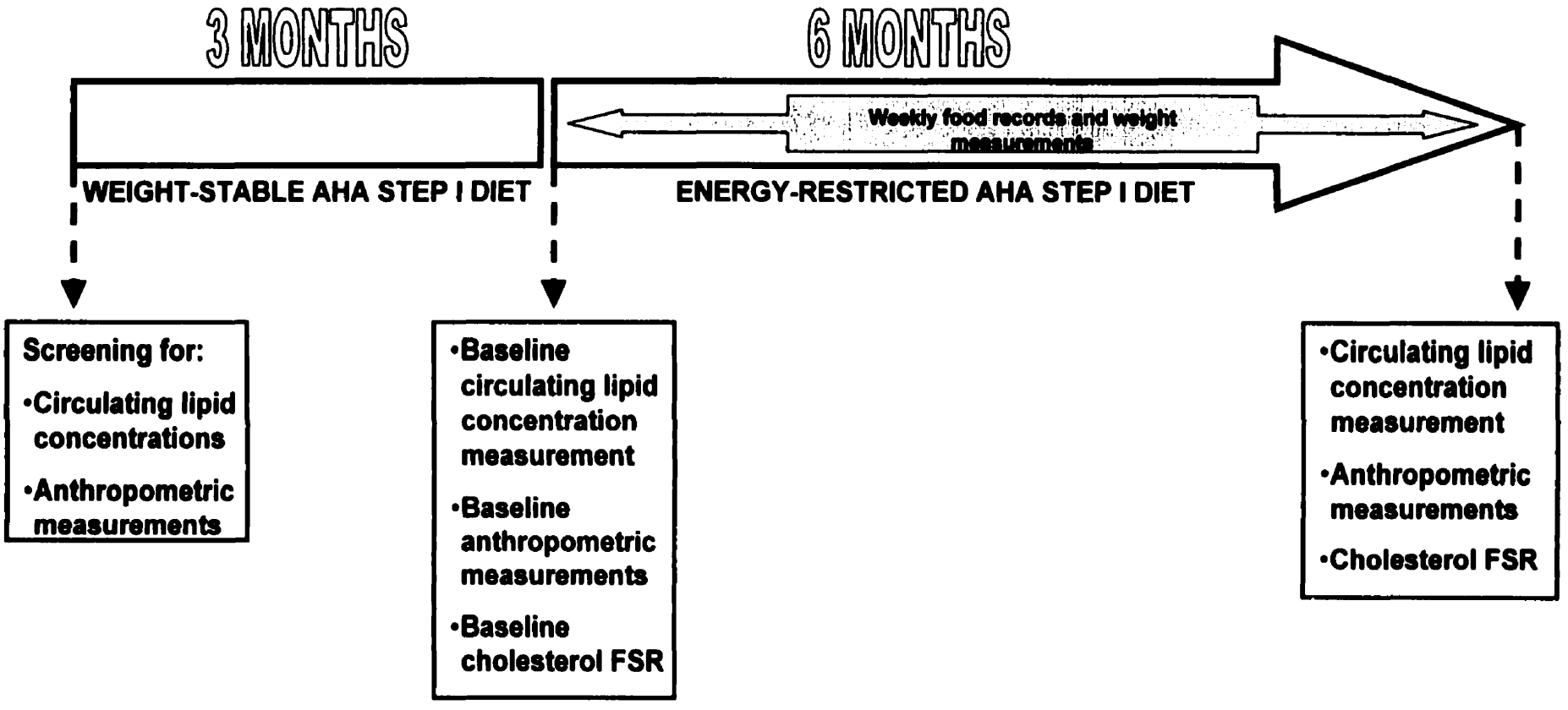


Figure 3.1. Experimental protocol

period. Subjects were instructed on how to make simple food substitutions or alterations to food preparation that would result in a 1000 $kJ \cdot day^{-1}$ reduction in energy intake without selectively reducing intake of a specific macronutrient. Compliance was monitored weekly using 3-day food records and weight loss progression.

Plasma Lipid Analysis

Serum lipid concentrations were determined at the start (preWL) and end (postWL) of phase 2 at the University of Maryland in Baltimore, MD with enzymatic techniques validated by the National Institutes of Health Lipid Research Clinics. Subjects were placed on a euenergetic diet during phase 1; therefore, they were weight stable at the end of the first phase. HDL cholesterol (HDL-C) level was determined after treatment of plasma with dextran sulphate and Mg^{++} using a Hitachi 717 autoanalyzer. Serum LDL cholesterol concentrations were calculated from serum total and HDL cholesterol concentrations using the equation formulated by Friedewald *et al.* (1972).

Cholesterol Biosynthesis Measurement

Cholesterol synthesis rates were determined at the start (preWL) and end (postWL) of phase 2. Subjects were weight stable at the time baseline measurements were made. Measurements were performed over 24 hours. On day 1, an 8mL fasting blood sample was taken from each subject in the morning in

order to determine baseline body water and cholesterol deuterium enrichment. Subjects then received a bolus oral dose of 1.2 $g \cdot kg^{-1}$ body water of deuterium oxide (D_2O, 99.96% deuterium; Isotech Inc., Miamisburg, OH). On day 2, a second fasting blood sample was obtained for measurement of body water and cholesterol deuterium enrichment.

Cholesterol FSRs were determined as the rate of incorporation of deuterium into erythrocyte membrane free cholesterol (Jones *et al.* 1993b). Erythrocyte lipids were extracted using a modified Folch extraction procedure (Folch *et al.* 1957) and dried under nitrogen. Free cholesterol was isolated by thin-layer-chromatography (TLC) on silica gel against a free cholesterol standard. Cholesterol bands were scraped from the TLC plates and the cholesterol was eluted from silica with hexane:chloroform:diethyl ether solution (5:2:1 vol:vol:vol). The cholesterol was transferred to Pyrex™ combustion tubes containing CuO and silver wire. Tubes were subsequently flame-sealed under vacuum and cholesterol was combusted to CO_2 and H_2O at 520°C for 4 hours. Water resulting from the combustion was cryogenically separated from CO_2 by distillation into Pyrex™ tubes containing 50 mg zinc under vacuum. Tubes were flame-sealed under vacuum and the water was reduced at 520°C for 30 minutes in order to obtain H_2/D_2 gas. Deuterium enrichment of the resultant gas was measured on a dual inlet isotope ratio mass spectrometer (VG Isogas 903D, Cheshire, England). Plasma-water enrichment was measured after dilution of 0

and 24 hour plasma samples with water of known isotopic abundance to bring the enrichment into the working range of the International Atomic Energy Agency mass spectrometer calibration standards.

Erythrocyte membrane free cholesterol deuterium enrichment values at 0 and 24 hours were expressed relative to the corresponding mean plasma water sample enrichment after correcting for the deuterium-protium ratio in cholesterol to yield FSRs (in %/day) for the free-cholesterol pool. The FSR index represents that fraction of the free portion of the rapidly turning over central cholesterol pool that is synthesized in 24 hours as per the formula (Jones *et al,* 1993a):

$$FSR(\%/day)=(\delta_{cholesterol}/\delta_{plasma}) \times 0.478 \times 100 \qquad \text{Eqn (1)}$$

where δ refers to deuterium enrichment above baseline over 24 hours. The factor 0.478 represents D_{max}, the maximum number of deuterium atoms incorporated per molecule of cholesterol over periods up to 48 h (Jones *et al,* 1993a).

Absolute synthesis rate (ASR) was calculated as per the formula (Goodman *et al,* 1980):

$$ASR\ (g/day)=FSR \times (M_1/2) \qquad \text{Eqn (2)}$$

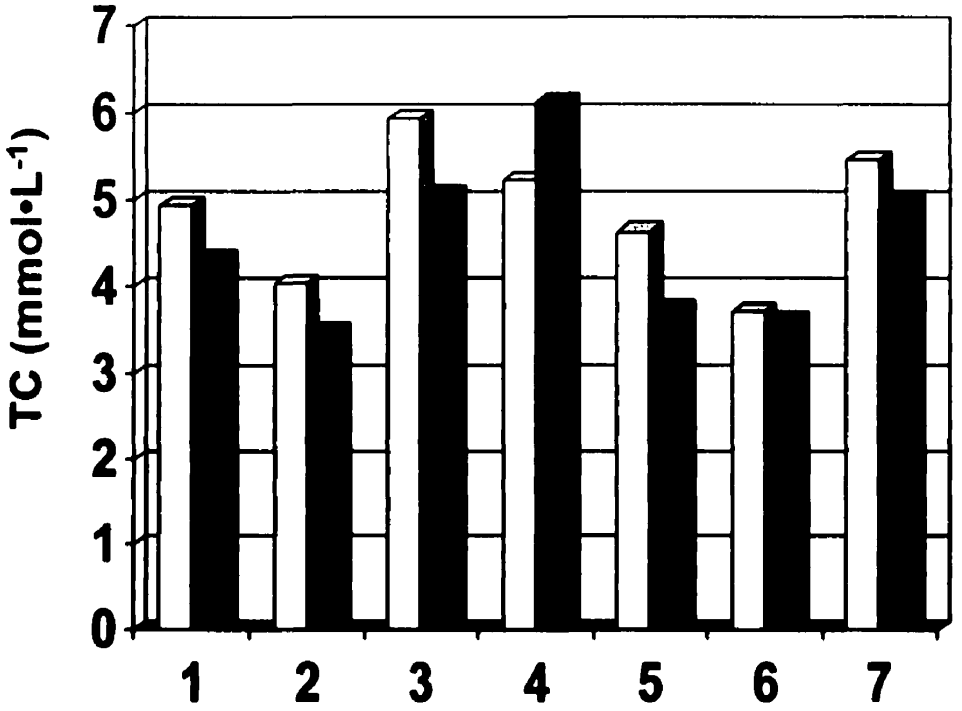
M_1 = 0.287 x body weight (kg) + 0.0358 x total cholesterol concentration (mg/dL) - 2.40 x TGGP. Where M_1 represents the size of the M_1 pool, and TGGP is a variable equal to 1, 2, or 3 depending on serum triglyceride concentration (<2.72, 2.72-3.41, or >3.41 mmol/L) .

Statistical Analysis

Paired t-tests were used to compare FSRs and plasma lipoprotein cholesterol concentrations measured at the start and end of phase 2 using SAS statistical software (SAS Institute Inc., Cary, NC). We tested for correlations between the change in FSR from preWL to postWL (ΔFSR), change in weight from preWL to postWL (ΔWL) and change in total cholesterol (TC) from preWL to postWL (ΔTC). Results were considered significant at a level of $p<0.05$.

3.4 RESULTS

Demographic data are summarized in Table 3.1. A difference was observed in mean weight following weight loss (postWL) as compared to preWL (85.6±12.3 kg vs. 91.3±12.5 kg). Individual weight loss ranged from 3 to 8 kg. BMI dropped from 30.5±3.4 $kg \cdot m^{-2}$ to 28.7±3.3 $kg \cdot m^{-2}$. Percent body fat as measured by dual emission X-ray absorptiometry (DEXA) was also reduced ($p<0.05$) from 33.0±3.6 % to 29.1±6.1 % from the preWL to the postWL period.



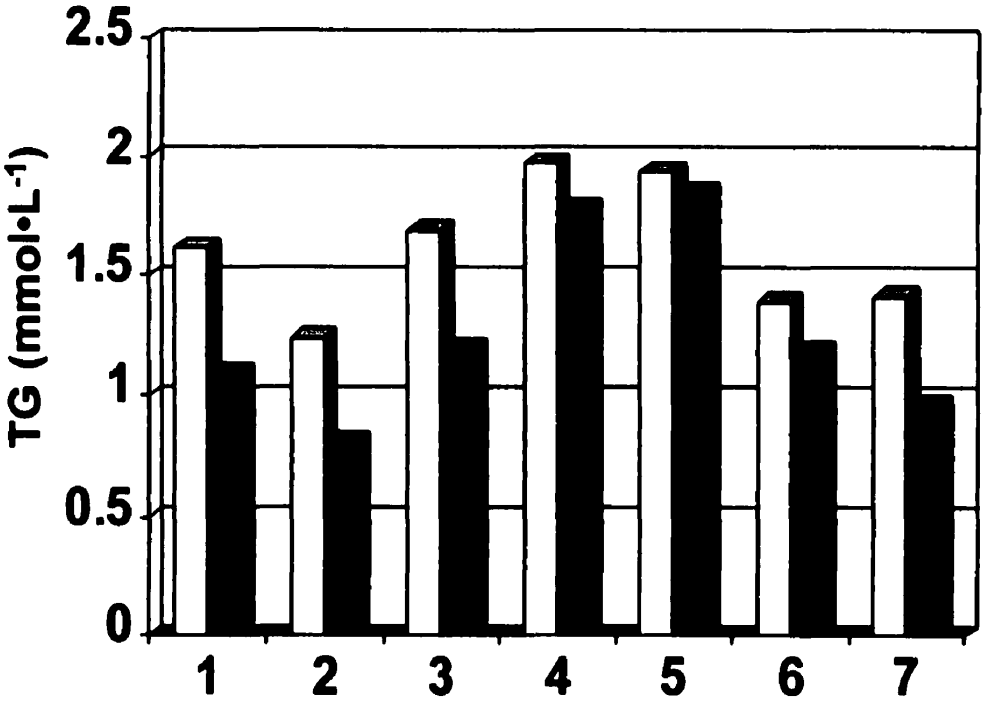
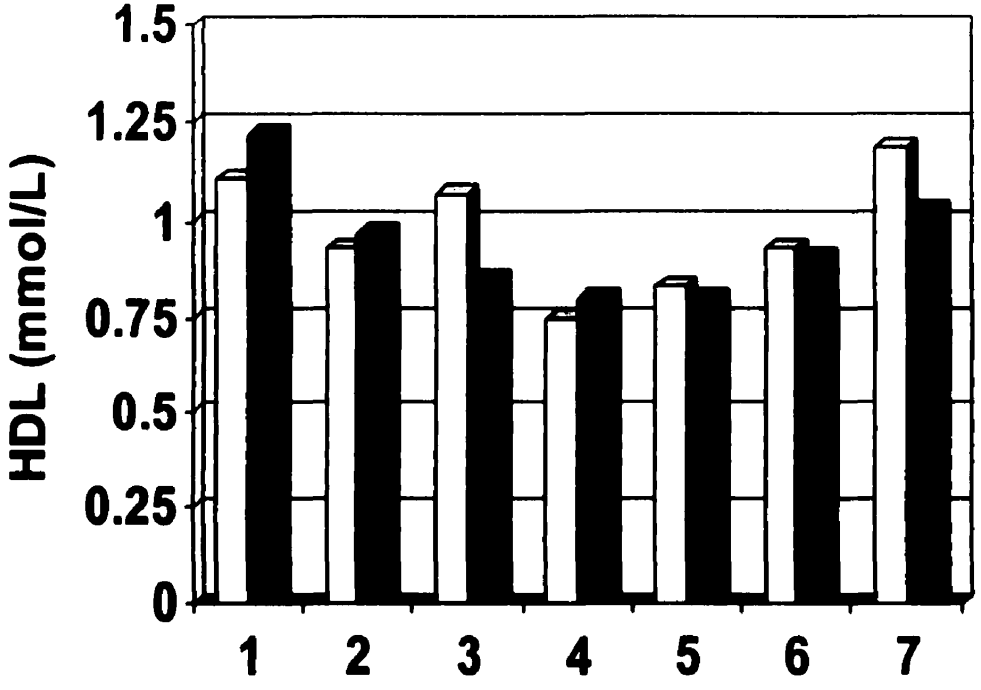


Figure 3.2. Total cholesterol (TC) and triglyceride (TG) concentrations for individual subjects preWL (□) and postWL (■). Mean TC concentrations did not change from the preWL (4.47±0.96 mmol/L) to the postWL (2.94±0.93 mmol/L) period. Mean TG concentrations decreased significantly ($p<0.05$) from the preWL (1.61±0.28 mmol/L) to the postWL (1.27±0.39 mmol/L) period.



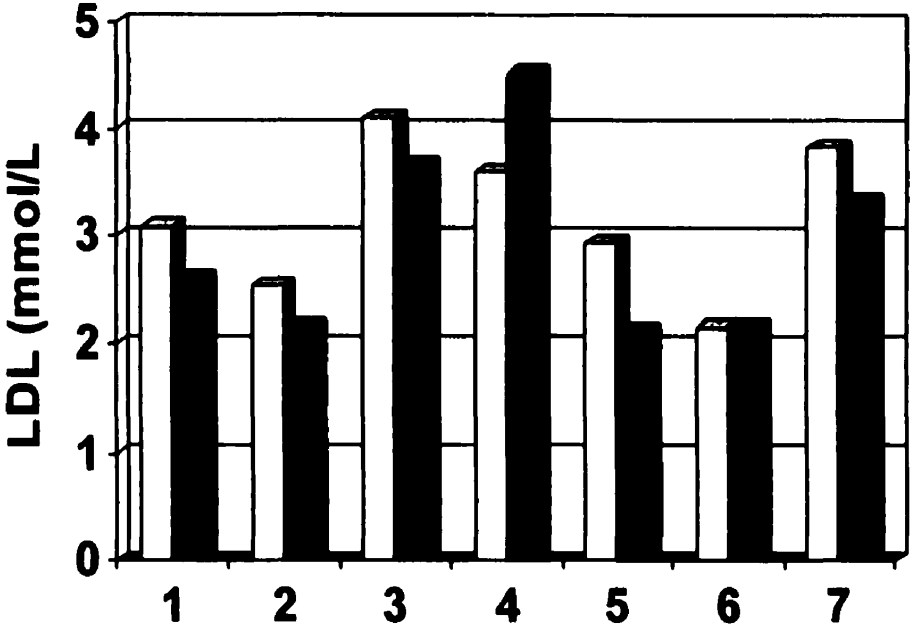


Figure 3.3. HDL and LDL-Cholesterol concentrations for individual subjects preWL (□) and postWL (■). Mean HDL concentrations did not change from the preWL (0.97±0.16 mmol/L) to the postWL (0.94±0.15 mmol/L) period. Mean LDL concentrations did not change from the postAHA (3.17±0.71 mmol/L) to the postWL (2.94±0.93 mmol/L) period.

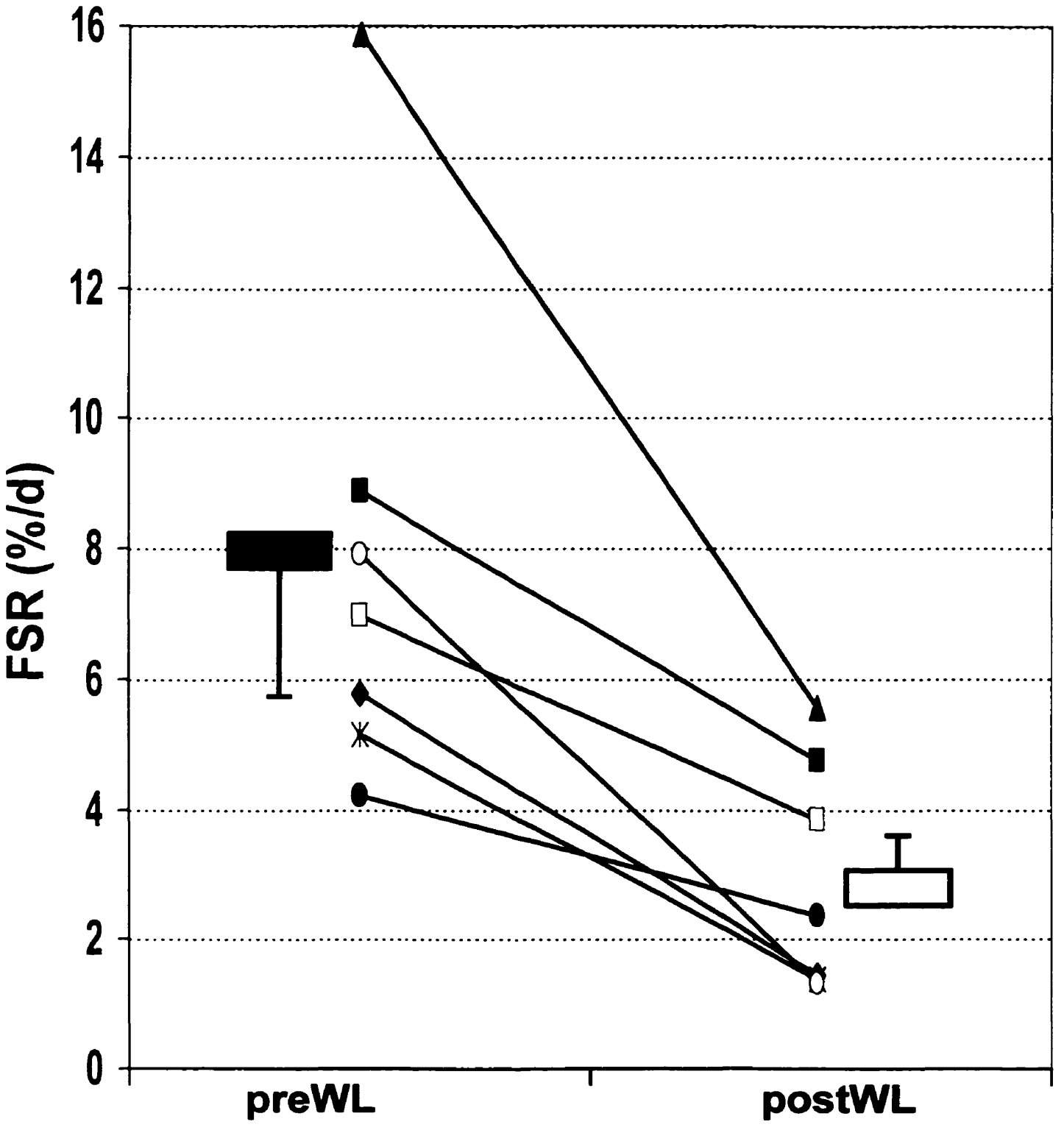


Figure 3.4. Fractional synthesis rate (FSR) of individual subjects before (preWL) and after (postWL) 6 mo on an energy restricted AHA Step I diet leading to weight loss. x±SD. FSR (%/d) for subjects was 7.80±3.61 preWL and 2.95±1.63 postWL (significantly different at $p<0.05$)

Compared to preWL values (1.61±0.28 mmol·L^{-1}) , subjects showed a decrease ($p<0.05$) in mean triglyceride (TG) concentrations postWL (1.27±0.39 mmol·L^{-1}). TC decreased (ns) to 4.47±0.96 mmol·L^{-1}, and LDL-C decreased (ns) 2.94±0.93 mmol·L^{-1} (Figure 3.2, 3.3). HDL-C on the other hand did not change from preWL (0.97±0.16 mmol·L^{-1}) to postWL (0.94±0.15 mmol·L^{-1}). Neither TC:HDL (5.06±1.03 to 4.88±1.46), nor LDL:HDL (3.31±0.83 to 3.22±1.31) changed between preWL and postWL. In all subjects cholesterol FSR values after weight loss (2.95±1.63 %·d^{-1}) were reduced ($p<0.05$) compared to those pre-weight loss (7.80±3.61 %·d^{-1}) (Figure 3.4). We also observed a decline ($p<0.05$) in ASR between preWL (1.17±0.58 g·d^{-1}) and postWL (0.40±0.22 g·d^{-1}). A non-significant correlation ($r=0.62$, $p=0.07$) was observed between change in cholesterol FSR and change in circulating TC concentrations from the preWL to postWL phase. No such correlation was found between change in FSR and change in weight from the preWL to postWL phase ($r=0.31$, $p=0.3$).

3.5 DISCUSSION

According to current Health Canada statistics, up to one half of Canadians aged 18 to 74 are overweight (Petrasovits, 1997). Obesity is characterized by several metabolic and hormonal changes including an elevated production of cholesterol, atherogenic dyslipidemia, hypertension, insulin resistance and glucose intolerance, and abnormalities in the coagulation system (McGarry, 1998). Sterol balance methods have been used to show that obese individuals

synthesize almost twice as much cholesterol per kilogram body weight as do non-obese individuals (Miettinen, 1971; Nestel *et al,* 1973). More recently, Ståhlberg *et al.* (1997) demonstrated marked elevations in both activity and mRNA expression of 3-hydroxy-3-methyl glutaryl Coenzyme A (HMG CoA) reductase in operative liver biopsies of obese versus non-obese patients. However, these studies failed to show whether weight loss could revert cholesterol synthesis levels seen in obese individuals to levels seen in normal individuals.

Our findings show for the first time that weight loss achieved through diet alone results in a substantial decrease in the fractional and absolute synthesis of cholesterol in men. The findings are consistent with observations made by Bennion and Grundy in 1975 who determined that obese subjects maintained on very-low-calorie-diets (~1000 Kcal·day^{-1}) while hospitalized for a period of 3-5 months secreted more cholesterol in bile following weight loss; however, the results obtained were not significant (Bennion & Grundy, 1975). Other studies have shown that energy restriction which does not result in weight loss is also capable of decreasing cholesterol synthesis in humans (Jones *et al,* 1988; Kudchodkar *et al,* 1977; Jones *et al,* 1993a; Jones *et al,* 1993b). The extent of weight loss we observed is similar to that seen in other trials evaluating the effects of weight loss through diet alone on various outcomes (Thompson *et al,* 1979; Wood *et al,* 1988; Dengel *et al,* 1995). Our data suggest that moderate energy

restriction is one of the most potent dietary inhibitors of cholesterol biosynthesis in humans (Figure 3.5).

Circulating cholesterol concentrations are determined to a large extent by the size of the body pool of cholesterol, which in turn is determined by endogenous cholesterol synthesis rates (Rudney & Sexton, 1986; Dietschy, 1984). Although we observed a significant decrease in circulating triglyceride (TG) concentrations between the preWL and postWL phases, there were no significant changes in either TC, LDL-C or HDL-C concentrations. Nonetheless, the changes observed in TC and LDL-C were very similar to those obtained in many other studies examining the influence of weight loss alone on circulating lipid concentrations in older subjects (Dattilo & Kris-Etherton, 1992). No changes were observed in TC:HDL and LDL:HDL either. This discrepancy may be corrected with a larger sample size; yet, Wing *et al.* (1987) also observed significant changes only in TG concentrations following 2.4 to 6.8 kg weight loss over a 1 year period. A recent study by Nicklas *et al.* showed that weight loss has no effect on TC and LDL-C concentrations in postmenopausal women who are first placed on a euenergetic AHA Step I diet; however, they concluded that the combined diet modification and weight loss led to substantial improvements in the lipid profiles of their subjects. There is evidence, nonetheless, that the suppression of cholesterogenesis leads to a reduction in circulating TC concentrations as demonstrated by the strong correlation between change in FSR and change in TC

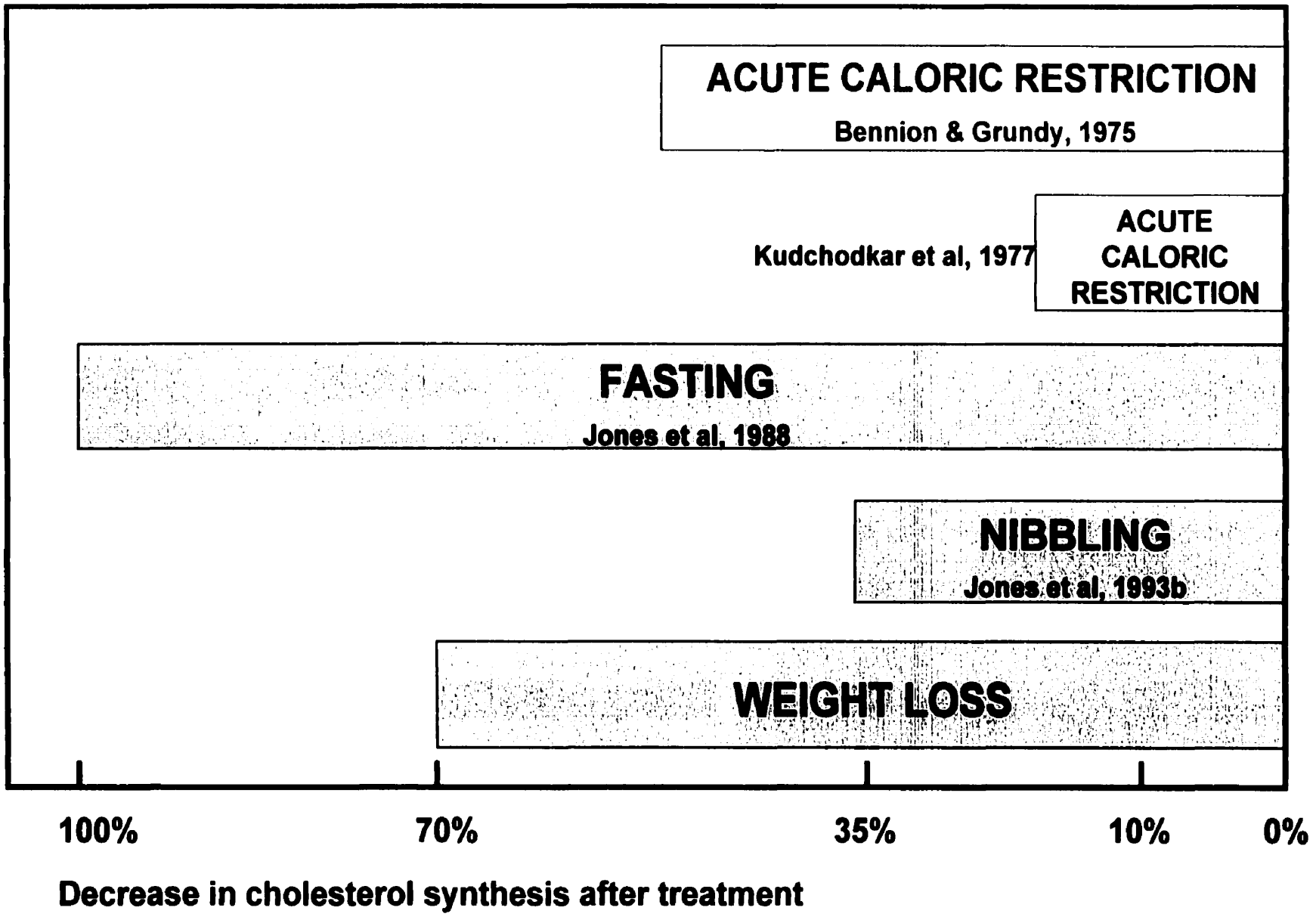


Figure 3.5. Effects of various forms of food restriction on human endogenous cholesterol biosynthesis as measured by sterol balance (white box) or deuterium uptake (grey box). Mild energy restriction leading to weight loss of only 6.27±2.61% initial body weight results in a greater than 70% reduction of cholesterol biosynthesis. Only complete fasting results in a greater reduction.

from the preWL to postWL phase. Nevertheless, decreasing cholesterol biosynthesis alone still has a beneficial effect in the prevention of CVD. Several studies have shown that isoprenoid metabolites formed during cholesterol biosynthesis play a role in the development of atherosclerosis (Corsini *et al.* 1996; Hughes, 1996). Furthermore, when the synthesis of these metabolites is arrested by administration of HMG CoA reductase inhibitors, smooth muscle cell migration and proliferation, which have been implicated in atherogenesis, is decreased. Suppression of the HMG CoA reductase enzyme through weight loss most likely has a similar effect.

A closer look at individual data reveals that one subject of the seven had higher TC and LDL-C concentrations following weight loss; all other subjects showed decreases in plasma lipids. If data for this subject are excluded, then TC and LDL-C both change significantly from the preWL (TC = 4.78±0.84 $mmol \cdot L^{-1}$, LDL-C= 3.08±0.75 $mmol \cdot L^{-1}$) to the postWL period (TC= 4.20±0.69 $mmol \cdot L^{-1}$, LDL= 2.67±0.67 $mmol \cdot L^{-1}$). The increase in this individual is not entirely abnormal. Vaswani (1983) showed that the response of serum cholesterol in subjects placed on a 12-week weight loss diet was biphasic, initially dropping but returning to pre-study concentrations by the end of the weight loss period. Exactly why this individual demonstrated higher serum cholesterol concentrations at the end of the study remains unclear but may be related to this biphasic "rebound" as described by Vaswani (1986).

As for HDL-C, Wing *et al.* (1987) have shown that changes in weight of 5% induced by diet alone significantly reduce TG concentrations but do not increase HDL-C. Similar results have been obtained more recently by Wood *et al.* (1991). Some subjects actually had lower HDL-C concentrations following weight loss than prior to weight loss. Thompson *et al.* (1979) have also seen this occur in women undergoing weight loss through caloric restriction and conclude that it is a physiologic effect of negative energy balance.

Our findings add strength to the reported position that weight loss of 5-15% initial body weight is beneficial for the reduction of cardiovascular risk factors (Van Gaal *et al,* 1997). We have shown that a moderate weight loss of approximately 8 kg over a 6-month period induced by mild energy restriction results in dramatic reductions in cholesterol fractional and absolute synthetic rates. These results confirm that moderate weight loss through energy restriction decreases the risk of CVD development in overweight older men through a decrease in weight and a reduction in cholesterol biosynthesis.

Acknowledgments

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Table 3.1. Individual and mean subject characteristics before (preWL) and after (postWL) a 6-month energy restricted weight loss diet.

Subject	Age (years)	Weight (kg)		BMI ($Kg \cdot m^{-2}$)		Body Fat (%)		VO_{2max} ($mL \cdot Kg^{-1} \cdot min^{-1}$)	
		preWL	postWL	preWL	postWL	preWL	postWL	preWL	postWL
1	77	79.8	72.2	27.6	25.0	28.8	21.5	22.2	23.9
2	64	87.2	79.2	28.6	26.8	31.7	21.7	25.5	28.2
3	77	83.0	80.0	27.8	26.8	30.6	28.3	21.1	20.9
4	70	103.0	100.0	34.5	33.4	34.8	35.6	21.8	21.6
5	73	104.6	97.4	34.8	32.4	37.2	32.3	22.0	23.2
6	67	76.4	72.8	27.4	26.1	30.2	28.0	24.9	26.7
7	67	104.8	97.4	32.5	30.2	37.7	36.4	23.4	26.4
Mean	71	91.3	85.6*	30.5	28.7*	33.0	29.1*	22.9	24.4
(±SD)	(±5)	(±12.5)	(±12.3)	(±3.4)	(±3.3)	(±3.56)	(±6.06)	(±1.7)	(±2.8)

*Significantly different. $p<0.05$.

GENERAL CONCLUSION

Despite the emergence of stable isotope techniques for the measurement of in vivo cholesterol biosynthesis in humans, their systematic evaluation against one another has not been previously conducted. We compared cholesterol synthesis measured by deuterium uptake and MIDA under temporal conditions typical of both techniques and determined that synthesis rates measured by deuterium incorporation did not differ from those measured by MIDA in adult humans. These findings show that deuterium enrichment of erythrocyte free cholesterol provides a reasonable estimate of central pool synthesis rate in adult humans, and that measurements are most accurate if taken over 24 h.

Although both deuterium incorporation and MIDA have been extensively used to measure cholesterol biosynthesis, the two methods had not been directly compared under temporal conditions typical of both techniques until now. Furthermore, MIDA, unlike deuterium incorporation, has not been validated against any of the widely accepted techniques of cholesterol synthesis measurement such as sterol balance or mevalonic acid excretion.

The results obtained in manuscript 1 add strength to those found in manuscript 2, mainly that weight loss through energy restriction suppresses *de novo* cholesterogenesis in overweight men. Evidence found in the literature suggest that weight loss resulting from energy restriction similar to that employed

by our group in manuscript 2 leads to significant reductions in circulating lipid concentrations. Our sample size of seven older men was too small to identify significant changes in some circulating lipid parameters; yet we were able to identify a drastic reduction in cholesterol biosynthesis. Although consistent with data currently found in the literature, this is the first known scientific evidence demonstrating the effects of weight loss upon cholesterol biosynthesis in humans. Without the use of deuterium incorporation as a reliable technique by which to accurately measure cholesterogenesis in free-living humans, these results would have been much more difficult to obtain.

The results outlined in manuscript 2 not only demonstrate that weight loss is an effective regulator of cholesterol synthesis *in vivo*, but add strength to the reported position of weight loss being beneficial in the prevention of cardiovascular disease. The recent evidence highlighting a cardio-protective role of HMG CoA reductase inhibitors independent of their serum lipid lowering effect is applicable to weight loss by means of energy restriction. Decreased synthesis by down-regulation of HMG CoA reductase leads to a decrease in isoprenoid metabolites known to play a role in the development of atherosclerotic plaques. Hence we conclude that weight loss through energy restriction suppresses cholesterol biosynthesis and decreases the risk of cardiovascular disease in older, overweight men.

In summary, we have shown that deuterium incorporation is the most practical stable isotope methodology currently available to study *in vivo* cholesterol biosynthesis in humans, and we subsequently used this technique to demonstrate that synthesis is sensitive to weight loss as achieved through energy restriction, thereby offering protection against cardiovascular disease in overweight, older men.

BIBLIOGRAPHY

Abate N, Garg A, Peshock RM, Stray-Gundersen J, Grundy SM. Relationship of generalized and regional adiposity to insulin sensitivity in men. *J Clin Invest.* 1995; 96:88-98

Andersen JM, Dietschy JM. Regulation of sterol synthesis in 16 tissues of rat. I. Effect of diurnal light cycling, fasting, stress, manipulation of enterohepatic circulation and action of chylomicrons and triton. *J Biol Chem.* 1979; 252:3646-3651

Andersen JM, Turley SD, Dietschy JM. Low and high density lipoproteins and chylomicrons as regulators of rate of cholesterol synthesis in rat liver in vivo. *Proc Natl Acad Sci USA.* 1979; 76:165-169

Ashley FW, Kannel WB. Relation of weight change to changes in atherogenic traits: the Framingham study. *J Chron Dis.* 1974; 27:103-114

Balasubramanian KA, Nalini S, Manohar M. Nonesterfified fatty acids and lipid peroxidation. *Mol Cell Biochem.* 1992; 111:131-135

Bandsma RH, Stellaard F, Vonk RJ, Nagel GT, Neese RA, Hellerstein MK, Kuipers F. Contribution of newly synthesized cholesterol to rat plasma and bile determined by mass isotopomer distribution analysis: bile-salt flux promotes secretion of newly synthesized cholesterol into bile. *Biochem J.* 1998; 329: 699-703

Bennion LJ, Grundy SM. Effects of obesity and caloric intake on biliary lipid metabolism in man. *J Clin Invest.* 1975; 56:996-1011

Bjorkhem I, Lutjohann D, Breuer O, Sakinis A, Wennmalm A. Importance of a novel oxidative mechanism for elimination of brain cholesterol. Turnover of cholesterol and 24(S)-hydroxysterol in rat brain as measured by 18O2 techniques in vivo and in vitro. *J Biol Chem.* 1997; 272: 30178-30184

Bland AE, Altman DG. Statistical methods for assessing agreement between two measures of clinical measurement. *Lancet.* 1986; I: 307-310

Bloch K. Sterol structure and membrane function. *Crit Rev Biochem.* 1983; 14:47-92

Bosello O, Armellini F, Zamboni M, Fitchet M. The benefits of modest weight loss in type II diabetes. *Int J Obes.* 1997; 21: S10-S13

Cella, L.K., Van Cauter, E., Schoeller, D.A. Effect of meal timing on diurnal rhythm of human cholesterol synthesis. *Am J Physiol.* 1995; 269: E878-E883

Connor WE, Witiak DT, Stone DB, Armstrong MO. Cholesterol balance and fecal neutral steroid and bile acid excretion in normal men fed dietary fats of different fatty acid composition. *J Clin Invest.* 1969; 48:1363-1375

Corsini A, Bernini F, Quarato P, Donetti E, Bellosta S, Fumagalli R, Paoletti R, Soma VM. Non-lipid-related effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Cardiology.* 1996; 87: 458-468

Cuchel M, Scahaefer EJ, Millar JS, Jones PJ, Dolnikowski GG, Vergani C, Lichtenstein AH. Lovastatin decreases de nov cholesterol synthesis and LDL Apo-B 100 production rates in combined-hyperlipidemic males. *Arterioscler Thromb Vasc Biol* 1997; 17: 1910-1917

D'Eramo-Melkus G. Obesity and weight loss. *Lippincott's Primary Care Practice.* 1997; 1:243-251

Dattilo AM, Kris-Etherton PM. Effects of weight reduction on blood lipids: a meta-analysis. *Am J Clin Nutr.* 1992; 56:320-328

DeFronzo RA. The triumvirate: b-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes.* 1988; 37:667-687

Dengel JL, Katzel LI, Goldberg AP. Effect of an AHA diet, with or without weight loss, on lipids in obese middle aged and older men. *Am J Clin Nutr.* 1995; 62:715-721

Dietschy JM. Regulation of cholesterol metabolism in man and other species. *Klin Wochenschr.* 1984; 62: 338-345

Dietschy JM, McGarry J D. Limitations of acetate as a substrate for measuring cholesterol synthesis in liver. *J Biol Chem.* 1974; 249:52-58

Dietschy JM. Regulation of cholesterol metabolism in man and other species. *Klin Wochenschr.* 1984; 62:338-345

Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res.* 1993; 34(10):1637-1659

Diraison F, Pachiaudi C, Beylot M. In vivo determination of plasma cholesterol and fatty acid synthesis with deuterated water: Determination of the average number of deuterium atoms incorporated. *Metabolism.* 1996; 45: 817-821

Eckel RH. Obesity and heart disease. A statement for healthcare professionals from the Nutrition Committee, American Heart Association. *Circulation.* 1997; 96:3248-3250

Empen K, Lange K, Stange EF, Scheibner J. Newly synthesized cholesterol in human bile and plasma: Quantitation by mass isotopomer distribution analysis. *Am J Physiol.* 1997; 272: G367-G373

Faix D, Neese R, Kletke C, Wolden S, Cesar D, Coutlangus M, Shackleton CH, Hellerstein MK. Quantification of menstrual and diurnal periodicities in rates of cholesterol and fat synthesis in humans. J Lipid Res. 1993; 34: 2063-2075

Feingold K, Grunfeld C. Tumor necrosis factor-alpha stimulated hepatic lipogenesis in the rat in vivo. *J Clin Invest.* 1987; 80: 184-190

Folch J, Lees M, Sloane SGH. A simple method for the extraction and purification of total lipids from animal tissues. J Biol Chem. 1957; 226: 497-509

Friedewald WT, Levy RI, Frederickson DS. Estimation of the concentration of low density lipoprotein without the use of preparative ultracentrifuge. *Clin Chem.* 1972; 18: 499-502

Garrison RJ, Castelli WP. Weight and thirty-year mortality of men in the Framingham study. *Ann Intern Med.* 1985; 103: 1006-1009

Goodman DS, Smith FR, Seplowitz AH, Ramakrishnan R, Dell RB. Prediction of the parameters of whole body cholesterol metabolism in humans. *J Lipid Res.* 1980; 21: 699-713

Gotto AM, La Rosa JC, Hunninghake D, Grundy SM, Wilson P, Clarkson B, Hay JW. Goodman DS. The cholesterol facts. *Circulation.* 1990; 81: 1721-1733

Green KL, Cameron R, Polivy J, Cooper K, Liu L, Leiter L, Heatherton T. Weight dissatisfaction and weight loss attempts among Canadian adults. *Can Med Assoc J.* 1997; 157: S17-S25

Grundy SM, Ahrens EH. Measurement of cholesterol turnover, synthesis, and absorption in man carried out by isotope kinetic and sterol balance methods. *J Lipid Res.* 1969; 10:91-107

Grundy SM, Mok HY, Zech L, Steinberg D, Berman M. Transport of very low density lipoprotein triglycerides in varying degrees of obesity and hypertriglyceridemia. *J Clin Invest.* 1979; 63: 1274-1283

Grundy SM. Multifactorial causation of obesity: implications for prevention. *Am J Clin Nutr.* 1998; 67(suppl):S563-S572

Hellerstein MK, Neese RA. Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am J Physiol.* 1992; 263: E988-E1001

Hellerstein MK. Methods for measurement of fatty acid and cholesterol metabolism. *Current Opinion in Lipidology.* 1995; 6: 172-181

Howard G, O'Leary DH, Zaccaro D, Haffner S, Rewers M, Hamman R, Selby JV, Saad MF, Savage P, Bergman R. Insulin sensitivity and atherosclerosis. *Circulation.* 1996; 93:1809-1817

Hughes AD. The role of isoprenoids in vascular smooth muscle: Potential benefits of statins unrelated to cholesterol lowering. *Journal of Human Hypertension.* 1996; 10: 387-390

Jeske DJ, Dietschy JM. Regulation of rates of cholesterol synthesis *in vivo* in the liver and carcass of the rat measured using [^{3}H] water. *J Lipid Res.* 1980; 21:364-376

Jones PJH, Scanu AM, Schoeller DA. Plasma cholesterol synthesis using deuterated water in humans: effects of short term food restriction. *J Lab Clin Med.* 1988; 111:627-633

Jones PJH. Use of deuterated water for measurement of short-term cholesterol synthesis in humans. *Can J Physiol Pharm.* 1990; 88:955-959

Jones PJ, Pappu AS, Illingworth DR, Leitch CA. Correspondance between plasma mevalonic acid levels and deuterium uptake in measuring human cholesterol synthesis. *Eur J Clin Nutr.* 1992; 22: 609-613

Jones PJH, Leitch CA, Pederson RA. Meal-frequency effects on plasma hormone concentartions and cholesterol synthesis in humans. *Am J Clin Nutr.* 1993; 57: 868-874

Jones PJH, Leitch CA, Li Z-C. Human cholesterol synthesis measurement using deuterated water: Theoretical and practical considerations. *Arterioscler Thromb.* 1993; 13: 247-253

Jones PJH. Regulation of cholesterol synthesis by diet in humans. *Am J Clin Nutr.* 1997; 66: 438-446

Jones PJH, Ausman LM, Croll DH, Feng JY, Schaefer EA, Lichtenstein AH. Validation of deuterium incorporation against sterol balance for measurement of human cholesterol biosynthesis. *J Lipid Res.* 1998; 39: 1111-1117

Kelleher JK, Kharroubi AT, Aldaghlas TA, Shambat IB, Kennedy KA, Holleran AL, Masterson TM. Isotopomer spectral analysis of cholesterol synthesis: Applications in human hepatoma cells. Am. J. Physiol. 1994; E384-E395

Kraemer H, Berkowitz R I, Hammer L D. Methodoligal difficulties in studies of obesity, I: measurement issues. *Ann Behav Med.* 1990; 12:112-118

Kudchodkar RJ, Sodhi HS, Mason DT, Borhani NO. Effects of acute caloric restriction on cholesterol metabolism in man. *Am J Clin Nutr.* 1977; 30:1135-1146

Landau BR, Fernandez CA, Previs SF, Ekberg K, Chandramouli V, Wahren M, Kalhan SC, Brunengraber H. A limitation in the use of mass isotopomer distributions to measure gluconeogenesis in fasting humans. Am. J. Phsiol. 1995; E18-E26

Lee W.N., Bassilian S, Ajie H.O., Schoeller D.A., Edmund J., Bergner E.A., Byerley L.O. In vivo measurement of fatty acids and cholesterol synthesis using D2O and mass isotopomer analysis. *Am J Physiol.* 1994; 266: E699-E708

Leenen R, Van der Kooy K, Meyboom S, Seidell JC, Deurenberg P, Westrate JA. Relative effects of weight loss and dietary modification on serum lipid levels in the dietary treatment of obesity. *J Lipid Res.* 1993; 34:2183-2191

London I.M., Schwarz H. Erythrocyte metabolism. The metabolic behavior of the cholesterol of human erythrocytes. *J Clin Invest.* 1953; 32: 1248-1252

McGarry JD. Glucose-fatty acid interactions in health and disease. *Am J Clin Nutr.* 1998; 67(suppl):S500-S504

Mansen JE, Willett WC, Stampfer MJ, Colditz GA, Hunter PJ, Harkinsen SE, Hennekens CH, Speizer FE. Body weight and mortality among women. *N Engl J Med.* 1995; 333: 677-685

Mazier MJ, Jones PJ. Diet fat saturation and feeding state modulate rates of cholesterol synthesis in normolipidemic men. *J. Nutr.* 1997; 127: 332-340

Miettinen TA. Cholesterol production in obesity. *Circulation.* 1971; 44:842-850

Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, Koh YO. A new predictive equation for resting energy expenditure in healthy individuals. *Am J Clin Nutr.* 1990; 51: 241-247

Muscelli E, Camastra S, Catalano C, Galvan AQ, Ciociao D, Baldi S, Ferrannini E. Metabolic and cardiovascular assessment in moderate obesity: effect of weight loss. *J Clin Endocrinol Metab.* 1997; 82: 2937-2943

Neese RA, Faix D, Kletke C, Wu K, Wang AC, Shackleton CH, Hellerstein MK. Measurement of endogenous synthesis of plasma cholesterol in rats and humans using MIDA. *Am J Physiol.* 1993; 264:E163-E147

Nervi FO, Dietschy JM. Ability of six different lipoprotein fractions to regulate the rate of hepatic cholesterogenesis in the rat. *Biochim Biophys Acta.* 1975; 398:315-324

Nestel PJ, Schreibman PH, Ahrens EH. Cholesterol metabolism in obesity. *J Clin Invest.* 1973; 52:2389-2397

Nicklas BJ, Katzel LI, Bunyard LB, Dennis KE, Goldberg AP. Effects of an American Heart Association diet and weight loss on lipoprotein lipids in obese, postmenopausal women. *Am J Clin Nutr.* 1997; 66: 853-859

Ostlund RE, Hsu F-F, Bosner MS, Stenson WF. Quantification of cholesterol tracers by gas chromatography-negative ion chemical ionization mass spectrometry. J of Mass Spectrometry. 1996; 31: 1291-1296

Petrasovits A. Obesity in Canada. *Obesity Research.* 1997; 5:S95 *(abstract)*

Popkin BM, Siega-Riz AM, Haines PS. A comparison of dietary trends among racial and socioeconomic groups in the United States. *N Engl J Med.* 1996; 335:716-720

Previs SF, Fernandez CA, Yang D, Soloviev MV, David F, Brunengraber H. Limitaions of the mass isotopomer distribution analysis of glucose to study gluconeogenesis. J Biol Chem. 1995; 19806-19815

Rabkin SW, Mathewson FA, Hsu PH. Relation of body weight to development of ischemic heart disease in a cohort of young North American men after a 26 year observation period: The Manitoba Study. *Am J Cardiol.* 1977; 39: 452-458

Randle PJ, Priestman DA, Mistry S, Halsall A. Mechanisms modifying glucose oxidation in diabetes mellitus. *Diabetologia.* 1994; 37:S155-S161

Roberts KD, Bandi L, Calvin HI, Drucker WD, Lieberman S. Evidence that steroid sulfates serve as biosynthetic intermediates. IV. Conversion of cholesterol sulfate *in vivo* to urinary C19 and C21 steroid sulfates. *Biochem.* 1964; 3:1983-1988

Rössner S. Defining success in obesity management. *Int J Obes.* 1997; 21: S2-S4

Rudney H, Sexton R C. Regulation of cholesterol biosynthesis. *Ann Rev Nutr.* 1986; 6:245-272

Schoenheimer R, Rittenberg D. Deuterium as an indicator in the study of intermediary metabolism. *J Biol Chem.* 1935; 111: 163-192

Schwartz MW, Brunzell JD. Regulation of body adiposity and the problem of obesity. *Arterioscler Thromb.* 1997; 17:233-238

Seidell J C. Obesity in Europe: scaling an epidemic. *Int J Obes.* 1995; 19:S51-S54

Selmer J, Grunnet N. Ethanol metabolism and lipid synthesis by isolated liver cells from fed rats. *Bicohim Biophys Acta.* 1976; 428: 123-137

Selwyn AP, Kinlay S, Creager M, Libby P, Ganz P. Cell dysfunction in atherosclerosis and the ischemic manifestations of coronary artery disease. *Am J Cardiol.* 1997; 79: 17-23

Shapiro DJ, Rodwell VW. Fine structure of the cyclic rhythm of 3-hydroxy-3methylglutaryl coenzyme A reductase. Differential effects of cholesterol feeding and fasting. *Biochem.* 1972; 11(6):1042-1045

Sørensen HT, Sabroe S, Gillman M, Madsen KM, Fischer P, Sørensen TIA. Continued increase in prevalence of obesity in Danish young men. *Int J Obes.* 1997; 21:712-714

Ståhlberg D, Rudling M, Angelin B, Björkhem I, Forsell P, Nilsell K, Einarsson K. Hepatic cholesterol metabolism in human obesity. *Hepatology.* 1997; 25:1447-1450

Stephens T, Fowler Graham D, Health and Welfare Canada. Canada's health promotion survey 1990: Technical report. *Minister of Supply and Services Canada.* 1992

Thompson PD, Jeffrey RW, Wing RR, Wood PD. Unexpected decrease in plasma high density lipoprotein cholesterol with weight loss. *Am J Clin Nutr.* 1979; 32: 2016-2021

Ulbricht TLV, Southgate DAT. Coronary heart disease: Seven dietary factors. *Lancet.* 1991; 338; 985-992

Van Gaal LF, Wauters MA, De Leeuw IH. The beneficial effects of modest weight loss on cardiovascular risk factors. *Int J Obes.* 1997; 21:S5-S9

Vartianinen E, Puska P, Jousilahti P, Korhonen HJ, Tuomilehto J, Nissinen A. Twenty year trends in coronoary risk factors in North Karelia and other areas of Finland. *Int J Epidemiol.* 1994; 23:495-504

Vaswani AN. Effect of weight reduction on circulating lipids: an integration of possible mechanisms. *J Am Coll Nutr.* 1983; 2:123-132

Warnick G.R., Benderson J., Albers J.J. Dextran sulfate-Mg2+ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin Chem.* 1982; 28: 1379-1388

Williamson DF. Intentional weight loss: patterns in the general population and its association with morbidity and mortality. *Int J Obes.* 1997; 21: S14-S19

Wing RR, Koeske R, Epstein LH, Nowalk MD, Gooding W, Becker D. Long-term effects of modest weight loss in type II diabetic patients. *Arch Intern Med.* 1987; 147: 1749-1753

Wood PD, Stefanick ML, Dreon DM, Frey-Hewitt B, Garay SC, Williams PT, Superko HR, Fortmann SP, Albers JJ, Vranizan KM, Ellsworth NM, Terry RB, Haskell WL. Changes in plasma lipids and lipoproteins in overweight men during weight loss through dieting as compared with exercise. *N Engl J Med.* 1988; 319: 1173-1179

Wood P, Stefanick ML, Williams PT, Haskell WL. The effects on plasma lipoproteins of a prudent weight-reducing diet, with or without exercise, in overweight men and women.
N Engl J Med. 1991; 325: 461-466

Zimmet P, Dowse G, Bennett PH. Hyperinsulinemia is a predictor of non-insulin-dependent diabetes mellitus. *Diabetes Metab Rev.* 1991; 17:101-108