CHOLESTEROL SYNTHESIS IN TYPE III HYPERLIPOPROTEINEMIC AND NON-HYPERLIPIDEMIC INDIVIDUALS

by

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ABSTRACT

The purpose of this study was to investigate whether increased endogenous cholesterol synthesis contributes to the elevated plasma cholesterol levels observed in type III hyperlipoproteinemia (type III HLP). Eight apolipoprotein (apo) E2 subjects with type III HLP and 8 apo E2 non-hyperlipidemic control subjects (controls) were given a priming bolus dose of deuterium oxide (D_2O) (0.7 g D_2O/kg body H_2O). Daily M1 (central) pool free cholesterol fractional synthetic rate (FSR) was calculated as the incorporation rate of deuterium from body water into plasma free cholesterol. Blood samples were collected one half hour prior to, and at 12 hour intervals over 48 hours following, the bolus D_2O dose. Drinking water labelled at 1.4 and 0.7 g D_2O/liter H_2O was given on the fed and fasted days, respectively. Over 0-24 hours, subjects consumed a diet of three isocaloric meals which, in composition, approximated average North American intakes. Subjects fasted over 24-48 hours. The deuterium enrichment of plasma free cholesterol and plasma water was determined by isotope ratio mass spectrometry. When all subjects were included, mean ($\pm$SEM) free cholesterol overall FSR in type III HLPs (0.031 $\pm$ 0.006 per day) was not significantly different from controls (0.037 $\pm$ 0.004 per day). Estimated M1 total cholesterol pool size in type III HLPs (26.1 $\pm$ 1.9 g) and controls (24.9 $\pm$ 0.6 g) was not significantly different. When free cholesterol net synthesis was calculated as the absolute amount of cholesterol synthesized per day, based on M1 total cholesterol pool size, overall free cholesterol net synthesis in type III HLPs (0.304 $\pm$ 0.034 g/day) was not significantly different from controls (0.364 $\pm$ 0.035 g/day). When all subjects were included, overall free cholesterol FSR and overall free cholesterol net synthesis were significantly greater ($p<0.001$) in the fed (0.066 $\pm$ 0.006 day^{-1} and 0.655 $\pm$ 0.048 g/day, respectively) as compared to the fasted state (0.001 $\pm$ 0.004 day^{-1} and 0.010 $\pm$ 0.037 g/day, respectively). In the fed state, type III HLPs tended to synthesize cholesterol at a lower rate and in a lower absolute amount as compared to controls, while the reverse was observed in the fasted state. These results suggest that: (1) the elevated plasma cholesterol levels observed in type III HLPs are not due to excess de novo cholesterol synthesis; (2) fasting significantly reduces cholesterol synthesis from the fed state.

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1. INTRODUCTION

Individuals with the apo E 2/2 phenotype, who comprise approximately 1-2% of the population (Brown et al. 1983b, Innerarity et al. 1986), display primary dysbetalipoproteinemia, a lipoprotein abnormality characterized by low levels of LDL and traces of fasting β-VLDL (Utermann et al. 1977). It is postulated that impaired lipoprotein clearance (Utermann 1986), due to the abnormal binding of the E2 apoprotein to hepatic lipoprotein receptors (Weisgraber et al. 1982, Schneider et al. 1981), is the primary basis for these plasma lipoprotein alterations. The resultant effect of such abnormal binding is normal or subnormal plasma cholesterol levels. The same processes occur in the approximately 10% of the population with the apo E3/2 phenotype (Davignon et al. 1988), yet to a more moderate degree, due to possession of only a single E2 apoprotein (Weintraub et al. 1987, Havel et al. 1986).

Although accurate prevalence data are scarce, it is estimated that 1 in 1,000 to 10,000 individuals have a confirmed diagnosis of type III hyperlipoproteinemia (type III HLP) (Breslow et al. 1986), a relatively rare fasting hyperlipidemia characterized by highly elevated concentrations of fasting β-VLDL, xanthomatosis and premature coronary artery disease (Mahley et al. 1984b). Untreated, plasma cholesterol levels may range from 7.8-26 mmol/l (Innerarity et al. 1986). Paradoxically, type III HLP, and the elevated cholesterol levels observed, most commonly develop in individuals with the apo E2/2 phenotype (Mahley et al. 1984b), yet individuals with other apo E phenotypes (Gregg et al. 1983, Havel et al. 1983, Smit et al. 1987, Rall et al. 1989) and apo E deficiency (Schaefer et al. 1986, Mabuchi et al. 1989) have been identified with this disorder.

The apo E2:receptor binding abnormality is considered the principle defect in type III HLP (Rall et al. 1983a). However, as this disease does not develop in all apo E2 individuals, a multifactorial etiology has been proposed (Utermann et al. 1986), suggesting an interaction of other genetic and/or environmental factors with the underlying apo E2 defect to precipitate the expression of type III HLP (Davignon et al. 1988).

It is hypothesized that excess primary de novo cholesterol synthesis may be a factor which interacts with the abnormal E2 apoprotein to cause the elevated plasma cholesterol levels observed in type III HLP. In order to test this hypothesis, two primary objectives were addressed:

1) to determine whether apo E2 type III hyperlipoproteinemic subjects (type III HLPs) synthesize cholesterol at an increased rate as compared to apo E2 non-hyperlipidemic control subjects (controls).

2) to determine whether cholesterol synthetic rate in apo E2 type III hyperlipoproteinemic subjects (type III HLPs) and apo E2 non-hyperlipidemic control subjects (controls) is influenced by feeding state.

2. LITERATURE REVIEW

2.1 FUNCTION OF APOLIPOPROTEIN E

Apolipoprotein E (apo E) is a 299 amino acid, 34,000 molecular weight glycoprotein (Mahley et al. 1984a), synthesized in humans primarily in the liver, while secondary sources include the brain, adrenals, kidney and spleen (Utermann et al. 1986, Davignon et al. 1988). Apo E was first identified in 1973 by Shore and co-workers (Shore et al. 1973). As a surface component of several plasma lipoproteins, apo E functions as a ligand which mediates the interaction between specific cellular lipoprotein receptors and the plasma lipoproteins (Brewer et al. 1983). This apoprotein plays a critical role in three major pathways of lipid transport (Mahley et al. 1988) including transport of dietary lipids to the liver, transport of endogenous lipids to peripheral cells, and transport of lipids from peripheral tissues to the liver.

2.1.1 Transport of Dietary Lipids to the Liver

Apo E is present in chylomicrons, which transport dietary cholesterol and triglyceride from intestinal mucosal cells through the thoracic duct lymph to the liver. Apo E is acquired by chylomicrons upon secretion into lymph. Following entry into the bloodstream, chylomicrons are metabolized to chylomicron remnants within extra hepatic tissues, through lipolytic action on chylomicron core triglycerides by the enzyme lipoprotein lipase (LPL).

Chylomicron remnants are rapidly extracted from the bloodstream by hepatocytes, via receptor-mediated endocytosis. Hepatic intracellular cholesterol metabolism is in turn regulated by the degradation of the incoming cholesterol and triglyceride containing lipoproteins, through effects on 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase activity, the rate limiting enzyme of the cholesterol biosynthetic pathway, and on the low density lipoprotein (LDL) receptor, or apo B,E receptor, synthesis and expression (Brown and Goldstein 1983a, Andersen et al. 1979, Brown et al. 1981). The binding of chylomicron remnants to the hepatic chylomicron remnant receptor, or apo E receptor, present only on hepatic cells, is mediated by apo E (Gregg et

al. 1986), yet the specific uptake mechanism is unknown (Mahley 1988). In contrast to the LDL (apo B,E) receptor, found on liver and extrahepatic cells, the activity of the remnant (apo E) receptor is unregulated (Brown and Goldstein 1983a, Mahley 1984b). Although apo E of chylomicron remnants may be recognized by the hepatic LDL (apo B,E) receptor, normally most chylomicron remnants are cleared by the liver through the remnant (apo E) receptor (Brown and Goldstein 1983a, Sutherland et al. 1988).

2.1.2 Transport of Endogenous Lipids to Peripheral Cells

Secondly, apo E is a constituent of very low density lipoproteins (VLDL). Endogenous triglycerides and cholesterol, synthesized in the liver, are transported by VLDL secreted from hepatocytes. Similar to the catabolic course of chylomicrons, VLDL remnants, also referred to as lipoproteins of intermediate density (IDL), are produced by LPL hydrolysis of VLDL core triglycerides. The subsequent processing of VLDL remnants diverges at this point from that of chylomicrons. Firstly, VLDL remnants (IDL) may be removed directly into the liver via either the remnant (apo E) receptor or the LDL (apo B,E) receptor. Secondly, VLDL remnants may be further catabolized via LPL to LDL. In normal human metabolism, most VLDL remnants undergo conversion to LDL (Utermann 1986, Gregg et al. 1986). Apo E appears to play a role in both of these catabolic routes of VLDL metabolism, yet the precise mechanism whereby apo E functions is undefined (Utermann 1986). LDL do not contain apo E, and 60-80% of these lipoproteins are therefore catabolized directly by the LDL (apo B,E) membrane receptors of hepatocytes or peripheral tissues (Meddings et al. 1987). The remaining 20-40% of LDL are removed from the plasma by nonspecific receptor-independent endocytosis in hepatic and extrahepatic tissues.

2.1.3 Transport of Lipids from Peripheral Tissues to Liver

In normal human plasma, the majority of reverse cholesterol transport is thought to occur via cholesterol ester transfer protein (CETP) transport of cholesterol esters from HDL to either

chylomicrons and VLDL (and/or their remnants), or primarily to LDL, followed by LDL (apo B,E) and remnant (apo E) receptor hepatic uptake (Havel 1988). Some of the typical high density lipoproteins (HDL) without apo E become enriched with unesterified cholesterol from peripheral tissue cell membranes and/or the surfaces of lipoproteins (Havel 1988), and acquire apo E, forming apo E-HDLc (Mahley et al. 1988). It has been speculated, that in humans, apo E-HDLc functions as an element in reverse cholesterol transport, carrying a fraction of the cholesterol obtained from peripheral tissues directly to the liver through an apo E:LDL (apo B,E) receptor interaction (Mahley et al. 1988).

Clearly, apo E is critically important as a component of chylomicron and VLDL remnants, as well as apo E-HDLc, in the recognition process of these plasma lipoproteins by lipoprotein receptors. Apo E is thus indirectly crucial to the control of intracellular lipid metabolism, as these lipoproteins are subsequently removed from the plasma. Any variation in the functional role of apo E, as a result of genetic alteration of this polypeptide, would have significant effects on cholesterol homeostatic control in humans.

2.2 APO E POLYMORPHISM

Utermann et al. (1977) were the first to demonstrate that human apo E exists as three major isoforms using the phenotyping technique of isoelectric focusing of VLDL apo E. In subsequent studies, Zannis and Breslow (1981) hypothesized that the polymorphic nature of apo E is due to genetic variation at the apo E gene locus. These investigators predicted that synthetic control of apo E is determined by three independent alleles of the gene for apo E, designated ε4, ε3, and ε2, each allele coding for a major gene product, referred to as the E4, E3 and E2 isoforms, respectively (Zannis et al. 1981). The three alleles result in six genotypes: 3 homozygous (ε4/4, ε3/3, and ε2/2) and 3 heterozygous (ε4/3, ε3/2, and ε4/2). Six corresponding phenotypes arise from the expression of any one of the genotypes: E4/4, E3/3, E2/2 and E4/3, E3/2, and E4/2 (Davignon 1988).

The relative frequency with which the three common alleles ε4, ε3, and ε2, occur displays within and between population variability. As reviewed by Boerwinkle (1987), frequencies ranging from 12-18%, 72-89% and 0-13% for the ε4, ε3, and ε2 alleles respectively, were estimated from 11 populations of differing ethnic origin or geographical location. Relative frequencies of these common alleles among Caucasians (weighted averages, from Germany, Scotland, Canada, Netherlands, France, Finland and New Zealand) and for Canadians (Ottawa) have been estimated by Davignon (1988) as 15.0%, 76.9% and 8.0% and 15.2%, 77.0%, and 7.8% for the ε4, ε3, and ε2 alleles, respectively. Recent studies have determined the approximate frequencies of 3.9%, 61.7%, 2.0%, 20.6%, 9.8%, and 2.0% for the E4/4, E3/3, E2/2, E4/3, E3/2, and E4/2 phenotypes, respectively, for a sample of Canadians living in Ottawa (Davignon et al. 1988).

2.3 STRUCTURAL BASIS FOR APO E POLYMORPHISM

As the homozygous E3/3 phenotype predominates within the population, the ε3/3 genotype is considered the wild type or parent apo E isoform, from which the aberrant E4 and E2 isoforms are derived (Mahley et al. 1984b). The structural basis for apo E polymorphism has been explained through amino acid sequencing of the major apo E isoforms (Rall et al. 1982b). Weisgraber et al. (1982) have demonstrated point mutations at residues 112 and 158, resulting in alterations of the primary structure of the apo E gene. Apo E4 differs from apo E3 by a single substitution of the basic amino acid arginine in place of the neutral amino acid cysteine at residue 112 in the apo E3 isoform, noted as "E4 Cys 112 --> Arg". This amino acid interchange to form the E4 mutant, results in a +1 charge difference relative to the E3 isoform. The most common form of apo E2 differs from apo E3 by a single substitution of the amino acid cysteine in place of arginine at residue 158 in the apo E3 isoform, noted as "E2 Arg 158 --> Cys". This amino acid interchange to form the E2 mutant, results in a -1 charge difference relative to the E3 isoform (Weisgraber et al. 1982). These cysteine-arginine interchanges in the parent apo E3 isoform, which is noted as "112 Cys, 158 Arg", provide the basis for separation of the three major isoforms

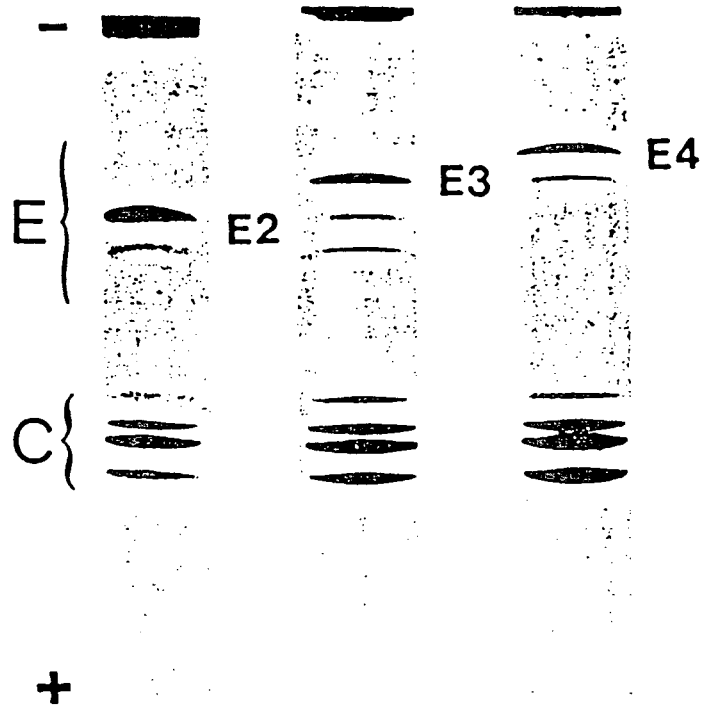
by polyacrylamide gel isoelectric focusing according to differing isoelectric points of 6.2 to 5.7, ranging from basic (E4) to acidic (E2) (Rall et al. 1986) (Figure 1).

In addition to the three major, common apo E isoforms, several rare apo E mutant isoforms have been described, among which include E2* (E2 145 Arg --> Cys) and E2** (E2 146 Lys --> Gln) (Breslow et al. 1986, Ehnholm et al. 1986). Homozygosity for the apo E2 phenotype may therefore result from a combination of any two of the $\varepsilon 2, \varepsilon 2^*$ or $\varepsilon 2^{**}$ alleles (Breslow et al. 1986).

2.4 IMPACT OF ALLELIC VARIATION ON FUNCTIONAL CHARACTERISTICS OF APO E

Recent studies by Weisgraber et al. (1982) and Schneider et al. (1981) have demonstrated that the apo E2 isoform exhibits abnormal binding activity to LDL (apo B,E) lipoprotein receptors, due to the cysteine-arginine substitution at residue 158. Innerarity et al. (1986) have described the molecular basis for this decreased binding affinity. Alterations in the molecular conformation of the apo E receptor binding domain, comprised of amino acid residues 140-150, may result from amino acid substitutions in regions outside the actual binding site, such as residue 158. These conformational alterations may be deleterious to normal binding activity.

Heterogeneity in the defective lipoprotein:receptor binding activity of individuals with the apo E2/2 phenotype (Rall et al. 1982b) may be explained by the genetic heterogeneity within this phenotype. Impaired binding activity is exhibited by all of the apo E2 mutant forms, varying from $<2\%$, 40%, and 45% of apo E3 normal binding for the E2, E2**, E2* mutants, respectively (Mahley et al. 1984a, Mahley et al. 1984b). The defective receptor binding of the apo E2 variants (E2** and E2*) may be explained by disruption of an apoprotein:receptor direct ionic interaction with the substitution of neutral for basic amino acids at residues 145 and 146 in the receptor binding domain (Lalazar et al. 1988).



	E2/2	E3/3	E4/4
Relative Charge	0	+1	+2
Residue 112	Cys	Cys	Arg
Residue 158	Cys	Arg	Arg

Figure 1 One-dimensional isoelectric focusing technique showing the three homozygous apo E phenotypes. The amino acid differences among the three major polymorphic forms of apo E are given for comparison (Rall et al. 1986).

The impact of this allelic variation on the functional role of apo E is manifest in defective receptor mediated transport of triglyceride and cholesterol containing lipoproteins in human plasma. A defect in the high affinity binding of apo E containing chylomicron remnants, VLDL remnants (IDL), and apo E-HDLc to both the hepatic LDL (apo B,E) receptor and the remnant (apo E) receptor would be expected to result in hyperlipidemia, due to an accumulation of these particles in the plasma (Utermann 1986, Utermann et al. 1984). Individuals homozygous for the E2/2 phenotype do display impaired lipoprotein clearance and accumulation of plasma cholesterol and triglyceride enriched chylomicron and VLDL remnants and apo E-HDLc (Davignon et al. 1988, Havel et al. 1980a), yet only to a moderate degree. Paradoxically, gross elevations in cholesterol and triglyceride levels in most of these subjects do not occur, while subnormal lipid levels are often observed (Utermann et al. 1977, Utermann et al. 1979a).

Utermann et al. (1979a), comparing serum lipid levels in the three phenotypic groups, apo E-N (apo E3/3), apo E-ND (apo E3/2), and apo E-D (apo E2/2), observed a hypocholesterolemic effect in individuals homozygous for the E2/2 phenotype, whose serum cholesterol levels were approximately 1.0 mmol/l (40 mg/dl) lower than E3/3 subjects. The effects of apo E polymorphism on normal plasma lipid and lipoprotein concentration were confirmed and extended through further investigation by Sing and Davignon (1985). Based on the previous finding that interindividual genetic variability accounts for 50% of the fluctuation in normal serum cholesterol levels (Sing et al. 1978), the fractional contribution of the common apo E alleles to plasma cholesterol levels was estimated to be 14% of the genetic variance, and 7% of the total variance for a population sample in Ottawa, Canada (Sing and Davignon 1985). These results, in combination with data from other studies (Utermann 1986, Bouthillier et al. 1983), have established the "cholesterol-lowering" effect of the $\varepsilon 2$ allele, measured against the wild type $\varepsilon 3$ allele, and the "cholesterol-raising" effect of the $\varepsilon 4$ allele (Davignon et al. 1988). A difference of approximately 1.6 mmol/l (60 mg/dl) has been observed between mean total cholesterol levels of individuals with the E2/2 and E4/4 phenotypes, averaging approximately 3.6 and 5.2 mmol/l (140 and 200 mg/dl), respectively (Utermann 1986). Individuals heterozygous for the apo $\varepsilon 2$ allele exhibit plasma

cholesterol levels between values obtained for ε2 homozygotes and individuals who do not possess the ε2 allele (Utermann et al. 1984). Therefore, the ε4, ε3 , and ε2 alleles in apo E homo- and heterozygotes effect an overlapping array of phenotypically related plasma cholesterol concentrations in the population (Utermann 1986). Mean plasma cholesterol levels for apo E phenotypes observed in a study in Ottawa, Canada (Davignon 1988) were 3.53 (E2/2), 4.17 (E3/2), 4.51 (E3/3), 4.62 (E4/2), 4.77 (E4/3), and 4.67 (E4/4) mmol/l.

The homozygous E2 phenotype occurs in approximately 1 (Brown et al. 1983b) to 2% (Innerarity et al. 1986) of Europeans and North Americans. As shown by Utermann, despite inheritance of this mutant form of apo E, most individuals displaying the E2/2 phenotype actually have subnormal cholesterol levels (Utermann et al. 1979a, Utermann et al. 1979b). The metabolic consequences of the apo ε2 allele may be summarized as: hypocholesterolemic, hypertriglyceridemic and dyslipoproteinemic (Utermann et al. 1979a). Most of the individuals who are homozygous for this allele display primary dysbetalipoproteinemia (Utermann et al. 1977), a lipoprotein abnormality characterized by the E2/2 phenotype, LDL deficiency, and evidence of β-VLDL, also known as "floating β-lipoproteins".

Three basic characteristics differentiate β-VLDL particles from normal VLDL, also referred to as α-VLDL. Firstly, β-VLDL exhibit β-mobility on electrophoresis, as opposed to the normal pre-β mobility of α-VLDL, hence the term dysbetalipoproteinemia. On most types of electrophoretic systems, α-VLDL usually migrate more rapidly than LDL, also referred to as β-lipoproteins. Carlson and Carlson (1975) have classified VLDL with slow electrophoretic mobility as "β-VLDL" or "slow pre-β" VLDL. Secondly, β-VLDL exhibit an increased ratio of cholesterol, mainly cholesterol ester, to triglycerides (Sata et al. 1972), compared to normal VLDL, which are triglyceride-rich. Thirdly, the total amount of apo E in β-VLDL is absolutely increased (Havel et al. 1973, Havel 1980b) and the ratio of apolipoprotein C:apo B is decreased (Brown et al. 1983b, Havel et al. 1973), relative to normal VLDL.

It has been shown that β-VLDL are composed of two distinct fractions (Fainaru et al. 1982). The first subfraction has been suggested to correspond to chylomicron remnants, as

evidenced by their supposed intestinal origin, large 700-800 angstrom diameter, and apo E, apo B-48 content. The second subfraction resembles VLDL cholesterol enriched remnants, identified by their supposed origin in the liver, smaller 400 angstrom diameter, and apo E, apo B-100 content.

These defective metabolic consequences exhibited in individuals who carry the mutant ε2 allele, have recently been confirmed by Weintraub et al. (1987) in a study of the effect of apo E polymorphism on dietary fat clearance. Apo E3/2 phenotypic individuals were documented to display, yet to a more moderate degree, the delayed chylomicron remnant clearance observed in E2/2 homozygotes. Havel et al. (1986) have also reported such moderate accumulations of β-VLDL in E3/2 heterozygotes.

2.5 METABOLIC CONSEQUENCES OF APO E2/2 PHENOTYPE - POSSIBLE MECHANISM

Utermann (1986) has proposed a model for the mechanism of apo E2 homozygosity effects on plasma cholesterol. Direct uptake of chylomicron remnants and apo E-HDLc into hepatocytes, is mediated by high affinity binding of their surface apo E ligand primarily to remnant (apo E) receptors, and secondarily to LDL (apo B,E) receptors. Possession of the mutant ε2 allele results in defective lipoprotein:lipoprotein receptor interaction and an increase in plasma chylomicron remnant, VLDL remnant and apo E-HDLc concentrations. As a result, the decreased uptake of cholesterol derived from the diet and peripheral tissues into the liver stimulates an up-regulation of hepatic LDL receptors. A corresponding decrease in the level of plasma cholesterol rich LDL particles, and therefore a decrease in plasma total cholesterol levels, results due to an increased LDL uptake proportional to the increased LDL receptor availability.

A second component of this model, which may explain the reduced LDL levels and presence of β-VLDL observed in apo E2 individuals, is the inhibitory role of apo E2 in the normal conversion of VLDL remnants to LDL (Ehnholm et al. 1984, Chait et al. 1977). It has been suggested that normal apo E3 is required for this interconversion, while the variant E2 impedes this process. The resultant effect on plasma lipid levels of these altered lipoprotein metabolic pathways is to increase the amount of remnant lipoproteins rich in cholesterol and triglyceride and

decrease the concentration of cholesterol rich LDL in the plasma. This corresponds to net lowered plasma cholesterol concentrations and prolonged raised plasma triglycerides postprandially in apo E2/2 individuals (Davignon et al. 1988).

2.6 ASSOCIATION OF THE APO E2/2 PHENOTYPE WITH TYPE III HYPERLIPOPROTEINEMIA

Paradoxically, of the 1-2% of the population homozygous for the ε2 allele, approximately 2-10% (Rall et al. 1989) develop a relatively rare fasting hyperlipidemia termed type III hyperlipoproteinemia (HLP). Type III HLP, also known as broad-beta disease, differs from the primary dysbetalipoproteinemia exhibited in most E2/2 homozygotes, in that the characteristic E2/2 phenotype and evidence of fasting plasma β-VLDL are accompanied by a quantitative increase in plasma lipoprotein remnants with resultant hypercholesterolemia and hypertriglyceridemia, and the appearance of distinguishable clinical signs.

2.7 HISTORICAL BACKGROUND OF TYPE III HYPERLIPOPROTEINEMIA

In 1952, Gofman et al. (1954) were the first to describe some of the clinical features of type III HLP, while in 1967, Fredrickson et al. instituted the nomenclature "type III hyperlipoproteinemia" to designate patients presenting with this disorder from the five other hyperlipoproteinemia classifications (Fredrickson et al. 1978). In these early clinical and biochemical studies, the diagnosis of type III HLP was based on two abnormal characteristics of VLDL from fasting blood: β-electrophoretic mobility, increased VLDL cholesterol:total triglyceride ratio greater than 0.30, and a measurement of 3.9-25.9 mmol/l (150-1000 mg/dl) for plasma triglycerides (Zannis 1986). The significance of the regulatory involvement of apolipoprotein E was first evidenced in 1975, by Havel and Kane's observation that the β-VLDL of type III HLP patients contained a greater total amount of this arginine-rich protein than that occurring in normal α-VLDL (Havel and Kane 1973). Utermann et al. in 1975, implementing the technique of

isoelectric focusing, demonstrated that type III HLP patients displayed the E2/2 phenotype (Utermann et al 1975).

In most of the studies recorded to date, a strong association of the E2/2 phenotype with type III HLP has been suggested, as 90% of individuals with this disorder have been determined homozygous for the ε2 allele (Breslow et al. 1982, Utermann 1987, Sutherland et al. 1988). The remaining 10% of individuals diagnosed with type III HLP, are not apo E2 homozygotes, but exhibit defective apo E:receptor binding, due to heterozygosity for the apo ε2 allele, or possession of an unusual and functionally abnormal variant of apo E (Utermann 1986, Rall et al. 1982b). Individuals with the E3/2 (Smit et al. 1987), and E3/3 (Havel et al. 1983, Rall et al. 1989, Havekes et al. 1984, Havekes et al. 1986) phenotypes have been identified with this disorder. Type III HLP has also been associated with apo E deficiency (Schaefer et al. 1986, Mabuchi et al. 1989).

2.8 CLINICAL AND PATHOLOGIC FEATURES OF TYPE III HYPERLIPOPROTEINEMIA

Characteristic features of type III HLP may be summarized as: hypercholesterolemia, hypertriglyceridemia, most commonly an E2/2 phenotype, presence of β-VLDL, increased plasma apo E levels, and clinical findings including xanthomatosis and atherosclerosis (Mahley et al. 1984b).

With respect to clinical biochemistry, typical plasma cholesterol levels observed in untreated type III HLP patients are 7.8 mmol/l (300 mg/dl) and may even extend as high as 26 mmol/l (1000 mg/dl), while triglyceride values typically range from 2.3 mmol/l (200 mg/dl), up to 9.0 mmol/l (800 mg/dl) (Mahley et al. 1984b, Innerarity et al. 1986).

In addition to plasma chylomicrons, α-VLDL, LDL and HDL normally present in type III HLP subjects, the most prevalent abnormal plasma lipoproteins in these individuals are β-VLDL particles (Brown et al. 1983b). These lipoproteins comprise the broad beta band observed on paper electrophoresis of whole plasma. Higher resolution techniques of starch block electrophoresis delineate both pre β-VLDL, or normal α-VLDL, and β-VLDL, in this fraction

(Innerarity et al. 1986). The β-VLDL are composed of two heterogeneous subgroups as described above. LDL levels are usually decreased, while the concentration of HDL in the plasma may be at or below normal levels (Mahley et al. 1984b).

The most prominent clinical manifestation of type III HLP is the development of xanthomas, characterized as slightly elevated, soft, rounded nodules formed by deposition of lipid in tissues. In a study of 115 patients, 50% exhibited these clinical features (Brewer et al. 1983). Xanthomata striata palmaris, lipid deposits in the creases of the palm, identified by an orange or yellow pigmentation, are the most common xanthomas, and are considered pathognomonic for type III HLP (Mahley et al. 1984b). Additional types of xanthomas observed include tuberoeruptive xanthomas, raised tuberous tissue on the elbows and knees, and xanthelasmas, slightly raised yellowish tumors occurring on the upper and lower eye lids, found in less than 25% of type III HLP patients.

These pathologic tissue characteristics have been suggested to occur as a result of macrophage accumulation of lipids. This accumulation is thought to result from discharge of massive loads of cholesterol to various tissue macrophages by intestinal and hepatic β-VLDL, resulting in induction of cholesterol ester synthesis and storage in these phagocytic cells (Fainaru et al. 1982). The foam cells of tuberous xanthomas are postulated to originate from such macrophages (Carlson et al. 1988, Assmann 1984).

Combined data from four major studies (Brewer et al. 1983, Zannis 1986) indicated that premature coronary artery disease occurred in 33% of type III HLP patients, while peripheral vascular atherosclerosis was detectable in one third of patients. Coronary artery disease appeared earlier in men, on average in the late thirties, and in women, in the late forties.

Age of onset of type III HLP has been documented to range from 16-95 years (Brown et al. 1983b), although cases of this disorder in adolescents have been reported (Mabuchi et al. 1989). Men tend to present at a mean age of 39, with women exhibiting onset of the disorder at a mean age of 49. Four major studies have demonstrated the occurrence of type III HLP in men as compared to women is 2:1 (Brewer et al. 1983).

2.9 GENETIC MODE OF INHERITANCE OF TYPE III HYPERLIPOPROTEINEMIA

The mode of inheritance for the type III HLP disorder is a subject of debate. Confusion has existed as to the autosomal recessive or dominant genetic nature of the disease (Utermann et al. 1979b, Morganroth et al. 1975). The proposal that type III HLP in most cases is inherited as an autosomal recessive trait has been supported by observations that individuals with the E2/2 phenotype and type III HLP are born from parents who possess the ε2 allele, but do not exhibit type III HLP. Also, children born from one parent with the E2/2 phenotype plus type III HLP, and one parent without the ε2 allele, have been shown to lack the apo E2/2 phenotype and be free from the type III HLP disease (Breslow et al. 1986). Therefore, as type III HLP is most commonly associated with inheritance of two apo ε2 alleles, it is typically thought of as a recessive trait.

Observance of type III HLP in patients heterozygous for the normal apo E3 protein and an apo E variant protein defective in lipoprotein receptor binding, suggests a truly autosomal dominant mechanism of transmission in some individuals (Breslow et al. 1986, Havekes et al. 1986, Rall et al. 1989, Smit et al. 1987).

2.10 TREATMENT OF TYPE III HYPERLIPOPROTEINEMIA

Of all the familial hyperlipoproteinemias, treatment of type III is the most successful. Successful treatment is determined by the reduction of plasma cholesterol and triglyceride levels to more normal concentrations (Brown et al. 1983b). Exacerbating secondary factors, such as hypothyroidism and obesity, must first be eliminated before effective therapy is begun. The hyperlipidemia is exceptionally responsive to diet control. Effective measures in the dietary management of this disorder include restriction of total caloric intake until ideal body weight is reached, as well as reduction of cholesterol, saturated fat and alcohol intake (Brown et al. 1983b, Mahley et al. 1984b, Brewer et al. 1983).

In addition, drug therapy is often implemented in the treatment of type III HLP patients, to achieve and maintain normal plasma lipid levels. Although β-VLDL will always be present in

individuals homozygous for the ε2 allele, the exceptional effects of two common medications administered to these patients, clofibrate (2 g/day) and nicotinic acid (2-3 g/day), are demonstrated by regression of xanthomatous lesions after a few weeks (Mahley et al. 1984b, Brown et al. 1983b, Brewer et al. 1983) and the subjective inhibition of atherosclerotic progression (Fredrickson et al. 1978).

2.11 FACTORS MODULATING THE EFFECTS OF THE APO E2 DEFECT IN THE PHENOTYPIC EXPRESSION OF TYPE III HYPERLIPOPROTEINEMIA

The apo E2:receptor binding abnormality is considered the principal defect in type III HLP (Rall et al. 1983a). However as all apo E2 individuals do not develop the gross hyperlipidemia and disease phenotypically expressed as type III HLP (Utermann 1987), additional factors must act to precipitate the pathogenesis of the disease.

Recent studies by Rall et al. (1983a) have supported the concept for a multifactorial etiology of type III HLP. It was speculated that apo E2 from hypo- and normolipidemic individuals may not exhibit the defective receptor binding activity observed in E2/2 homozygotes with type III HLP. Rall demonstrated, however, a 158 cysteine residue and a functional abnormality in apolipoprotein E2 in all hypo-, normo- and hypercholesterolemic subjects. Apo E:LDL receptor binding in vitro was equally defective in all cases.

Utermann et al. (1979b) have proposed a permissive role for the mutant apo ε2 allele in individuals who develop type III HLP. The apo ε2 allele, or apo E variant with similar defective receptor binding, while necessary, is not sufficient to cause this disease. Additional genetic or environmental factors must act to modulate this inborn metabolic error and precipitate the phenotypic expression of type III HLP (Utermann 1987). Several precipitating factors have been suggested to promote type III HLP, when superimposed most commonly on the ε2/2 genotype (Davignon et al. 1988, Utermann et al. 1979b, Utermann 1987).

Firstly, coincidental inheritance of a gene for hyperlipidemia, such as familial-combined hyperlipoproteinemia or familial hypertriglyceridemia, has been suggested to modulate the effect

of the apo E2 defect and result in the phenotypic expression of type III HLP. Although the molecular mechanism is poorly understood, inheritance of an additional defect in lipid metabolism may overload the already compromised degradative pathway of VLDL conversion to VLDL remnants and LDL (Utermann et al. 1979b), with resultant elevations in plasma lipid levels.

Secondly, endogenous endocrine perturbations such as decreases in circulating estrogen levels with menopause or hypothyroidism, may augment the apo E2 metabolic abnormality and precipitate type III HLP disease (Davignon et al. 1988, Utermann et al. 1979b, Utermann 1987). Estrogens have been shown in animals to increase the affinity or number of hepatic LDL (apo B,E) receptors which bind apo E, therefore increasing VLDL remnant catabolism and lowering plasma lipid levels (Brewer et al. 1983, Stuyt et al. 1986). Hypothyroidism is often associated with type III HLP. The pathophysiological relationship of hypothyroidism to type III HLP may occur through the diminished receptor-mediated lipoprotein removal observed in the hypothyroid state (Mahley et al. 1984b, Thompson et al. 1981), as a result of decreased hepatic LDL (apo B,E) receptor activity (Mahley et al. 1985).

Thirdly, elements such as obesity, another clinical characteristic often associated with type III HLP may unmask or exacerbate the effects of the apo E genetic disorder (Brewer et al. 1983), although the physiological association of obesity with the pathogenesis of type III HLP has yet to be determined.

Fourthly, metabolic alterations occurring with age (Sutherland et al. 1988, Rall et al. 1983a, Mahley et al. 1984b, Mahley et al. 1985, Meddings et al. 1987) may be responsible for the manifestation of type III HLP. Hepatic LDL (apo B,E) receptor expression in animals has been shown to be age-dependent. Adult animals, in contrast to their young, express hepatic LDL receptors at very low levels (Mahley et al. 1984b). Although the biochemical basis for the age-related expression of type III HLP has not been definitely resolved, age dependent hepatic apo B,E receptor expression may be responsible for influencing β-VLDL concentration in type III HLP (Sutherland et al. 1988, Mahley et al. 1984b).

This multifactorial model for the phenotypic expression of type III HLP, relating the interaction of the primary molecular defect of apo E2 with secondary genetic and/or environmental triggering factor(s), has been summarized graphically by Davignon et al. (1988) (Figure 2).

A final possible mechanism for the paradoxical effects of homozygosity for the $\varepsilon 2$ allele and the development of the clinical expression of type III HLP from primary dysbetalipoproteinemia, is excessive primary de novo synthesis of cholesterol by the liver (Mahley et al. 1984b, Innerarity et al. 1986, Rall et al. 1983a, Gregg et al. 1983).

The basis for excessive primary de novo synthesis of cholesterol in type III HLP individuals may relate to the rate determining step in hepatic cholesterol biosynthesis, the conversion of HMG CoA to mevalonic acid. This step is catalyzed by the enzyme HMG CoA reductase. The activity of this enzyme, and therefore the rate of hepatic cholesterogenesis, may be increased in apo E2 type III HLPs as compared to apo E2 non-hyperlipidemic individuals.

Additional possible explanations for increased rates of hepatic cholesterogenesis, however, include secondary causes such as obesity (Mahley et al. 1984b, Rall et al. 1983a). Several studies have demonstrated elevated hepatic cholesterol biosynthesis in obese humans (Angelin 1985), although mechanisms are unknown. Using a chromatographic sterol balance technique, Meittinen has demonstrated that increasing body weight is significantly correlated with increased cholesterol synthetic rate (Meittinen et al. 1971).

2.12 MEASUREMENT OF CHOLESTEROL SYNTHESIS

Precision measurement of the rate of human cholesterol synthesis necessitates an accurate, quantitative, non-hazardous methodology. Three types of procedures for determination of lipid synthetic rates have been studied extensively in experimental animals. The first of these methods, the determination of HMG-CoA reductase activity, a well established indicator of cholesterol synthesis, has not been widely applied to human study, as fresh liver biopsy tissue is required (Sodhi et al. 1979) and, as a qualitative method, only relative rates of cholesterol synthesis are obtained (Dietschy et al. 1984).

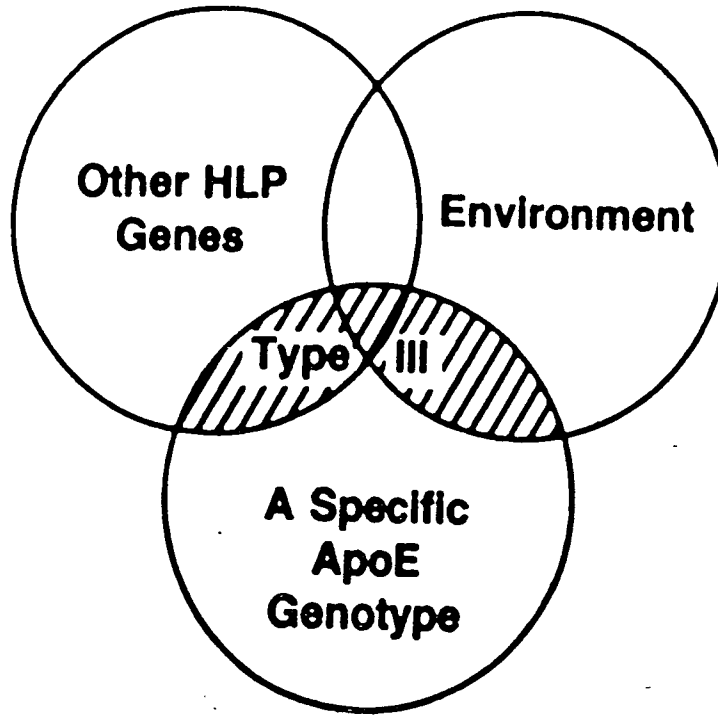


Figure 2 Pathogenesis of type III HLP: interaction between genes, environment, and a specific apo E genotype (Davignon et al. 1988).

Secondly, measurement of the incorporation rate of ^{14}C-labelled cholesterol precursors such as acetate (Turley et al. 1976), glucose or pyruvate (Dietschy et al. 1984), has been carried out in animal models. However, the utility of this technique is limited in humans, due to the radioactivity associated with this isotope of carbon and the potential underestimation of cholesterol synthetic rate measurements due to dilution of the acetyl CoA intracellular pool specific activity, through mixing of labelled precursor or the acetyl CoA produced from the precursor, with unlabeled substrates. Rates of incorporation of various ^{14}C-labelled substrates therefore provide only relative, rather than absolute, rates of sterol synthesis.

Thirdly, measurement of the incorporation of tritium atoms from tritiated water in the endogenous synthesis of cholesterol (Jeske et al. 1980) and fatty acids (Jungas 1968, Wadke et al. 1973) has been successfully implemented in animals to determine lipid biosynthesis, but the radiation hazard associated with this technique eliminates its wide use in studies using humans.

Available methods to determine de novo cholesterol biosynthesis in humans include measurement of plasma and 24-h urinary mevalonic acid (MVA) concentration (Parker et al. 1984). Measurement of MVA formed from HMG-CoA in an irreversible reaction catalyzed by HMG-CoA reductase and NADPH, provides an accepted estimate of cholesterol synthesis, as it is generally concluded that most MVA produced is converted to cholesterol (Sodhi et al. 1979).

Sterol balance techniques (Nestel et al. 1973, Bennion et al. 1975) have been used, based on the premise that fecal cholesterol and its metabolites indicate whole body net cholesterol synthesis when exogenous cholesterol intake is eliminated in the steady state, assuming the feces are the major route of elimination of endogenous cholesterol and its metabolites from the body.

Cholesterol turnover and synthesis have also been measured by decay of plasma cholesterol specific activity after injection of ^{14}C-labelled cholesterol, followed by compartmental analysis using three pool theoretical modelling (Goodman et al. 1980).

Of the above methods available for measuring human cholesterol synthesis, the former procedure provides only a qualitative, indirect measure of cholesterol synthesis, while the two

latter techniques are laborious and lengthy, with the experimental period often spanning several months (Sodhi et al. 1979).

2.13 DEUTERIUM INCORPORATION METHODOLOGY

Direct, short-term measurement of human plasma cholesterol synthesis has been accomplished by deuterium incorporation methodology (Jones et al. 1988, Jones et 1990). The technique involves oral administration of deuterium oxide, an isotope of water occurring naturally in the body. Deuterium oxide contains non-radioactive, deuterium or "heavy hydrogen" atoms, so named by virtue of a single neutron in the atomic nucleus, in place of isotopic protium or "light hydrogen" atoms, containing no neutrons in the atomic nucleus. The incorporation of deuterium atoms into endogenously synthesized cholesterol molecules in place of protium atoms, permits quantitative determination of cholesterol fractional synthetic rate (FSR) over short time periods, without radiation risk.

Initial studies employing deuterium labelling for measuring human de novo cholesterol synthesis were carried out by Taylor et al. in 1966. Deuterium enrichment of body water was maintained at 5.0-6.0 g deuterium oxide/kg body water, above normal deuterium body water baseline levels which are 0.145 g deuterium oxide/kg body water. This high level of deuterium enrichment was required to adequately label de novo synthesized cholesterol because of the lack of precision of isotope ratio mass spectrometers then available. Approximately 40 days were required to achieve maximum deuterium oxide enrichment. Also, toxic side affects, such as severe dizziness, were observed in subjects given such large amounts of deuterium oxide (140-250 g).

Recent methodological advances have increased the precision of protium and deuterium relative abundance measurements in plasma cholesterol samples analyzed by differential isotope ratio mass spectrometry. Assessment of cholesterol deuterium enrichment within 12 hours post oral dosage of deuterium, and reduction in the required level of deuterium enrichment of body water, which eliminates toxic side effects, are now possible (Jones et al. 1988, Jones et al. 1990, Schoeller et al. 1983). Labelling of the body water pool at 0.5 g deuterium oxide/kg body water

has been used successfully to detect cholesterol deuterium enrichment over a 12 hour period (Jones et al. 1988).

2.14 ASSUMPTIONS OF DEUTERIUM INCORPORATION METHODOLOGY

Whole body cholesterol turnover has been characterized by a three pool mathematical model which describes the rates at which the three major groups of exchangeable cholesterol in the body equilibrate with plasma cholesterol (Goodman et al. 1973). This quantitative methodology involves periodic evaluation of plasma cholesterol specific activity (SA) over a period of several months following a labeled cholesterol injection. The decay curve of the radioactive cholesterol, obtained by plotting SA versus time, is analyzed by a multicompartmental system which suggests that the long-term disappearance of the tracer can best be explained by a three pool model. The three cholesterol pools do not describe physiological compartments within the body, rather they separate the exchangeable cholesterol within body tissues into three categories, based on how quickly the SA of the tissue cholesterol equilibrates with plasma cholesterol. The rapidly exchangeable central pool, pool 1 (M1), which is estimated to be 24 grams in size in non-hyperlipidemic individuals (Goodman et al. 1980), consists mainly of plasma, liver, intestine, red blood cell, pancreas, spleen, kidney and lung cholesterol. Side pools 2 (M2) and 3 (M3) consist of tissue cholesterol which equilibrates at intermediate and slow rates with plasma cholesterol, respectively. Pool 2 is comprised of some visceral tissue cholesterol and a portion of peripheral tissue cholesterol. Pool 3 includes mostly peripheral tissue cholesterol such as skeletal muscle, adipose tissue, connective tissue and arteries (Goodman et al. 1980).

The three pool cholesterol model assumes that losses of cholesterol from the body, by catabolism to bile acids or direct excretion through the feces, occur only via pool 1 (Goodman et al. 1973, Sodhi et al. 1979). It is also assumed that the introduction of new cholesterol occurs solely through pool 1, via absorption of dietary cholesterol or endogenous cholesterol synthesis (Dietschy et al. 1970, Goodman et al. 1973) and that exchange of cholesterol between pools 2 and 3 is

conducted only through pool l (Goodman et al. 1973). In the three pool model, all de novo cholesterol synthesis is therefore assumed to take place in the tissues of the M1 central pool.

The deuterium incorporation methodology states that cholesterol FSR may be validly determined through measurement of deuterium enrichment of plasma free cholesterol and plasma water contained within the M1 pool, based on Goodman's three pool model and the following rationale. Upon administration of the priming bolus deuterium oxide dose, deuterium oxide equilibrates with the extracellular plasma water pool. After rapid migration across all cellular membranes, deuterium atoms of deuterium oxide equilibrate with the protium atoms of intracellular water pools, present within cholesterol synthetic tissues (Dietschy et al. 1984), mainly the liver and intestine of the M1 pool, which synthesize approximately 90% of total body cholesterol (Sodhi et al. 1979). In subsequent cellular de novo cholesterol synthesis, constituent hydrogen atoms are drawn from the stable isotope labelled intracellular water pool, and deuterium isotopes, rather than protium isotopes, are permanently incorporated into the molecular structure. Unesterified, deuterium labelled, de novo synthesized cellular cholesterol molecules, and those incorporated into lipoproteins, exchange rapidly with other plasma lipoproteins, and quickly equilibrate with the extracellular plasma free cholesterol component of the M1 pool (Norum et al. 1983). Therefore, applying the three assumptions of Goodman's three-pool model (Goodman et al. 1973), with the additional assumption that movement of free cholesterol to and from the M2 and M3 side pools will be slow (Jones et al. 1988), due to the slow rates of equilibration of these pools with plasma cholesterol (M1 pool) (Goodman et al. 1973), the fraction of the total M1 pool synthesized de novo in a day (cholesterol FSR), may be calculated from the free cholesterol and water deuterium enrichment of a sample of plasma from the M1 pool.

2.15 SUMMARY

At present, the factors which interact with the genetically abnormal E2 apolipoprotein to precipitate the clinical manifestations of type III HLP are speculative. It is suggested that environmental factors, such as an increase in the rate of de novo cholesterol synthesis,

superimposed on this genetic background, may be responsible. Recent methodological advances have made available the non-hazardous technique of deuterium incorporation for short-term measurement of plasma cholesterol synthetic rate. It is anticipated that analysis of cholesterol synthetic rate in both apo E2 type III HLP and non-hyperlipidemic individuals, and comparison of these values determined in both the fed and fasted state, will provide information contributing to elucidation of mechanisms responsible for elevated cholesterol levels observed in type III HLP.

3. METHODS

3.1 EXPERIMENTAL DESIGN

3.1.1 Phase I: Initial Subject Screening

Two subject groups, test (n=8) and control (n=8), were identified. The test group consisted of hyperlipidemic individuals with the apo E2/2 or E3/2 phenotype, diagnosed with type III HLP (type III HLPs). The control group consisted of non-hyperlipidemic individuals with the apo E2/2 or E3/2 phenotype, without type III HLP disease and one non-hyperlipidemic type III HLP individual (controls).

The type III HLP test subjects were recruited from the University Hospital, Shaughnessy Site Lipid Disorders Clinic. Over 300 patient charts were reviewed to identify potential test subjects. Contact with 53 selected patients was initiated by letter (Appendix One). Forty replies were returned by mail, and 30 interested respondents were invited by phone to a follow-up informational meeting. Eighteen individuals attended the meeting, and 8 of the most appropriate were selected for the experimental trial. The following selection criteria were used, based on information obtained from patient charts and a completed subject information sheet (Appendix Two): (i) male or female, (ii) 35-75 years of age, (iii) non-obese, defined as weight not greater than 20% above ideal weight (Burtis et al. 1988), (iv) free of restrictive food allergies or diet limitations, (v) easy access to UBC campus, (vi) free of chronic ailments, (vii) diagnosed with type III HLP, (viii) willingness to discontinue use of lipid lowering drug treatment or any other medication 4 weeks before and during the experimental trial (Grundy 1978, Carlson 1988, Kuo 1988).

Few type III HLP subjects met all of the above criteria. Thus selection was modified to include subjects with the following less desirable characteristics: (i) obesity (NN, ES, MKu, DP), (ii) taking medication for high blood pressure (WJ, EK) and hypothyroidism (MKu) (maintained during the experimental trial) and (iii) heterozygous apo E2 phenotype (JB, AT, DP, EK).

The normolipidemic control subjects were recruited in 4 ways. Firstly, one apo E2 homozygote previously identified at the Lipid Disorders Clinic with type III HLP, yet maintaining

normal lipid levels through diet control, was asked to participate. Secondly, one subject previously identified as an apo E2 homozygote in studies at the University of British Columbia, was contacted, rescreened, and asked to participate. Thirdly, six first degree relatives of type III HLP patients were contacted and four were screened. Fourthly, to match the controls with the type III HLPs for age and sex, advertisements for study volunteers were placed at local senior citizens' centers. Over 70 respondents were prescreened by phone, and 32 were selected for screening. Six more of the most appropriate subjects screened were selected as control subjects for the experimental trial, based on information obtained from laboratory results and a completed subject information sheet (Appendix Three), against the following criteria: (i) male or female, (ii) 35-75 years of age, (iii) homozygous for the apo E2 phenotype, (iv) fasting plasma total cholesterol levels between 2.80-6.35 mmol/l (110-245 mg/dl) and triglyceride levels between 0.79-2.26 mmol/l (70-200 mg/dl) (Hoeg et al. 1987, Brown et al. 1987), (v) no previous history of raised plasma cholesterol or triglyceride levels, (vi) non-obese, (vii) willingness to discontinue use of any medication 4 weeks before and during the experimental trial, (viii) free of reported chronic ailments, (ix) free of reported restrictive food allergies or diet limitations, and (x) easy access to the UBC campus.

Due to the relatively rare occurrence of apo E2/2 homozygotes in the population (1-2%) (Brown et al. 1983b), apo E3/2 heterozygous individuals, which comprise approximately 10% of the population (Lenzen 1986) were necessarily included in the control group (JG, DD, EM, RS, HD, MW). Additionally, 2 subjects taking hypothyroid medication (JG, MW) (maintained during the experimental trial), and one obese subject (MW), were included, due to the difficulty of age-sex matching the elderly type III HLP subjects with elderly individuals who are free of all medications and at normal body weight.

3.1.2 Phase II: Experimental Trial

Subjects were divided into groups of 3 or 4. Each group underwent a 3 day experimental trial. Two weeks before commencement of the trial, subjects met at the UBC Human Nutrition

Metabolic Laboratory, for orientation. At this time, height, weight and daily activity information was obtained, consent forms (Appendix Four) were signed, and a description of the research project protocol (Appendix Five) and food record instructions (Appendix Six) were given.

Subjects recorded intake of all foods and beverages consumed for seven days preceding the trial. Subjects were instructed to determine portion sizes from household measuring devices such as measuring cups, spoons, and scales, as well as recording product package weights. Recipes were included when appropriate and available. During the trial, food records were reviewed with subjects, and any clarification needed regarding brands, types, portions, preparation methods or omissions of foods, was obtained.

Three food record days (Thurs., Fri., and Sat.) (Stuff et al. 1983) were selected for determination of carbohydrate (CH0), protein, total fat, alcohol, energy, and polyunsaturated:saturated fat (P:S) intake, as an estimate of habitual dietary consumption. Computerized nutritional analysis was carried out using the PC Nutricom V2.0 program (1986, Smart Engineering Ltd., Vancouver, B.C.). Foods were entered into the program using Canadian Nutrient File food descriptions and Nutrition Canada food codes (Canadian Nutrient File, Food Name Subfile, 1986, Department of National Health and Welfare). Data for cholesterol, polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) not available in Nutricom for some foods entered, were obtained from food composition tables (Pennington et al. 1985, Nutrient Value of Some Common Foods, 1987).

The diet fed to subjects during the experimental trial was intended to approximate the average North American consumption of 40%, 45% and 15% total energy intake from fat, CHO and protein respectively, 200 mg cholesterol/1000 kcal, and a P:S ratio of 0.4 (US Dept of Health and Human Services, 1986). A standard 3000 kcal diet consisting of isocaloric breakfast, lunch and dinner meals, previously designed by a research dietitian using data from the Canadian nutrient file (Verdier et al. 1984), was modified slightly in the present study (Appendix Seven) for practicality of packaging meals for take-out eating. The modified trial diet consisted of 40% fat, 45% CHO, 15% protein, 220 mg cholesterol/1000 kcal and a P:S ratio of 0.7, as computed on PC

Nutricom. The caloric value of the diet was adjusted to meet the estimated energy requirements determined for each subject, by multiplication of each food item by the appropriate conversion factor (estimated energy need (kcal)/3000 kcal).

Subjects' daily caloric requirements were estimated (Appendix Eight) with the aid of the Mayo Clinic Nomogram (Mayo Clinic Diet Manual, 1961). Body surface area (m^2) was first determined from the nomogram using ideal weight and height. The nomogram was then used to obtain basal metabolic rate (BMR) (kcal/24 hrs) from body surface area, age and sex. Individual daily energy requirements (kcal/24 hrs) were then calculated by multiplying BMR by an activity factor of 1.5 or 1.7, depending on subject reported activity (Bogert et al. 1973, Bell et al. 1985).

Non-perishable menu items were bought in bulk and frozen or stored, while perishable items were purchased from a local grocer before each experimental trial. Careful weighing of food items, meal preparation and cooking of meals was carried out before each trial in the UBC Human Nutrition Metabolic Kitchen. Breakfast and dinner meals were served in the Metabolic Kitchen, while the lunch meal was packaged for take-out.

On day 0, the day before the experimental trial began, subjects were asked to fast after consuming the evening meal. At 7:00 am on day 1 of the trial, subjects reported to the Division of Human Nutrition Metabolic Laboratory, UBC, when the first 28 ml blood sample was drawn for determination of baseline plasma water and cholesterol deuterium enrichment. All blood samples taken during the experimental trial were drawn by a registered laboratory technician. At 7:30 am, a "priming" bolus dose of deuterium oxide (99.9 atom % excess deuterium (A%E)), MSD Isotopes, Montreal, Canada and Cambridge Isotope Labs, Woburn, Mass.) was administered orally to each subject at a level of approximately 0.7 g D_2O/kg estimated total body water (Appendix Nine), followed by 50 ml distilled H_2O as a rinse. Individual total body water determinations were calculated based on a body water content estimation of 60% of body weight (Gamble et al. 1954). At 8:00 am, (hr 0 post bolus D_2O dose), timing of four 12 hr intervals began (12, 24, 36, and 48 hr post bolus D_2O dose). A 28 ml blood sample was drawn at each interval. Drinking water labelled at approximately 1.4 and 0.7 g D_2O/l H_2O was given on day 1 and day 2,

respectively, to maintain deuterium body water enrichment (Appendix Nine). The amount of deuterium label in the drinking water on the feeding day was double that on the fasting day to compensate for dilution of deuterium body water enrichment by the unlabelled water consumed in the experimental diet.

At hr 48, additional 28 ml blood samples were drawn from the type III HLPs for apo E re-phenotyping. This was done to validate the phenotype determined previously for these individuals by the University Hospital, Shaughnessy Site Lipid Clinic, using the same method. Additional 28 ml blood samples were also drawn from 6 subjects for thyroxine (T4) and thyroid stimulating hormone (TSH) assays, in order to determine thyroid status at the time of the experimental trial. Thyroid status tests were not done on all subjects as 3 experimental trials had been completed before the decision to acquire this data was made.

Throughout the 3 day trial, subjects consumed only the test meals provided, and were weighed daily. At 8:00 am on day 1 (feeding day) subjects were fed the experimental trial breakfast meal. Subjects were given the packaged lunch meal and permitted to leave the Metabolic Laboratory until 7:00 pm, at which time they returned and consumed the dinner meal. Subjects were instructed to eat all of the food provided at each meal. Subjects reported to the Metabolic Laboratory at 8:00 am on day 2 (fasting day), and remained there under supervision, involved in various quiet activities, until 8:00 pm. Subjects consumed no food on day 2, although calorie free, caffeine free soft drinks were permitted. On the final day of the study, day 3, subjects returned to the metabolic unit at 8:00 am after 36 hours of fasting.

3.2 ANALYTICAL PROCEDURES

3.2.1 Phase I: Initial Subject Screening

A single 12 hr fasting 28 ml blood sample was drawn from each subject screened into test tubes containing EDTA (Becton Dickinson, Miss., Ont.) by a registered laboratory technician at the Department of Laboratory Medicine, University Hospital, UBC campus. Blood samples were

stored for less than 4 hrs at 4 °C before plasma was obtained by centrifugation for 15 min at 3000 rpm. Plasma samples were stored at -20 °C. Total cholesterol and triglyceride level analyses were carried out for each subject at the University Hospital, Shaughnessy Site Lipid Clinic. All analyses were performed on the automated Technicon RA-500 (Technicon Instruments, Corp., Tarrytown, NY). Plasma samples (300-400 μl) were assayed for single determination of total cholesterol and triglyceride concentration. Samples were assayed in batches of approximately 30 with two controls (Technicon Diagnostics Testpoint Assayed Chemistry Controls 1 and 2, Technicon Instruments Corp., Tarrytown, NY) run at the beginning and end of each batch assay. Within-run variability reported for cholesterol assays performed on this machine approximates 1.6%.

An enzymatic colorimetric method (Cholesterol C-system, Boehringer Mannheim, Montreal, Quebec) was used for the determination of total plasma cholesterol. Free cholesterol was liberated from cholesterol esters by cholesterol esterase. Delta-4-cholestenone and hydrogen peroxide was then produced from the oxidation of free cholesterol by cholesterol oxidase. Hydrogen peroxide, 4-aminophenazone and phenol, in the presence of peroxidase, combined to form a chromagen, 4-phenazone, which absorbed maximally at 500 nm. The concentration of total cholesterol in the sample was directly proportional to the intensity of the color produced (Katterman et al. 1984).

Triglycerides were assayed using the Technicon RA-500 systems triglycerides enzymatic method. Plasma triglycerides were hydrolyzed to glycerol and free fatty acids by LPL. Glycerol-3-phosphate was formed by glycerol kinase and ATP. Hydrogen peroxide was then produced by reaction with glycerol phosphate-oxidase. The chromagen produced by the reaction of hydrogen peroxide with 4-chlorophenol and 4-aminoantipyrine in the presence of peroxidase, had maximal absorbance at 500 nm. The triglyceride concentration of the sample was directly proportional to the intensity of the color produced (Fossati et al. 1982).

The apo E phenotype of each subject screened was determined by isoelectric focusing on a single polyacrylamide cylindrical gels, according to the methods of Bouthillier et al. (1983) and

Warnick et al. (1979). Density gradient preparative ultracentrifugation of 10-12 ml plasma was used to separate out the VLDL fraction in each sample. Duplicate samples for each subject of 5-6 ml plasma were injected below 3.0 ml of 0.15 mole/l NaCl into a 10 ml ultracentrifuge tube, using a 21 gauge 1 1/2" needle. The tube was filled to a final volume of 9.0 ml by carefully layering saline on top. VLDL was isolated by ultracentrifugation in a Beckman L3-50 ultracentrifuge with type 50 rotor (Beckman Instruments, Palo Alto, CA) at 16 ^{o}C for 18-20 hrs at 40,000 rpm. The duplicate VLDL samples for each subject were removed by pipetting off the top milky layer and pooled for a second wash in a third ultracentrifuge tube, with final volume adjusted to 9.0 ml using 0.15 M/l saline. The VLDL was then recentrifuged under the same conditions. The washed VLDL was recovered by pipetting off the top 3 ml into a 12 ml culture tube and freezing at -20 ^{o}C until delipidation. Delipidation of VLDL was carried out by dropwise transfer of VLDL into 10 ml of acetone:ethanol (1:1 v/v). The mixture was stored for 4 hrs or overnight at -20 ^{o}C, after which the protein was sedimented by centrifugation for 15 min at 3000 rpm at 4 ^{o}C. The solvent was discarded, the protein pellet resuspended in another 10 ml of ethanol:acetone by thorough vortexing, and the mixture was stored for 2 hrs at -20 ^{o}C. After centrifugation at 3000 rpm for 15 min, the solvent was discarded and the protein pellet resuspended in 5 ml of cold diethyl ether by thorough vortexing and stored for 1 hr at -20 ^{o}C. The solvent was discarded after a final centrifugation at 3000 rpm for 15 min and the protein dried under nitrogen gas (N_2) before storage at -20 ^{o}C.

Single polyacrylamide gel isoelectric focusing was carried out at the University Hospital, Shaughnessy Site Lipid Clinic. Polyacrylamide gel solution for 10 samples, including one previously phenotyped control sample, was prepared for each focusing experiment by combining 1.50 g acrylamide (electrophoresis purity, BDH, Toronto, Ont.), 40 mg BIS, 9.6 g urea, and 1.0 ml carrier ampholytes, pH 4-6 (LKB Bromma, Fisher Scientific, Toronto, Ont.) made up to a final volume of 20 ml with distilled water in a graduated cylinder. The polymerization reaction was activated by the addition of 10 μl TEMED and 40 μl ammonium persulfate. The gel solution was quickly pipetted into gel tubes sealed with parafilm at one end, to a level of 8.0 cm. To form a

smooth interface, the gel solution was overlaid with 1 drop of distilled H_2O and left to polymerize for 1/2 hr. 200μl of a solubilization buffer consisting of 2.4 g urea, 16 mg DTT and 5.0 ml of 10 mM Tris-HCl was added to each dried protein sample, vortexed thoroughly and stored at 4 ^{o}C for 1.5 hrs. Meanwhile the polymerized gel tubes were loaded into the electrophoresis chamber (Model 150 A, Bio-Rad Laboratories) containing a 10 mM orthophosphoric acid buffer in the lower anode reservoir and 20 mM NaOH buffer in the upper cathode reservoir. After 1 hr pre-focusing at 110 V, the upper reservoir buffer was discarded, the protein samples were loaded carefully into the tube gels and overlaid with 200μl of sample overlay buffer containing 1.0 ml solubilization buffer, 1.0 ml distilled H_2O and 20μl ampholytes, pH 4-6. The upper cathode reservoir was refilled with 20 mM NaOH buffer and the samples were focused for 16 hrs at 250 V, followed by an additional hr at 450 V. The focused gel tubes were removed from the electrophoresis chamber and the lower end of each gel (positive [anode], pH 6) was identified by inserting a needle containing Indian Ink 0.5 cm into the gel. The gels were removed from the gel tubes by a 22 gauge spinal needle with water spray into screw cap tubes containing 10 ml of Coomassie Brilliant Blue G250 staining solution (Bio-Rad Laboratories) and rotated end-over-end for 2 hrs. The gels were destained in 7.5% glacial acetic acid in distilled H_2O by rotation for 48-72 hrs and stored in fresh 7.5% glacial acetic acid in corked vials.

Individual apo E phenotypes were determined from visual identification of the relative proportions of apo E isoforms present against a known phenotype control sample, as per Bouthillier et al. (1983). The relative densities of the banding patterns were also inspected visually, in order to identify possible minor, weaker staining bands arising from sialic acid posttranslational glycosylation of the major apo E3, E4, and E2 isoforms, which may confound true phenotypic identity. This interpretation was supplemented by a quantitative determination of the E3/2 ratio by densitometric scan at 595 nm (Appraise, Beckman Instruments, Palo Alto, CA). Ratios less than 0.5, 0.5-1.2, and greater than 1.2 defined apo E2/2 homozygotes, apo E3/2 heterozygotes, and apo E3/3 homozygotes, respectively.

3.2.2 Phase II: Experimental Trial

The 28 ml blood samples taken during the experimental trial, were drawn into EDTA containing tubes. Plasma was obtained immediately by centrifugation of blood at 3000 rpm for 15 min at 4 ^{o}C. Each plasma sample was aliquoted for triplicate determination of free cholesterol deuterium enrichment and duplicate determination of plasma water deuterium enrichment (Jones et al. 1988).

A preliminary recovery study was carried out to determine the efficiency of the total lipid extraction and thin layer chromatography (TLC) procedure. The recovery of ^{14}C-labelled cholesterol was measured after direct addition of ^{14}C-labelled cholesterol solution to scintillation vials for counting, and addition to plasma for complete total lipid extraction and TLC plating and removal from silica. A 35.5% (CV = 8.8%) loss of ^{14}C-label was observed for the procedure. This was considered acceptable as the final analysis of cholesterol synthesis is based on a relative rather than absolute quantitative measure of deuterium present in the cholesterol molecule.

Total plasma lipids were extracted by combining 4 ml plasma, 8 ml methanol and heating for 15 min at 55 ^{o}C. Hexane-chloroform (4:1 vol/vol) was added, the mixture shaken for 15 min, combined with 2 ml H_2O, and shaken again for 10 min. Following centrifugation at 3000 rpm for 10 minutes, the upper hexane-chloroform/lipid phase was removed and the extract dried under N_2. Another 24 ml aliquot of hexane-chloroform (4:1, v/v) was added to the lower methanol/protein phase, the mixture was shaken for 15 min, then centrifuged for 15 min. The upper phase was removed, pooled with the first lipid extract and dried under N_2.

In preparation for separation of the total lipid extract components by TLC, the dried extract was dissolved in approximately 500μl of chloroform. The TLC plates (silica gel 60 A, 20 cm X 20 cm, 250 um, Whatman Inc., Clifton, N.J.) were activated by heating at 100 ^{o}C for 30 min and the lipid extract applied, 3 samples per plate, 2.5 cm from the bottom of the plate. Plates were run in a solvent of petroleum ether-ethyl ether-acetic acid (135:15:1.5, v/v/v) for 1-2 hrs. After drying, individual lipid bands were visualized by developing in an iodine vapor tank for approximately 5 min.

The free cholesterol bands were scraped from the TLC plates after identification against a free cholesterol control (Sigma Chemical Co.) dissolved in chloroform and a lipid standard (Supelco, Toronto, Ontario) spotted on either side of the plate. To remove the free cholesterol from the silica gel, 6 ml hexane-chloroform-ether (5:2:1, v/v/v) was added, shaken for 10 min, centrifuged for 10 min at 3000 rpm, and the free cholesterol containing solvent removed. Another 4 ml hexane-chloroform-ether was added to the silica gel, shaken for 10 min, centrifuged for 10 min, the solvent pooled with the first free cholesterol extract, and dried under N_2.

Streaking of the origin of the total lipid application into the separated individual lipid component bands was observed in some of the initial TLC samples run from type III HLP subjects. This streaking was thought to be the result of sample overload, due to the elevated plasma lipid levels observed in these individuals. Approximately 65% of the dried total lipid extract was plated for the remaining type III HLP samples, and streaking of the origin was eliminated. In samples where TLC streaking had been observed, the free cholesterol extract was replated and reextracted from the silica gel to reduce potential lipid contamination.

Free cholesterol extracts were then prepared for combustion by solubilization in a few drops of chloroform, and transfer to 15 cm x 6 mm lengths of vycor tubing (Corning Glass Works, Corning, NY) containing 0.5 g copper oxide (BDH) and a 2.5 cm x 1 mm length of sterling silver wire. The sample was frozen in liquid N_2 (LN_2) and the chloroform solvent boiled off under vacuum by gentle warming with a hair dryer. The combustion tube was evacuated and sealed under vacuum with a gas/oxygen torch.

The above procedures were carried out in duplicate for each subject for triglycerides also. Triglyceride extracts in combustion tubes were not processed beyond this point for combustion, reduction and analysis by isotope ratio mass spectrometry, due to time constraints.

Combustion, reduction and analysis of cholesterol deuterium enrichment by isotope ratio mass spectrometry was conducted by the Stable Isotope Laboratory, University of Chicago, Dr. Dale Schoeller, Director, for all samples except one subject. The free cholesterol extracts were combusted at 725 ^{o}C for 2 hrs to water and carbon dioxide (CO_2). The CO_2 was removed and the

combustion water transferred to quartz reduction tubes (Corning Glass Works) containing 60 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington, Indiana) via vacuum distillation. The combustion water was reduced to hydrogen gas by heating the reduction tubes at 500 ^{o}C for 30 min (Schoeller et al. 1980). The ratio of deuterium:protium isotopes present in the sample was analyzed by the direct introduction of the reduction tube into a differential isotope ratio mass spectrometer (Nuclide 3-60 H/D; MAAS, Bellefonte, Pa.). Deuterium enrichment of free cholesterol was calculated against two standards using electrical H^{3+} compensation. (Schoeller et al. 1983). The above steps were carried out for one subject within the Division of Human Nutrition at the University of British Columbia.

Analysis of plasma samples for plasma water deuterium enrichment by isotope ratio mass spectrometry was carried out, within the Division of Human Nutrition at the University of British Columbia. Plasma samples from one time point (hr 24) were diluted 1:5 with Vancouver tap water (200μl plasma:1000μl water) to obtain a sample deuterium enrichment comparable to that of the standards. Duplicate samples were transferred in 2μl capillary tubes (Drummond Microcaps, Fisher Scientific, Montreal, Quebec) by direct addition to 15 cm x 6 mm pyrex reduction tubes (Corning Glass Works) containing 60 mg zinc reagent. Reduction tubes were evacuated until pressure returned to baseline, and sealed with a gas/oxygen torch. Plasma water contained within the sample was reduced to hydrogen gas after heating at 510 ^{o}C for 30 min. The ratio of deuterium:protium isotopes present in the sample was analyzed by the direct introduction of the reduction tube into a differential isotope ratio mass spectrometer (VG Isogas 903D, Cheshire, England). Deuterium enrichment of plasma water was calculated against two standards using electrical H^{3+} compensation.

Apo E re-phenotyping of the type III HLP subjects was carried by single polyacrylamide gel electrophoresis at the University Hospital, Shaughnessy Site Lipid Clinic.

Assays for T4 and TSH were carried out on 6 subjects by the Department of Pathology and Laboratory Medicine, University Hospital, Shaughnessy Site.

3.3 FREE CHOLESTEROL FRACTIONAL SYNTHETIC RATE AND NET SYNTHESIS CALCULATIONS

Free cholesterol FSR was determined for four successive 12 hr intervals during the experimental trial, based on the methods of Dietschy and Spady (1984) and Jones et al. (1988), with several inclusive assumptions.

Firstly, it was assumed that the same number of deuterium atoms were incorporated in the de novo synthesis of all cholesterol molecules, in all cholesterol synthetic tissues, independent of metabolic state.

Secondly, the absolute value of the ratio of the deuterium (^2H) to carbon (C) atoms incorporated into the cholesterol molecule was applied to obtain an absolute rate of cholesterol synthesis. One molecule of cholesterol contains 27 carbon atoms and 46 hydrogen atoms. The latter are derived from water and may be introduced into cholesterol from one of three sources: directly from water itself, from NADPH, or from acetyl CoA units containing water H atoms. The second assumption, therefore, was that deuterium atoms incorporated into de novo synthesized cholesterol molecules in place of the constituent protium atoms, were derived from water and NADPH which had fully equilibrated with deuterated water, while the deuterium label was not incorporated into acetyl CoA. In theory, this represents 22 atoms of deuterium for each cholesterol molecule, resulting in a $^2H/C$ incorporation ratio of 0.81 (Dietschy and Spady 1984).

The daily central pool free cholesterol fractional synthetic rate, that fraction of free cholesterol in the M1 cholesterol pool synthesized in 24 hours, was computed using the equation (Jones et al. 1988):

$$\text{FSR (per day)} = \frac{\delta_{\text{cholesterol}}\ (^0/_{00})}{\delta_{\text{plasma water}}\ (^0/_{00}) \times 0.81\ ^2H/C \times 27C/46H}$$

where $\delta_{cholesterol}$ was the change in cholesterol deuterium enrichment over the 24 hr interval and

$\delta_{plasma\ water}$ was the corrected plasma water deuterium enrichment at hr 24 post bolus deuterium oxide dose, stated as parts per thousand (‰) versus standard mean ocean water (SMOW). Parts per thousand were defined as:

$$\text{parts per thousand} = \delta = \frac{\frac{^2H}{^1H}_{sample} - \frac{^2H}{^1H}_{standard}}{\frac{^2H}{^1H}_{standard}} \times 1000\ (‰)$$

The absolute C/H ratio of the cholesterol molecule was corrected for by multiplying by 27C/46H.

Individual free cholesterol net synthesis (g/day) was calculated based on the individual size of the M1 total cholesterol pool (g) in control and type III HLP subjects. The size of the M1 total cholesterol pool was estimated based on the equation derived by Goodman et al. (1980), where:

M1 pool (grams) = [0.287 x Wt. (kg)] + [0.0358 x total cholesterol (mg/dl)] - [2.40 x TGGP] - 1.72

where TGGP is a variable equal to 1, 2 or 3, corresponding to serum triglyceride levels of less than 200, 200-300 or greater than 300 mg/dl, respectively.

Individual free cholesterol net synthesis was then calculated for each subject using the equation:

Free cholesterol net synthesis (g/day) = size of M1 total cholesterol pool (g) x free cholesterol FSR corresponding to time interval during experimental trial x 0.4.

The factor 0.4 was used as it is estimated that approximately 40% of the M1 total cholesterol pool is free cholesterol (Jones et al. 1988).

3.4 STATISTICS

Results were tabulated and analyzed (with the exception of Q-tests) utilizing the Systat PC software package (Systat, Inc., Evanston, IL, 1989). P values <0.05 were considered to indicate a significant difference.

Subject triplicate values for plasma free cholesterol deuterium enrichment for each time interval during the experimental trial were treated with the Q-test for the elimination of possible outlying results (Eckschlager 1969).

Independent Student's *t*-tests were performed to determine the significance of differences in the means for the plasma lipid profile, anthropometric data, usual dietary intakes, total cholesterol levels during the experimental trial, body weight fluctuations during the experimental trial, and M1 total cholesterol pool size, for control and type III HLP subjects.

Paired Student's *t*-tests were used to determine the significance of differences in the means within subject groups for estimated daily caloric requirements and usual mean energy consumption per day (determined by 3 day food records), and for total cholesterol determined during screening and total cholesterol averaged over the three days of the experimental trial.

Apo E phenotype and plasma lipid concentration results for subjects screened for the control group were analyzed by one-way ANOVAs.

Cholesterol FSR and cholesterol net synthesis based on M1 cholesterol pool size results were analyzed by two-way repeated measures analysis of variance (RANOVA). The two independent variables analyzed were group and feeding state. Each variable was comprised of two levels: control versus type III HLP subjects and fed state versus fasted state, respectively (Keppel 1973). A comparison of simple main effects for cholesterol FSR in both the fed and fasted state, to investigate the significant interaction observed between group and feeding state, was carried out by independent Student's *t*-test (Huck et al. 1974).

4. RESULTS

4.1 PHASE I: INITIAL SUBJECT SCREENING

Apo E phenotype determined by polyacrylamide gel isoelectric focusing, plasma lipid concentration, age and sex results from subjects screened for the control group are listed in Appendix Ten and summarized in Table 1. Four phenotypic groups consisting of both males (n = 18) and females (n = 18) were identified, with 63.9 % of the subjects possessing the E3/2 phenotype. One-way ANOVA demonstrated that mean (±SEM) total cholesterol and mean (±SEM) triglyceride concentration were not significantly different among groups, analyzed as E4/3, E3/3, and E3/2 plus E2/2, due to the single subject comprising the E2/2 group. Highest total cholesterol levels were observed in the E3/2 group (5.7 ± 0.2 mmol/l) (n = 23), followed by the E4/3 group (5.1 ± 0.3 mmol/l) (n = 9). Lowest cholesterol levels were observed in the E2/2 group (4.1 mmol/l) (n = 1). Highest triglyceride levels were observed in the E4/3 group (1.7 ± 0.4 mmol/l), followed closely by the E3/2 group (1.6 ± 0.1 mmol/l). Lowest triglyceride levels were observed in the E3/3 group (1.1 ± 0.3 mmol/l). Mean (±SEM) and median ages of all subjects screened were 51.7 ± 2.4 years and 54.5 years, respectively. Subjects ages ranged from 26-73 years. Mean age was not significantly different among groups. The highest median age, 60.0 years, was found for the E3/2 group.

Table 2 displays the apo E and plasma lipid profile of the 8 most appropriate apo E2/2 and E3/2, non-hyperlipidemic subjects selected for the experimental trial from those screened for the control group (controls), and their 8 age-sex matched apo E2/2 and E3/2 test subjects, diagnosed with type III hyperlipoproteinemia (type III HLPs). The control group consisted of 6 males and 2 females, 2 apo E2/2 homozygotes and 6 apo E3/2 heterozygotes, with an average apo E3/2 ratio of 0.92 ± 0.03. Subject MKa was selected for the control group as he is an apo E2/2 homozygote who was previously identified at the University Hospital, Shaughnessy Site Lipid Disorders Clinic with type III HLP, yet he was maintaining normal lipid levels through diet control. The average (±SEM) age for this group was 56.0 ± 5.0 years, with a range of 35-73 years.

Table 1 Summary of apolipoprotein E phenotype and plasma lipid concentration results in subjects screened for control group.

Phenotype Group	Sex Ratio	Frequency	Relative Frequency	Mean Total Cholesterol	Mean Triglyceride	Age Mean	Age Median
	(M:F)		(%)	(mmol/l)	(mmol/l)	(yrs)	
E 2/2	1:0	1	2.8	4.1	1.2	35.0	35.0
E 3/2	12:11	23	63.9	5.7 (0.2)[1]	1.6 (0.1)	54.7 (3.1)	60.0
E 3/3	2:1	3	8.3	4.9 (0.3)	1.1 (0.3)	43.7 (9.3)	37.0
E 4/3	3:6	9	25.0	5.1 (0.3)	1.7 (0.4)	48.7 (3.8)	42.0
				$F=1.19^2$ $p=0.32$	$F=0.69$ $p=0.51$	$F=0.96$ $p=0.40$	
All groups	18:18	36	-	5.4 (0.2)	1.6 (0.1)	51.7 (2.4)	54.5

[1](±SEM); [2]One way ANOVA F ratio, where groups were E2/2 + E3/2, E3/3, and E4/3

Table 2 Apolipoprotein E and plasma lipid profile of age-sex matched control and type III HLP subjects selected for experimental trial.

Subject by Group	Sex	Apo E Phenotype	Age	E3:2 Ratio	Total[1] Cholesterol	Triglyceride[1]
			(years)		(mmol/l)	
Controls:						
CD	M	2/2	35	-	4.12	1.19
MKa	M	2/2	38	-	4.86	2.29
JG	M	3/2	68	0.91	4.89	1.04
DD	M	3/2	53	0.83	5.66	1.08
EM	M	3/2	62	0.97	6.07	1.38
RS	M	3/2	67	1.03	4.87	2.11
HD	F	3/2	52	0.97	6.35	1.11
MW	F	3/2	73	0.82	4.76	2.25
Mean	(6:2)	(2:6)	56.0	0.92	5.20	1.56
±SEM	(M:F)	(E2/2:E3/2)	5.0	0.03	0.27	0.20
Type III HLPs:						
NN	M	2/2	44	-	5.55	3.34
WJ	M	2/2	48	-	7.27	2.57
ES	M	2/2	74	-	6.02	3.15
JB	M	3/2	54	0.70	8.63	8.80
AT	M	3/2	64	0.81	7.73	3.10
MKu	F	2/2	65	-	12.49	4.55
DP	F	3/2	51	0.93	8.98	4.73
EK	F	3/2	71	0.81	4.09	3.27
Mean	(5:3)	(4:4)	58.9	0.81	7.60	4.19
±SEM	(M:F)	(E2/2:E3/2)	3.9	0.05	0.91	0.71
			p=0.66[2]	p=0.09	p=0.02	p=0.003
All Subjects:						
Mean	(11:5)	(6:10)	57.4	0.87	6.40	2.87
±SEM	(M:F)	(E2/2:E3/2)	3.1	0.03	0.55	0.49

[1]controls=screening values, type III HLPs=most recent values prior to study obtained from patient charts; [2]independent *t*-test

Mean (±SEM) total cholesterol and triglyceride levels were 5.20 ± 0.27 mmol/l and 1.56 ± 0.20 mmol/l, respectively for the control group.

The type III HLP group consisted of 5 males and 3 females, 4 apo E2/2 homozygotes and 4 apo E 3/2 heterozygotes, with an average apo E3/2 ratio of 0.81 ± 0.05. The average age for this group was 58.9 ± 3.9 years, with a range of 44-74 years. Mean total cholesterol and triglyceride levels were 7.60 ± 0.91 mmol/l and 4.19 ± 0.71 mmol/l, respectively for the type III HLP group. These values were the most recent ones recorded prior to the experimental trial in the patient charts. No significant differences were observed for age or apo E3/2 ratio between the two subject groups. Significant differences ($p < 0.05$) were observed for mean total cholesterol ($p = 0.02$) and triglyceride ($p = 0.003$) levels between the two groups.

The anthropometric, medication and hormonal profile of the control and type III HLP subjects selected for the experimental trial is shown in Table 3. No significant differences were observed between groups for mean height or weight. Wrist circumference was measured to determine frame size (ratio of height [cm]:wrist circumference [cm]) (Burtis et al. 1988) when this information could not be obtained from patient charts. Ideal weight, determined as the mean of ideal weight range from the 1983 Metropolitan Height-Weight Tables, using height and frame size (B.C. Diet Manual 1984), was not significantly different between groups, nor was percent ideal weight. One control subject, MW, was defined as obese, that is weight greater that 20% of normal weight for height, with a body weight 27% greater than her ideal weight. Four type III HLP subjects, NN, MKu, ES, and DP, were defined as obese, with body weights 22%, 25%, 32% and 85% greater than ideal weight.

Table 3 Anthropometric, medicinal and hormonal profile of control and type III HLP subjects selected for experimental trial.

Subject by Group	Wt.	Ht.	Wrist Circ.	Frame Size[1]	Ideal Wt.[2]	% Ideal Wt.	Meds[3]	Thyroid Hormones T4	TSH
	(kg)	(cm)	(cm)		(kg)			(nmol/l)	(mU/l)
Controls:									
CD	83.2	176.5	17.1	medium	73	+14	none	-	-
MKa	79.5	173.4	-	medium	70	+14	none	-	-
JG	78.3	178.4	18.3	medium	73	+7	synthroid	102	-
DD	79.6	172.7	17.8	medium	70	+14	none	124	-
EM	75.5	174.8	18.3	medium	71	+6	none	136	3.0
RS	74.9	184.2	17.8	medium	76	-1	none	115	4.0
HD	57.7	165.1	16.2	medium	62	-7	none	115	-
MW	77.6	162.0	15.3	medium	61	+27	thyroid	153	0.4
Mean	75.8	173.4			69.5	9.3			
±SEM	2.7	2.5			1.9	3.7			
Type III HLPs:									
NN	84.2	177.8	-	medium	69	+22	niacin	-	-
WJ	91.3	182.9	-	large	79	+16	sectral,lopid	-	-
ES	85.7	172.7	-	medium	65	+32	lopid	-	-
JB	84.2	172.0	18.7	large	75	+12	lopid	-	-
AT	84.7	180.3	-	medium	71	+19	lopid	-	-
MKu	71.3	165.1	-	medium	57	+25	thyroxine	-	-
DP	109.3	170.1	-	medium	59	+85	none	-	-
EK	54.9	157.5	-	medium	50	+10	questran,lopid blocadren	-	-
Mean	83.2	172.3			65.6	27.6			
±SEM	5.5	2.9			3.5	8.6			
	p=0.25[5]	p=0.78			p=0.34	p=0.07			
All Subjects:									
Mean	79.5	172.8			67.6	18.4			
±SEM	3.1	1.9			2.0	5.1			

[1]determined from ratio of ht (cm)/wrist circumference (cm) (Burtis et al. 1988) or obtained from patient charts ; [2]mean of ideal weight range, determined from 1983 Metropolitan Height-Weight Tables using height and frame size (B.C. Diet Manual 1984); [3]lipid lowering=niacin, lopid, questran, discontinued 4 weeks prior to and during study. Hypothyroid=synthroid, thyroid, thyroxine. Hypertension=sectral, blocadren; [4]T4=thyroxine, TSH=thyroid stimulating hormone;[5]independent *t*-test

Two control subjects, JG and MW, maintained their medication (synthroid, thyroid) for hypothyroidism during the experimental trial, while the remainder of the control subjects were free of any chronic ailments and medications. One type III HLP subject, MKu, took medication (thyroxine) for hypothyroidism, and two subjects, WJ and EK, took medication (Sectral and Blocadren) for hypertension, during the experimental trial. The lipid lowering medications taken by the type III HLP subjects included niacin, Lopid, and Questran, and were discontinued 4 weeks prior to and during the experimental trial. Two subjects, MKu and DP, were not currently taking lipid lowering medication four weeks prior to the experimental trial, although these subjects had consumed lipid lowering medication prior to this time.

Thyroid hormone assays for T4 and TSH, indicated that subjects who were analyzed for thyroid status were basically euthyroid at the time of the experimental trial (normal T4 levels = 58-148 nmol/l, normal TSH levels = 0.4-3.5 mU/l).

4.2 PHASE II: EXPERIMENTAL TRIAL

Analysis of control and type III HLP subjects' usual dietary intake, as reported by 3 day food records completed just prior to the experimental trial, is summarized in Table 4. The mean intakes (grams) of CHO, protein and total lipid for each subject over the three recording days were multiplied by the physiological fuel values 4 kcal/g, 4 kcal/g and 9 kcal/g, respectively, to obtain the average energy (kcal) intake for these macronutrients. Energy from alcohol consumed was calculated by determining total energy intake for all alcoholic beverages consumed, subtracting any energy contributed by CHO or protein, and averaging over 3 days. Total energy intake was then summed, and the percentage of energy (% kcal) derived from CHO, protein, total lipid and alcohol was calculated. The mean total consumption of cholesterol per day (mg) was converted to mg/1000 kcal, to standardize for differences in total daily energy intakes. The P:S ratio was calculated by dividing grams total polyunsaturated fatty acids by grams total saturated fatty acids consumed.

Table 4 Analysis of control and type III HLP subjects' usual dietary intake as reported by 3 day food records.

Subject by Group	CHO	Protein	Total Lipid	Alcohol	Energy	Cholesterol		P/S[1] Ratio	Apo E Phenotype
		Mean Consumption per Day							
		(% kcal)			(kcal)	(mg)	(mg/1000 kcal)		
Controls:									
CD	47.6	13.0	39.4	0.0	3401.0	413.0	121.4	0.4	2/2
MKa	45.2	17.0	37.8	0.0	2522.0	459.0	182.0	0.4	2/2
JG	52.9	14.6	28.9	3.6	2742.3	191.0	69.6	0.7	3/2
DD	41.1	13.6	39.4	5.9	2441.4	350.0	143.4	0.6	3/2
EM	47.9	19.8	32.3	0.0	2347.6	258.0	109.9	0.6	3/2
RS	59.5	15.4	21.6	3.5	2830.6	467.0	165.0	0.4	3/2
HD	40.7	12.4	43.7	3.2	1563.2	315.0	201.5	0.6	3/2
MW	44.1	10.7	45.2	0.0	2410.2	346.0	143.6	1.0	3/2
Mean	47.4	14.6	36.0	2.0	2532.3	349.9	142.0	0.6	
±SEM	2.2	1.0	2.8	0.8	183.4	33.9	14.9	0.1	
Type III HLPs:									
NN	51.5	14.9	24.6	8.9	2602.2	230.0	88.4	0.7	2/2
WJ	45.1	20.2	26.2	8.5	2057.4	299.0	145.3	0.5	2/2
ES	35.1	16.9	44.1	3.9	2839.1	484.0	170.5	0.4	2/2
JB	50.5	25.0	24.5	0.0	2192.4	467.0	213.0	0.3	3/2
AT	44.4	21.6	30.3	3.7	1983.4	351.0	177.0	0.6	3/2
MKu	29.7	27.3	43.0	0.0	1564.3	264.3	169.0	0.6	2/2
DP	47.5	15.8	36.7	0.0	1389.4	149.0	107.2	0.3	3/2
EK	69.3	16.3	14.4	0.0	1287.4	85.8	66.6	1.2	3/2
Mean	46.6	19.8	30.5	3.1	1989.5	291.3	142.1	0.6	
±SEM	4.2	1.6	3.6	1.4	197.2	49.7	17.7	0.1	
	p=0.88[2]	p=0.02	p=0.24	p=0.50	p=0.06	p=0.35	p=1.00	p=0.88	
All Subjects:									
Mean	47.0	17.2	33.3	2.6	2260.9	320.6	142.1	0.6	
±SEM	2.3	1.1	2.3	0.8	147.8	30.1	11.2	0.1	

[1]polyunsaturated fatty acid/saturated fatty acid;[2]independent *t*-test

Carbohydrate, protein and total lipid consumption for the control subjects accounted for 47.4 ± 2.2 %, 14.6 ± 1.0 % and 36.0 ± 2.8 %, respectively, of mean total energy intake (2532.3 ± 183.4 kcal), with alcohol contributing a final 2.0 ± 0.8 % kcal. Mean cholesterol intake for controls was 142.0 ± 14.9 mg/1000 kcal, while the mean P:S ratio for this group was 0.6 ± 0.1. For the type III HLPs, 46.6 ± 4.2 %, 19.8 ± 1.6 %, 30.5 ± 3.6 %, and 3.1 ± 1.4% of mean total energy intake (1989.5 ± 197.2 kcal) was obtained from CHO, protein, total lipid, and alcohol, respectively. Mean cholesterol intake for type III HLPs was 142.1 ± 17.7 mg/1000 kcal, while the mean P:S ratio for this group was 0.6 ± 0.1. Significant differences between groups for mean intakes of the macronutrients, alcohol and energy, were observed only for protein ($p=0.02$). No significant differences were observed for P:S ratio or cholesterol mean intakes between groups.

Estimated daily caloric requirements for the control (2506.4 ± 117.9 kcal/24 hrs) and type III HLP subjects (2370.6 ± 151.9 kcal/24 hours) administered on the feeding day of the experimental trial were not significantly different (Appendix Eight).

Paired Student's *t*-test revealed no significant difference ($p=0.85$) between mean consumption of energy per day determined from 3 day food records and estimated daily caloric requirements, in the control subjects, and a significant difference ($p=0.044$) in the type III HLP subjects.

Throughout the experimental trial, subjects were weighed daily (Appendix Eleven). Differences in weight between the two groups were not significantly different on each respective day (Appendix 11), nor were weight change differences before and after the feeding day (day 2 - day 1). All subjects lost weight from day 1 to day 3, although total weight change (day 3 - day 1) was significantly different ($p=0.02$) between groups, with type III HLPs losing more.

Table 5 shows the plasma total cholesterol determinations in the control and type III HLP subjects over the experimental trial. Day to day fluctuations in total cholesterol levels were not significantly different between groups, with a coefficient of variation for controls of 2.83 ± 0.54%, and 3.52 ± 0.74% for type III HLPs. However, the total cholesterol level for each group was significantly different on each day, with a three day average in total cholesterol for the controls of

Table 5 Plasma total cholesterol determinations in control and type III HLP subjects during the experimental trial.

Subject by Group	Baseline Day 1 (am) TC	Day 2 (am) TC	Day 3 (am) TC	3 day Mean TC	TC[1] CV	Apo E Phenotype
		(mmol/l)			(%)	
Controls:						
CD	4.36	4.20	3.97	4.18	4.69	2/2
MKa	6.24	6.32	5.89	6.15	3.72	2/2
JG	4.85	4.70	5.13	4.89	4.46	3/2
DD	6.09	5.71	6.03	5.94	3.44	3/2
EM	6.78	6.67	6.90	6.78	1.70	3/2
RS	5.64	5.50	5.65	5.60	1.50	3/2
HD	6.43	6.47	6.48	6.46	0.41	3/2
MW	4.67	4.83	4.93	4.81	2.72	3/2
Mean	5.63	5.55	5.62	5.60	2.83	
±SEM	0.32	0.32	0.33	0.32	0.54	
Type III HLPs:						
NN	5.75	5.88	5.88	5.84	1.29	2/2
WJ	9.69	9.18	9.04	9.30	3.68	2/2
ES	8.48	8.11	7.47	8.02	6.37	2/2
JB	7.87	7.72	7.27	7.62	4.10	3/2
AT	7.20	7.53	8.21	7.65	6.74	3/2
MKu	6.14	6.29	6.40	6.28	2.08	2/2
DP	8.36	8.22	8.03	8.20	2.02	3/2
EK	6.83	6.58	6.70	6.70	1.87	3/2
Mean	7.54	7.44	7.38	7.45	3.52	
±SEM	0.46	0.39	0.37	0.40	0.74	
	p=0.004[2]	p=0.002	p=0.003	p=0.003	p=0.465	

[1]total cholesterol; [2]independent *t*-test

5.60 ± 0.32 mmol/l, and 7.45 ± 0.40 mmol/l for the type III HLPs ($p=0.003$). Paired Student's *t*-tests revealed that when subject Mka is excluded, as his screening value was obtained from patient charts, the mean total cholesterol level for the control group determined during screening is not significantly ($p=0.06$) different from the 3 day average total cholesterol level during the experimental trial. For the type III HLP subjects, the mean screening value for total cholesterol obtained from patient charts as the most recent value prior to the study, and the 3 day average total cholesterol level over the experimental trial, were not significantly different ($p=0.89$).

The deuterium enrichment relative to SMOW of the plasma water (parts per thousand [$^{0}/_{00}$]) during the experimental trial, determined in the blood sample drawn at hour 24 post bolus deuterium oxide dose, is recorded in Appendix Twelve. Baseline measures for natural deuterium levels in plasma water samples were assigned a theoretical value based on Vancouver tap water deuterium enrichment (-100 $^{0}/_{00}$). Experimental determination of these baseline values was not possible due to contamination of the samples.

The corrected plasma water deuterium enrichment values, used in the cholesterol FSR calculation, adjusted for dilution of the plasma samples with Vancouver tap water, and for baseline deuterium enrichment (see sample calculation), are listed.

Plasma free cholesterol deuterium enrichment relative to SMOW of the blood samples drawn from the control and type III HLP subjects during the experimental trial at hrs 0, 24, and 48, stated as parts per thousand ($^{0}/_{00}$), is shown in Appendix Thirteen. A sample calculation for cholesterol FSR is also included. Table 6 displays the cholesterol fractional synthetic rate calculated for each control and type III HLP subject over the two 24 hr time intervals during the experimental trial. The effect of group (controls versus type III HLPs), for all subjects, on cholesterol FSR is illustrated in Figure 3. The summary of the repeated measures analysis of variance (RANOVA) for cholesterol FSR for all subjects, shown in Table 9, indicates no significant difference ($p=0.389$) in the FSR of cholesterol for controls and type III HLPs. The controls tended to have a higher FSR than the type III HLPs in the fed state, while the reverse was observed in the fasted state.

Table 6 Cholesterol fractional synthetic rate in control and type III HLP subjects during experimental trial.

Subject by Group	Fed State 0-24 hr	Fasted State 24-48 hr	0-48 hr	Apo E Phenotype
		(FSR[1] [per day])		
Controls:				
CD	0.051	-0.009	0.021	2/2
Mka	0.070	-0.015	0.028	2/2
JG	0.067	0.021	0.044	3/2
DD	0.069	-0.002	0.034	3/2
EM	0.092	-0.025	0.033	3/2
RS	0.086	-0.003	0.041	3/2
HD	0.079	-0.006	0.037	3/2
MW	0.087	0.024	0.055	3/2
Mean	0.075	-0.002	0.037	
±SEM	0.005	0.006	0.004	
Type III HLPs:				
NN	0.042	-0.001	0.021	2/2
WJ	0.063	-0.008	0.028	2/2
ES	0.050	0.003	0.027	2/2
JB	0.050	0.011	0.031	3/2
AT	0.067	-0.009	0.029	3/2
MKu	0.024	0.003	0.014	2/2
DP	0.037	0.021	0.029	3/2
EK	0.121	0.015	0.068	3/2
Mean (all subjects)	0.057	0.004	0.031	
±SEM	0.010	0.004	0.006	
Mean (no subj. EK)	0.048	0.003	0.026	
±SEM	0.006	0.004	0.002	
	p=0.002[2]	p=0.532	p=0.028	
All Subjects:				
Mean	0.066	0.001	0.034	
±SEM	0.006	0.004	0.003	

[1]Fractional Synthetic Rate; [2]comparison of simple effects by independent student's *t*-test

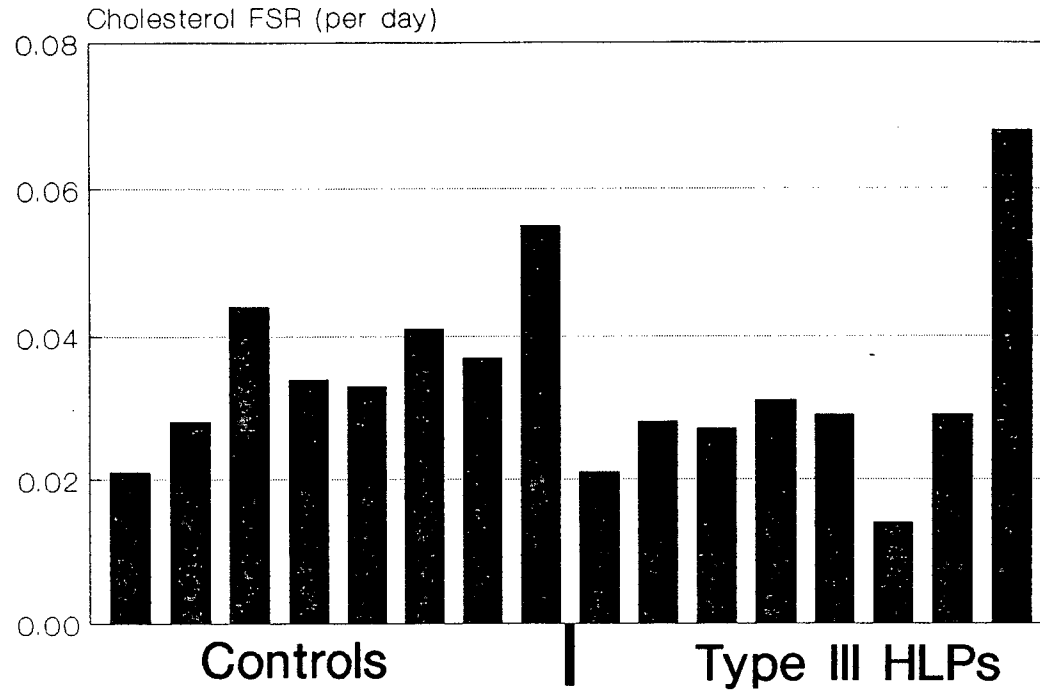


Figure 3 Effect of group (controls versus type III HLPs) on free cholesterol fractional synthetic rate (0-48 hr) for all subjects (p = 0.389).

Evaluation of the individual FSR values in Table 6 revealed however, a strikingly high FSR for one type III HLP subject (EK) during the fed state (0-24 hr) (0.121) and overall (0-48 hr) (0.068), as compared to the remainder of the individuals in this group. There is no methodological or statistically valid reason for excluding this subject from the analyses as a suspect value, however use of the antihypertensive medication Blocadren (Timolol), a β-adrenergic blocker, by this subject, may have had an effect on lipid metabolism, and perhaps an influence on cholesterol FSR. Data were therefore additionally analyzed without this subject.

RANOVA for cholesterol FSR for all subjects excluding subject EK (Table 9) revealed a significant main effect of group on cholesterol FSR ($p=0.026$). A significant interaction between group and feeding state ($p=0.015$), was also observed. In order to interpret this interaction, a comparison of simple main effects, by independent student's *t*-tests, of cholesterol FSR for each group in both the fed and fasted states was made. Results of these analyses (Table 6) are illustrated in Figure 4. A significant difference in cholesterol FSR was seen between controls and type III HLPs, for the 0-24 hr ($p=0.002$) interval or fed state.

The size of the M1 (central) total cholesterol pool (grams) was estimated, based on the equation derived by Goodman et al. (1980), for each subject (Table 7). Mean body weights, total cholesterol measurements and triglyceride measurements for days 1, 2 and 3 of the experimental trial, were used in the equation. The triglyceride concentration determined for the controls (1.34 ± 0.22 mmol/l) during the experimental trial was significantly different from that of the type III HLPs (3.73 ± 0.42 mmol/l) ($p<0.001$). As illustrated in Figure 5, subject EK, previously shown to have the highest cholesterol FSR of all subjects, has the smallest estimated M1 pool size. The mean (±SEM) M1 cholesterol pool size for the controls (24.9 ± 0.6 g) was not significantly different from that of the type III HLPs (26.1 ± 1.9 g).

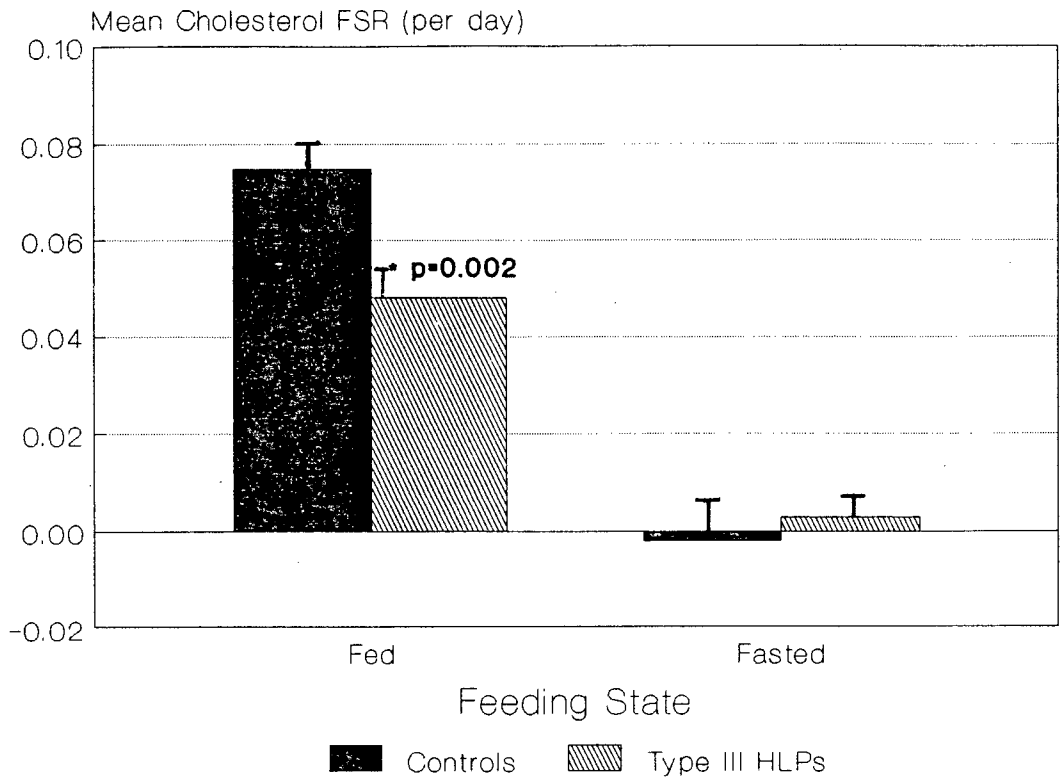


Figure 4 Effect of group (controls versus type III HLPs) on free cholesterol fractional synthetic rate when subject EK is excluded.

Table 7 Estimation of M1 (central) total cholesterol pool size in control and type III HLP subjects.

Subject by Group	Weight[1]	Total Cholesterol[2]		Triglycerides[3]			M1 Pool[4]	Apo E Phenotype
	(kg)	(mmol/l)	(mg/dl)	(mmol/l)	(mg/dl)	(TGGP)[5]	(g)	
Controls:								
CD	81.7	4.18	161.3	0.95	84.1	1	25.1	2/2
MKa	81.4	6.15	237.4	2.65	234.5	2	25.3	2/2
JG	76.7	4.89	188.8	0.82	72.6	1	24.7	3/2
DD	79.7	5.94	229.3	0.90	79.7	1	27.0	3/2
EM	74.1	6.78	261.7	1.34	118.6	1	26.5	3/2
RS	73.5	5.60	216.2	1.29	114.2	1	24.7	3/2
HD	58.5	6.46	249.4	0.95	84.1	1	21.6	3/2
MW	76.8	4.81	185.7	1.81	160.2	1	24.6	3/2
Mean	75.3	5.60		1.34		1.1	24.9	
±SEM	2.6	0.32		0.22		0.1	0.6	
Type III HLPs:								
NN	82.2	5.84	225.4	2.02	178.8	1	27.5	2/2
WJ	90.7	9.30	359.0	3.59	317.7	3	30.0	2/2
ES	85.8	8.02	309.6	3.76	332.8	3	26.8	2/2
JB	82.4	7.62	294.1	5.71	505.3	3	25.3	3/2
AT	84.3	7.65	295.3	3.23	285.9	2	28.3	3/2
MKu	67.7	6.28	242.4	2.75	243.4	2	21.6	2/2
DP	107.4	8.20	316.5	5.12	453.1	3	33.2	3/2
EK	53.9	6.70	258.6	3.69	326.6	3	15.8	3/2
Mean	81.8	7.45		3.73		2.5	26.1	
±SEM	5.6	0.40		0.42		0.3	1.9	
	p=0.31[6]	p=0.003		p<0.001		p<0.001	p=0.58	

[1]mean of body weights on study days 1, 2 and 3; [2]mean of total cholesterol measurements on study days 1, 2 and 3; [3]mean of triglyceride measurements on study days 1, 2 and 3; [4]M1 total cholesterol pool size (g)=[0.287 x Wt. (kg)] + [0.0358 x total cholesterol (mg/dl)] - [2.40 x TGGP] - 1.72, from Goodman et al. 1980; [5]variable equal to 1, 2, or 3 corresponding to serum triglyceride level of less than 200, 200-300, or greater than 300 mg/dl; [6]independent *t*-test.

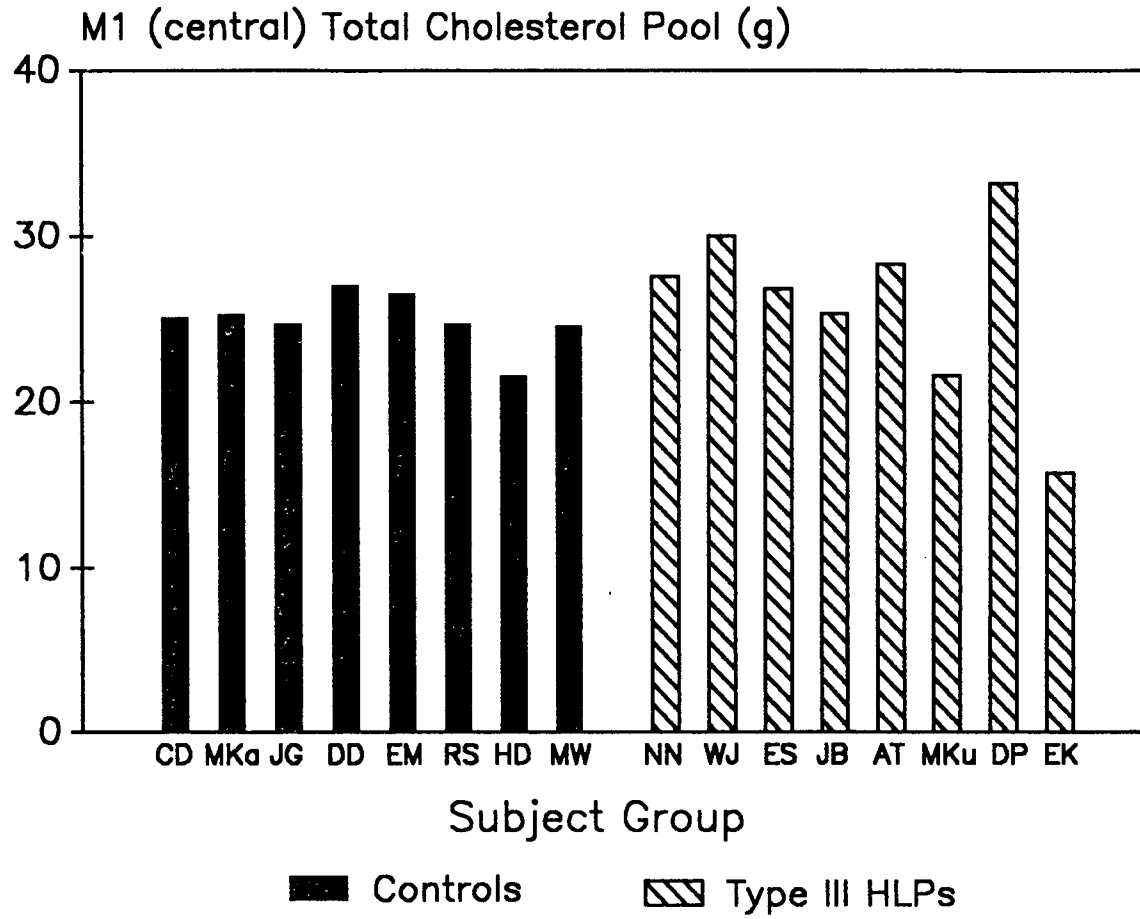


Figure 5 M1 (central) total cholesterol pool size in controls and type III HLPs.

Based on the individual M1 total cholesterol pool size calculated, individual free cholesterol net synthesis (g/day) was calculated for each control and type III HLP subject (Table 8). RANOVAs for free cholesterol net synthesis summarized in Table 9, reveal that for all subjects, there is no significant difference in the grams of free cholesterol synthesized per day between controls and type III HLPs ($p=0.227$) (Figure 6). The mean ($\pm$SEM) free cholesterol net synthesis in the fed state (0-24 hr) for controls was 0.749 ± 0.050 g/day, while that of type III HLPs was 0.560 ± 0.068 g/day. When subject EK is excluded, the lack of statistical significance between groups with respect to total grams of free cholesterol synthesized per day, is maintained (Table 9).

For both free cholesterol FSR and free cholesterol net synthesis, when all subjects are included or when subject EK is excluded, a significant main effect within subjects of feeding state was observed ($p<0.001$), as indicated by the summary of RANOVAs in Table 9. As illustrated in Figure 7, for free cholesterol net synthesis, values are significantly greater in the fed versus the fasted state. For both free cholesterol FSR when all subjects are included (Figure 8) and free cholesterol net synthesis when all subjects are included (Figure 9) or when subject EK is excluded, the type III HLPs tended to have a lower value than the controls in the fed state, while the reverse was observed in the fasted state.

Deuterium enrichment of plasma free cholesterol, cholesterol fractional synthetic rate and cholesterol net synthesis at hrs 12 and 36 were also obtained, and are listed in Appendices Fourteen, Fifteen, and Sixteen, respectively, for reference only, and were not included in any statistical analyses as they represent differences in cholesterol deuterium enrichment between day and night time intervals, and were not required in the analyses of the effect of group or feeding state on cholesterol FSR. Appendices Seventeen and Eighteen display the data in Appendices Fifteen and Sixteen, respectively, in an illustrative manner.

Table 8 Individual free cholesterol net synthesis per day based on individual M1 (central) total cholesterol pool size in control and type III HLP subjects.

Subject by Group	M1 Pool[1]	Fed State 0-24 hr	Fasted State 24-48 hr	0-48 hr	Apo E Phenotype
		(g free cholesterol synthesized per day)[2]			
Controls:					
CD	25.1	0.512	-0.090	0.211	2/2
MKa	25.3	0.708	-0.152	0.283	2/2
JG	24.7	0.662	0.207	0.435	3/2
DD	27.0	0.745	-0.022	0.367	3/2
EM	26.5	0.975	-0.265	0.350	3/2
RS	24.7	0.850	-0.030	0.405	3/2
HD	21.6	0.683	-0.052	0.320	3/2
MW	24.6	0.856	0.236	0.541	3/2
Mean	24.9	0.749	-0.021	0.364	
±SEM	0.6	0.050	0.060	0.035	
Type III HLPs:					
NN	27.5	0.462	-0.011	0.231	2/2
WJ	30.0	0.756	-0.096	0.336	2/2
ES	26.8	0.536	0.032	0.289	2/2
JB	25.3	0.506	0.111	0.314	3/2
AT	28.3	0.758	-0.102	0.328	3/2
MKu	21.6	0.207	0.026	0.121	2/2
DP	33.2	0.491	0.279	0.385	3/2
EK	15.8	0.765	0.095	0.430	3/2
Mean (all subj.)	26.1	0.560	0.042	0.304	
±SEM	1.9	0.068	0.044	0.034	
Mean (no subj. EK)	27.5	0.531	0.034	0.286	
±SEM	1.4	0.071	0.050	0.033	
All Subjects:					
Mean	25.5	0.655	0.010	0.334	
±SEM	1.0	0.048	0.037	0.025	

[1]see Table 7; [2]free cholesterol net synthesis per day (g) = size of M1 total cholesterol pool (g) x cholesterol FSR corresponding to time interval x 0.4. Factor 0.4 used as it is estimated that approximately 40% of the M1 (central) total cholesterol pool is free cholesterol (Jones et al. 1988).

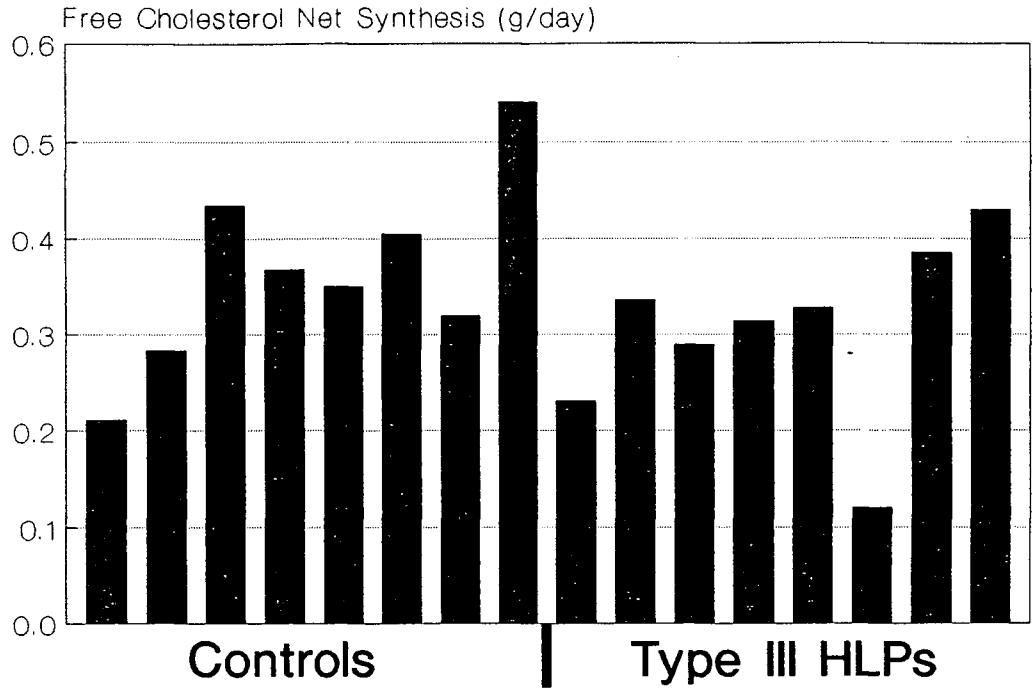


Figure 6 Effect of group (controls versus type III HLPs) on free cholesterol net synthesis (0-48 hr) for all subjects ($p = 0.227$).

Table 9 Summary of repeated measures analysis of variance for free cholesterol fractional synthetic rate and free cholesterol net synthesis based on M1 (central) total cholesterol pool size in control and type III HLP subjects.

Source of Variation		FSR[1] (per day) (all subjects)	FSR (per day) (excluding subject EK)	NET SYN[2] (g per day) (all subjects)	NET SYN (g per day) (excluding subject EK)
Between Subjects Effects:					
Group[3]	F-ratio[5]	0.790	6.292	1.600	2.683
	p value	0.389	0.026	0.227	0.125
Within Subjects Effects:					
F/F[4]	F-ratio	96.255	111.447	107.039	92.465
	p value	0.000	0.000	0.000	0.000
Group x F/F	F-ratio	3.487	7.842	4.076	4.295
	p value	0.083	0.015	0.063	0.059

[1]cholesterol fractional synthetic rate; [2]free cholesterol net synthesis; [3]normals versus type III HLPs; [4]fed versus fasted state; [5]two-way RANOVA .

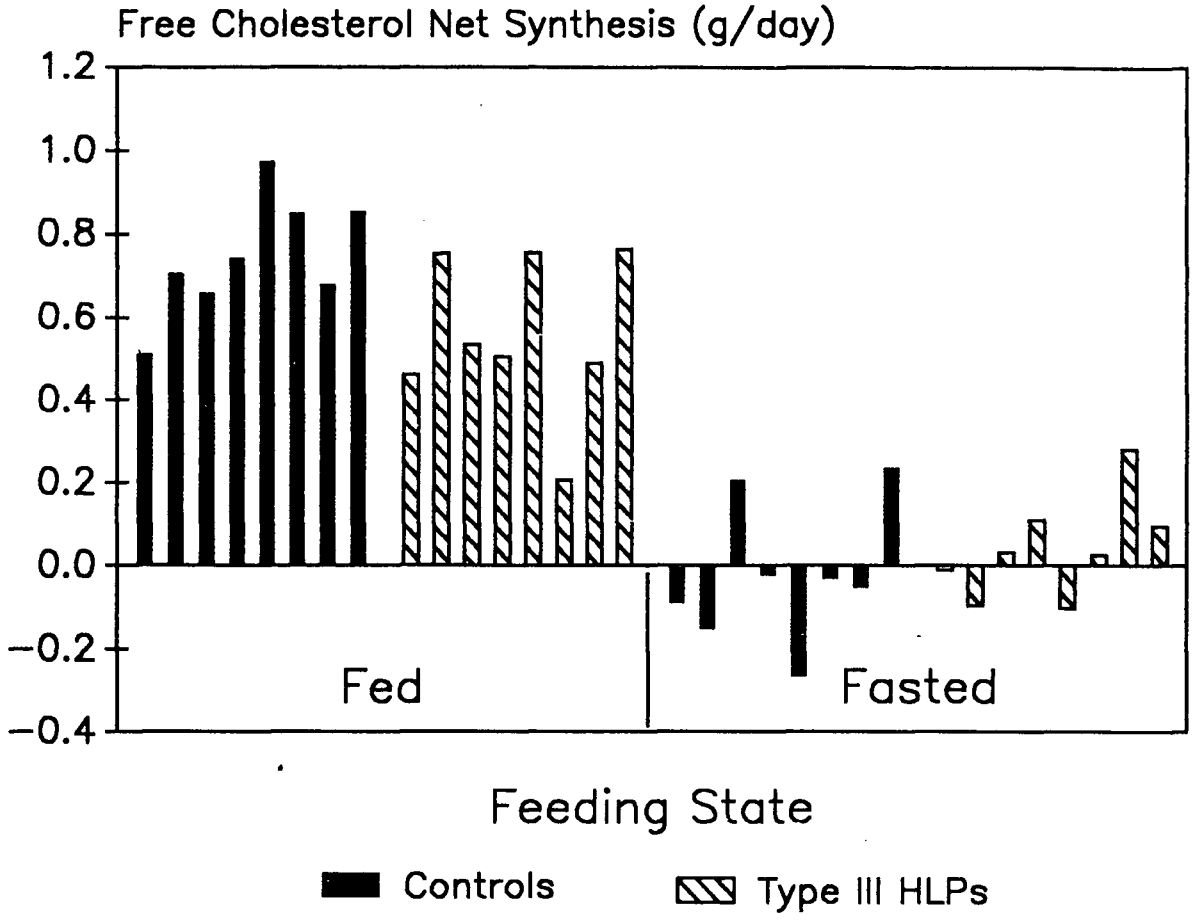


Figure 7 Effect of feeding state on free cholesterol net synthesis for all subjects ($p < 0.001$).

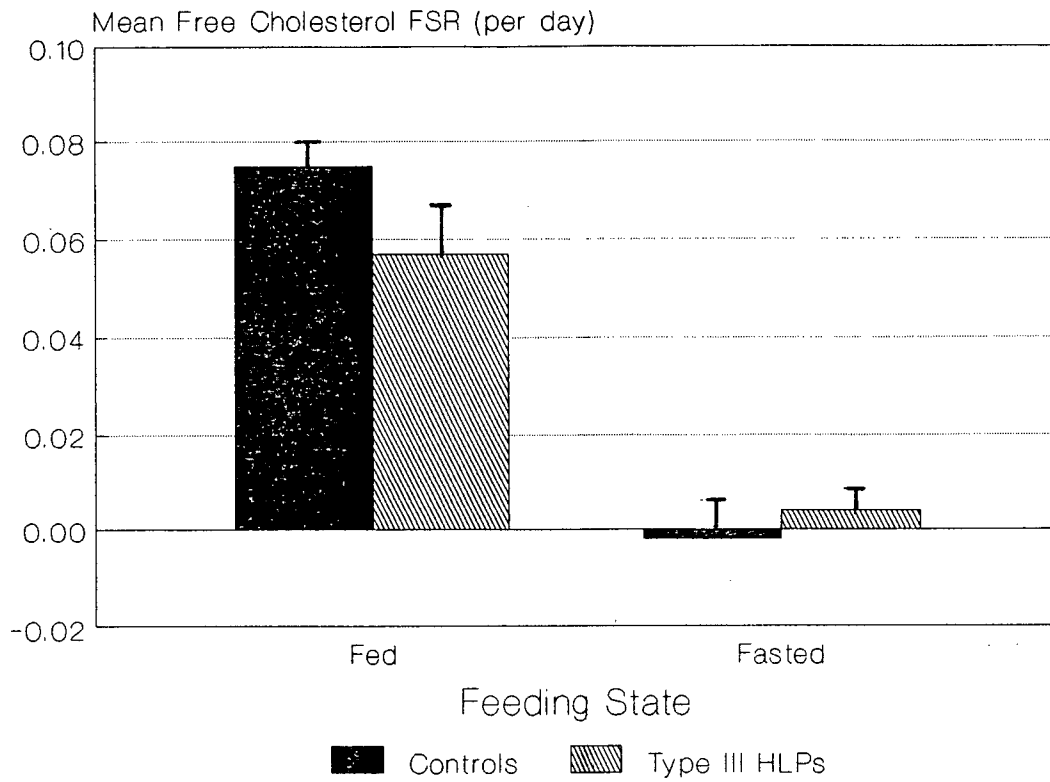


Figure 8 Mean free cholesterol fractional synthetic rate for all control and type III HLP subjects during the fed and fasted state.

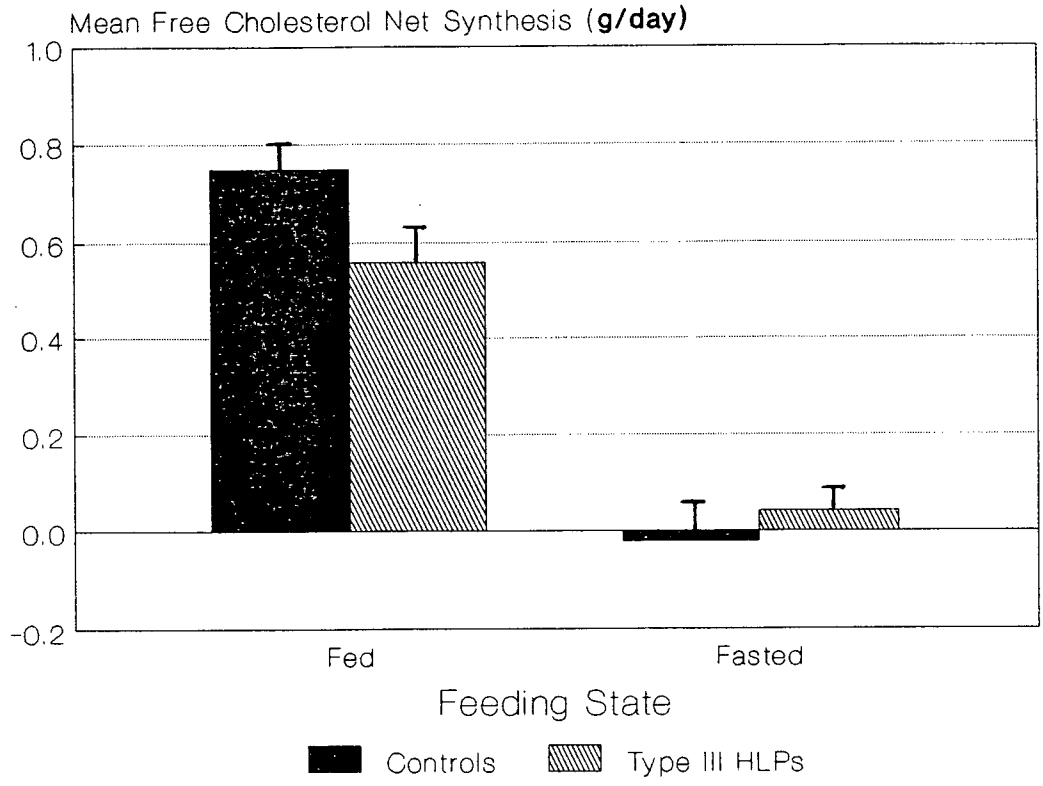


Figure 9 Mean free cholesterol net synthesis for all control and type III HLP subjects during the fed and fasted state.

5. DISCUSSION

5.1 PHASE I: INITIAL SUBJECT SCREENING

The relative frequencies of apo E phenotypes observed in the subjects screened from Vancouver, B.C. for the control group cannot be compared directly with those for other Canadians reported by Davignon (1988) as the subjects screened in the present study were not wholly randomly selected. Seven of the subjects possessing at least one ε2 allele, and one subject with two ε2 alleles were solicited for screening based on previous knowledge of low cholesterol levels, possible genetic inheritance of an ε2 allele as at least one parent was known to posses this allele, and previous phenotypic identification with an ε2 allele.

The increase in mean cholesterol level from the E2/2 (3.5 mmol/l) to E3/2 (4.2 mmol/l) to E3/3 (4.51) phenotype reported by Davignon in the Ottawa, Canada study, and similar stepwise increases in cholesterol level for the corresponding apo E phenotypes observed in other studies (Davignon 1988), were not observed in the present study. A mean total cholesterol level for the E3/2 phenotypes (5.7 mmol/l) not intermediate to, but greater than both the E2/2 (4.1 mmol/l) and E3/3 (4.9 mmol/l) phenotypes was observed, although the common trend of a lower cholesterol level for the E2/2 versus the E4/3 phenotype was maintained in this study. The mean total cholesterol levels for the E3/2 (n=23) and E3/3 (n=3) phenotypes observed may be altered with increased sample size, perhaps by diluting values such as those for two subjects with the E3/2 phenotype above the 90th (7.46 mmol/l) and 95th (8.36 mmol/l) percentiles for their age and sex, and adding observations to the E3/3 group. The only similarity in the pattern of increasing mean levels for triglycerides to that of cholesterol was from E2/2 to E4/3. There is no consistent evidence, however, for a polymorphic effect of apo E on plasma triglyceride concentration (Davignon 1988), although the E2 isoform has been shown to have a significantly greater frequency in hypertriglyceridemic patients (Utermann 1984).

Subject groups were age- (mean group age not significantly different) and almost completely sex-matched, in order to reduce variability in cholesterol FSR as a result of the variables age and sex.

A factor which may modulate the expression of type III HLP in the female as compared to male subjects, is a fluctuation in the level of circulating estrogen with menopause. Demonstration of up-regulation of hepatic LDL (apo B, E) receptors in animal models upon administration of estrogen (Brewer et al. 1983, Stuyt et al. 1986), with possible resultant effects on plasma β-VLDL levels, and the observation that in women, type III HLP usually does not occur before menopause (Mahley et al. 1985), illustrate the potential for an effect on plasma cholesterol concentrations in response to estrogen levels in female versus male subjects, in addition to de novo cholesterol synthesis. Although subjects were not questioned as to menstrual status, the average age of onset of menopause is currently 52 years (Ganong 1985), and it is therefore likely that most female subjects were postmenopausal. Also, the hypertriglyceridemic and paradoxical hypolipidemic response to exogenous estrogens of control and type III HLP females, respectively (Stuyt et al. 1986) may indicate a differential response in lipid metabolism between the postmenopausal control and type III HLP females in the present study. Reductions in estrogen with menopause may effect decreases in triglyceride concentrations in control women, yet increased cholesterol and triglyceride levels in the female type III HLP subjects.

Data on the age-sex specific distribution of plasma lipids in 10 North American cities have been compiled by the Lipid Research Clinics Program Epidemiology Committee (1980). Cholesterol or triglyceride levels in excess of the 95th percentile, identified with high risk of developing cardiovascular disease, have been defined hyperlipidemic (Brown et al. 1987). The total cholesterol values determined during screening for the female control subjects selected for the experimental trial were less than the 75th percentile, defined as moderate risk for developing cardiovascular disease, for women over 50 years of age (6.53 mmol/l, 252 mg/dl). For male controls, all subjects had a total cholesterol level below the 75th percentile for men over 50 years of age (5.96 mmol/l, 230 mg/dl) except for EM, whose total cholesterol level (6.07 mmol/l) was slightly above the 75th percentile, but below the 95th percentile (7.25 mmol/l, 280 mg/dl) (Hoeg et al. 1987). Triglyceride levels in all male and female control subjects were below the 95th

percentiles for sex and age (e.g. males, 50-59 years, 3.33 mmol/l or 295 mg/dl; females, 50-59, 2.88 mmol/l or 255 mg/dl) respectively.

The mean screening value for total cholesterol for the type III HLP subjects selected for the experimental trial (7.60 mmol/l) was slightly below the level of plasma total cholesterol commonly observed in untreated type III HLP patients of 7.8 mmol/l (300 mg/dl). The mean triglyceride level recorded for the type III HLP subjects (4.19 mmol/l) was within the range of values typically seen in this untreated disease (2.3-9.0 mmol/l or 200-800 mg/dl). The type III HLP subjects were all patients of the University Hospital, Shaughnessy Site Lipid Clinic and were receiving various treatments in attempts to modify their lipid levels at the time values for cholesterol and triglycerides were obtained. Thus, they cannot be compared directly to values determined in untreated subjects. Both sets of type III HLP lipid levels, however, were significantly greater than those of their apo ε2 counterparts without type III HLP disease.

The two control subjects who maintained use of medications during the experimental trial for hypothyroidism, were both determined to be euthyroid at the time of the experimental trial, on the basis of T4 and TSH hormone assays. Hypothyroidism has been characterized by elevated plasma cholesterol levels as a result of LDL hypocatabolism, possibly due to LDL receptor deficiency (Thompson et al. 1981). Defective hepatic LDL uptake would be expected to produce a corresponding increase in hepatic de novo cholesterol synthesis. This receptor deficiency appears to be reversible upon treatment with thyroid hormones, as evidenced by normalization of LDL cholesterol levels (Thompson et al. 1981). It is assumed that as a euthyroid state existed in subjects JG and MW, thyroid hormone deficiency did not confound plasma cholesterol or cholesterol FSR levels obtained. Hypothyroidism also existed in one type III HLP subject, who maintained use of hypothyroid medication during the experimental trial. As thyroid status assays were not carried out on this individual at the time of the experimental trial, it is possible that thyroid deficiency could have contributed to an elevation of plasma cholesterol and cholesterol FSR values in this subject.

Additionally, the use of two antihypertensive drugs, Sectral (Acebutolol HCL) and Blocadren (Timolol), by subjects WJ and EK, respectively, during the experimental trial, must be considered for their possible effects on lipid metabolism. Although both medications are effective β-adrenoceptor blocking agents used to lower elevated blood pressure, they have differing effects on blood lipids. Acebutolol has been shown to have no significant effects on total, VLDL, LDL or HDL cholesterol or total triglycerides (Miller et al. 1987). Timolol has been shown to significantly increase VLDL and LDL cholesterol and total triglycerides, while decreasing HDL (Leren et al. 1988). As the high FSR values observed for subject EK may be related to altered lipid metabolism in this type III HLP subject in response to the drug Blocadren, the data were analyzed with and without this subject.

Estimates of obesity for three (NN, ES, DP) of the four type III HLP subjects classified as obese may be exaggerated, as they were based on ideal weight for a medium frame size, reported in the patient charts. Elsewhere in the patient charts for these individuals, ideal weights corresponding to large frame sizes were also reported. If the large frame size and corresponding ideal weight had been selected, percent ideal weight values for these three subjects would have been lower, at +12%, +24% and +72%, respectively. Therefore obesity may be confirmed in three type III HLP subjects (ES, MKu, and DP) and one control subject (MW). Several in vivo studies have demonstrated increased cholesterol production in human obesity (Angelin et al. 1985). As a clinical characteristic often associated with type III HLP, some studies have reported up to 70% of subjects with type III HLP as obese (Brewer et al. 1983). It is possible, therefore, that cholesterol synthetic rates in the type III HLP and control subjects who are obese (NN, ES, DP, MW) may have been secondarily influenced by the effect of obesity on hepatic cholesterol production, and may not reflect the true primary de novo synthetic rate. If obesity influenced cholesterol synthetic rate, it would likely increase synthesis, therefore removal of this effect could result in an increase in the difference between controls and type III HLPs, possibly making the differences in FSR significant.

5.2 PHASE II: EXPERIMENTAL TRIAL

The mean usual dietary intakes for the control group may be compared with that of the average North American diet: total energy intake comprised of 40% fat, 45% CHO and 15% protein, 200 mg cholesterol/1000 kcal, and a P:S ratio of 0.4 (US Dept. of Health and Human Services, 1986). As a group the controls consumed about the same amount of protein (14.6 % kcal), but less fat (36.0% kcal) and more CHO (47.4 % kcal), less cholesterol/1000 kcal (142.0 mg/1000 kcal) and had a higher P:S ratio (0.6) than the average North American.

The mean usual dietary intakes for the type III HLP group may be compared with those of the dietary modifications recently proposed for Canadians with elevated blood lipid levels at the 1988 Canadian Consensus Conference on Cholesterol (CCCC) (Canadian Consensus Conference on Cholesterol 1988): total energy intake comprised of less than 30% fat, greater than 55% CHO, 10-15% protein, less than 10% saturated fat, less than 10% polyunsaturated fat, and less than 300 mg cholesterol/day for some hyperlipidemics. As a group the type III HLP subjects consumed more protein (19.8% of kcal), approximately an acceptable amount of fat (30.5% of kcal), less CHO (46.6% of kcal), and less total cholesterol (291.3 mg/day) than the current Canadian dietary intervention recommendations for individuals with increased blood lipids. It was anticipated that the type III HLP subjects would follow a more prudent diet than the controls, due to increased awareness of the effect of diet on blood lipid levels through dietetic counselling for their lipid disorder. However, significant differences between the two groups were found only for mean intakes of protein, although the type III HLPs tended to consume less total lipid, less total cholesterol and less total energy per day.

Generally, increases in plasma cholesterol levels have been shown in humans and animals on a low protein diet (5% total energy intake) (Debry et al. 1987). Experimental data in animals have shown that a high protein diet (20% and 40%) does not cause blood cholesterol to increase (Debry 1987). Implications for an effect of dietary protein on blood lipids center primarily on the more hypercholesterolemic effect of animal protein versus plant protein, observed in studies comparing casein to soy protein (Debry 1987). As protein intakes for both groups were within a

normal range of 10-20%, and the contribution of protein from animal and vegetable sources was not determined, and based on the need for further studies to define more clearly the relationship between food proteins and cholesterol metabolism, it is assumed that the significant differences in protein intake between the two groups had a minimal differential effect on cholesterol metabolism in control and type III HLP subjects, compared to the other dietary components which have a more potent effect on plasma cholesterol.

Although high CHO diets, containing over 65% CHO, have been shown to decrease LDL, they also result in a corresponding increase in VLDL and plasma triglycerides, and a decrease in HDL (Katan 1987). At moderate levels of CHO consumption, such as those observed in the control and type III HLP subjects, there appears to be little effect of CHO on plasma cholesterol levels.

Increases in plasma HDL and triglycerides with moderate and high intakes of alcohol, respectively have been reported (Katan 1987). As alcohol contributed only 2-3% of total calories for control and type III HLP subject groups, these low-moderate intakes were not considered to have important effects on total cholesterol levels.

The significance of dietary cholesterol level on cholesterol metabolism remains controversial. The basis for this controversy stems from the fact that humans, in contrast to animals, do not show increasing hypercholesterolemia with increased cholesterol feeding, and may therefore be inherently resistant to dietary cholesterol intakes. In a clinical setting, under carefully controlled conditions, increases in plasma cholesterol levels have been demonstrated with increasing cholesterol intake, approximating 0.26 mmol/l per 100 mg cholesterol per 1000 kcal (Grundy et al. 1988). It is suggested that differences in cholesterol intake between 0-500 mg per day have a more potent impact on plasma cholesterol levels than do differences in dietary cholesterol intakes in excess of 500 mg/day (Grundy 1988). This response may be linear or curvilinear from 0-500 mg cholesterol per day. Nonetheless, there appears to be quite a variability in individual response to dietary cholesterol (McNamara et al. 1987). Overall, the

effect of cholesterol as a dietary constituent on the concentration of cholesterol in the plasma appears to be relatively minor (McNamara et al. 1987) in most individuals.

Although the individual cholesterol intakes in the two subject groups ranged from 85.8 - 484.0 mg/day, within the range in which plasma cholesterol level is most sensitive, and recognizing that there is room for individual variability in response of cholesterol metabolism to dietary cholesterol intake, it could be assumed that as mean cholesterol intakes between the two groups were not significantly different, that similar effects on cholesterol metabolism, if any, would result. This assumption may not be valid if differences in percent absorption of dietary cholesterol occurred between the two groups.

In humans, plasma cholesterol seems to be the most responsive to the quality of dietary fat (McNamara et al. 1987, Grundy et al. 1988a). Substitution of polyunsaturated fatty acids for saturated fatty acids in the diet has been shown to result in reductions in total and LDL plasma cholesterol (Katan 1987) in most individuals, however to varying degrees (McNamara 1987), possibly through a mechanism of preferential use of PUFA for oxidation rather than VLDL synthesis (Beynen et al. 1985). Some studies have shown that the composition or amount of dietary fat does not modify the effect of dietary cholesterol on plasma cholesterol in subjects with normal lipid levels (Kestin et al. 1989), however this may not hold true for hypercholesterolemics. As the P:S ratio in control and type III HLP subjects was virtually identical, it is assumed that the resultant effect of this ratio on cholesterol metabolism may be similar for both groups.

The usefulness of three day food records as accurate indicators of the control and type III subjects' usual dietary intake may be partially assessed by comparison with studies of Basiotis et al. (1987), who evaluated the number of days of recording food intake which were necessary to estimate nutrient intakes in groups of individuals with an accuracy $\pm$ 10% of usual intake ($p<0.05$). Three to fifteen days were required to estimate the true average intake for groups of individuals, depending on the nutrient and sex of the group. Energy, protein, CHO, fat and cholesterol required 3, 4, 4-5, 6, and 13-15 days, respectively. White et al. (1981) have reported that 9 days of food record keeping are needed to obtain reliable estimates of dietary cholesterol

intakes. In both of these studies, the individuals used to generate the data were all trained in dietary record keeping and instructed on how to assess portion sizes accurately.

A varying degree of motivation and regard for accurately reporting diet intake over the record days was observed among the control and type III HLP subjects. Details on food preparation, extra food items, recipes etc, were variable from subject to subject, despite identical preliminary instructions on record keeping. However, after completion of the food records, each day's intake was reviewed, and the subject was questioned regarding portions, composition of entrees if specific ingredients not listed, possible omissions, to improve accuracy. As the mean estimated daily caloric requirement and the mean reported usual energy consumption per day, determined from 3 day food records were not significantly different for the control subjects, but differed only by 25.9 kcal, it is estimated that three day food records were adequate in accurately determining food energy in these individuals. The significant difference observed between these two estimates for energy in the type III HLP group may not reflect less accurate recording by these individuals, but perhaps overestimation of daily caloric requirements through the use of too large an activity factor for some of the more elderly and less active individuals.

The analysis of control and type III HLP subjects' usual dietary intakes as reported by 3 day food records, are somewhat questionable in degree of accuracy particularly for cholesterol intake, but provide, however, a general guideline as to trends in nutrient consumption between the two study groups. These trends may be used to approximate the effect of the subjects' usual diet on the parameters measured for cholesterol during the experimental trial, as subjects were not placed on a stabilization diet before the study began.

It is possible that the significantly greater weight loss in type III HLP subjects versus controls over the course of the three day trial may have had a differential effect on cholesterol synthesis in the two subject groups. As increasing body weight has been demonstrated to be significantly correlated with increased cholesterol synthetic rate (Meittinen et al. 1971), the significantly greater body weight loss in type III HLPs may have lowered cholesterol FSR in these subjects, as compared to controls.

The relatively stable plasma total cholesterol determinations in all subjects over the experimental trial reflect the minimal effect of fasting on plasma cholesterol levels. The individual variability for total plasma cholesterol measures in all subjects reflects the total intra-person coefficient of variation (CVp) (intra-individual biological coefficient of variation [CVb] + analytical coefficient of variation [CVa]) of total cholesterol in each individual (Cooper et al. 1988). The range in total intra-person coefficient of variation for total cholesterol of 0.41 - 6.74 % observed in the present study for samples taken one day apart, was lower than the average total intra-person coefficient of variation of total cholesterol determined by the Lipid Research Clinics for paired fasting samples taken 2.5 months apart of approximately 8% (Cooper et al. 1988). The mean CVp of the three samples collected within one week for both control (2.83%) and type III HLP subjects (3.52%) fell below the within-month CV estimated for samples collected two times a week of 4.8% (Cooper et al. 1988). Increasing the number of samples drawn from one individual and shortening the time frame during which they are taken, decreases the CVp (Cooper et al. 1988). The lower average CVps observed in the present study would therefore be expected.

The lack of statistical significance between the mean total cholesterol levels determined for the control group during screening and during the experimental trial further demonstrates the limited variability in the cholesterol measurements for these subjects.

Additionally, the mean total cholesterol level for type III HLPs determined during the experimental trial was still slightly below the lower level for plasma total cholesterol commonly observed in untreated type III HLP patients (7.8 mmol/l or 300 mg/dl), although lipid levels in any given patient may be quite variable (Mahley et al. 1984b). The type III HLP subjects taking various lipid lowering medications on a regular basis had been instructed to discontinue their use 4 weeks prior to and during the experimental trial; this withdrawal period is expected to have been sufficient for plasma lipid levels to revert to pretreatment concentrations (Levy et al. 1972). Nonetheless, plasma total cholesterol levels were significantly greater in type III HLP versus control subjects.

Although a statistically significant difference was not observed between the mean total cholesterol levels determined for the type III HLP group during screening and during the experimental trial, it was expected that the mean values for the experimental trial would have been greater than those determined at screening, as the subjects taking lipid lowering medications had discontinued their use. However, in addition to the above explanation, between the time the screening values for each subject were determined by the Lipid Clinic and the time of the experimental trial, subjects may have lost weight or altered the type or dosage of lipid lowering medication, therefore altering their baseline lipid levels.

5.3 EFFECT OF GROUP ON CHOLESTEROL FSR AND CHOLESTEROL NET SYNTHESIS

The first major objective of the research project was to determine whether a significant difference could be observed in cholesterol synthesis between the apo E2 control and type III HLP subjects. When all subjects were included, lack of a significant main effect of group ($p=0.389$) demonstrated that cholesterol FSR did not differ significantly between control and type III HLP subjects. With the exclusion of subject EK, the main effect of group achieved statistical significance ($p=0.026$), suggesting the potentially confounding effect of the potentially lipid altering antihypertensive medication taken by this subject. Interpretation of the main effect of group on FSR when subject EK was excluded was qualified by the statistically significant interaction between group and feeding state. Tests for simple main effects showed that cholesterol FSR was significantly lower ($p=0.002$) in type III HLP subjects versus controls in the fed state (0-24 hrs) only.

The use of the individual absolute rates of cholesterol synthesis determined in the control and type III HLP subjects, by calculation of cholesterol FSR, as a measure of the differential cholesterol production per day in these two groups of individuals, is based on the assumption that the size of the M1 total cholesterol pool is identical in the control and type III HLP subjects. The validity of cholesterol FSR is therefore questionable in situations of differing pool size. In an individual whose actual M1 pool size was smaller than that assumed, FSR would be

overestimated, and vice versa, due to the fact that the cholesterol FSR calculation is based on the ratio of deuterium labelled to unlabeled free cholesterol in the M1 pool. An individual with a smaller M1 pool size than assumed would have a decreased amount of unlabeled cholesterol to which the labelled de novo cholesterol synthesized that day is compared, therefore making cholesterol FSR appear relatively larger. Therefore, in order to accurately assess the net de novo daily cholesterol synthesis, the individual size of the M1 total cholesterol pool must be considered, especially in subjects with significantly different plasma cholesterol levels. As plasma cholesterol is a component of the M1 pool, plasma cholesterol levels would be expected to reflect M1 pool total size.

Goodman et al. (1980) have determined a highly significant and confirmed multiple regression prediction equation which expresses the relationship of the size of the M1 total cholesterol pool to the independent physiological variables weight, serum total cholesterol level and serum triglyceride level. The coefficient of multiple correlation, r, for the equation was 0.87. The coefficient of determination, r^2 (0.756), calculated from this value, states that this equation accounts for at least 75.6% of the total variation in M1 pool size. Additionally, each of the independent physiological variables in the equation are highly significantly related to M1 pool size ($p<0.01$). A test for the relative importance, or β-weight, of each variable in predicting the size of the M1 pool revealed values of 0.82, 0.74 and -0.40 for weight, total cholesterol and triglyceride concentration, respectively. Therefore increases in M1 pool size are most influenced by increases in body weight, followed by increasing cholesterol level, while increasing triglyceride level is associated with a smaller decrease in M1 pool size. The applicability of the M1 pool prediction equation to individuals such as those in the present study, besides those subjects in which the equation was originally developed, was confirmed through cross-validation. Goodman's heterogeneous study population, consisting of normolipidemic and hyperlipidemic subjects, was divided into two mixed groups: the regression equation was generated in the first group and the significance of the regression coefficients tested for in the second group. Therefore, this equation may be used with confidence to predict the M1 pool size in the hyperlipidemic and non-

hyperlipidemic subjects in the present study. Although the mean difference in the M1 pool size calculated by Goodman's equation was not significantly different between the control and type III HLP groups, the individual estimated values for M1 varied within the range of 15.8-33.2 grams.

Calculation of daily free cholesterol de novo net synthesis by multiplying free cholesterol FSR by individual M1 total cholesterol pool size and adjusting this value for approximately 40% free cholesterol in the total cholesterol M1 pool, allows direct comparison of daily cholesterol synthesis between subjects. When daily free cholesterol net synthesis (g/day) is compared between groups, a lack of significant difference between groups is confirmed, with or without subject EK. Relative cholesterol synthesis/day by subject EK is reduced due to small estimated M1 pool size, therefore exclusion of this subject from analyses based on potential effects of lipid altering anti-hypertensive medication is subjective.

Estimates of whole body cholesterol synthesis reported by other authors range from 0.7 - 0.9 g/day for a normal 70 kg human (Grundy et al. 1978, Jones et al. 1988, Jones et al. 1990). The mean value obtained for the control group in the fed state falls within this range, while that of the type III HLP group is below.

As endogenous cholesterol synthesis rates were not significantly altered in the two subject groups tested in the present study when M1 pool size was considered, the elevated plasma cholesterol levels observed in the apo E2 type III HLP subjects tested appear to be influenced by other factors. A possible mechanism to explain the absolute increase in the cholesterol levels observed in apo E2 type III HLP individuals may relate to increased rates of intestinal cholesterol absorption as compared to apo E2 non-hyperlipidemic subjects. Possibly, in the fed state, an increase in the amount of dietary cholesterol absorbed would lead to increased plasma cholesterol levels, via a quantitative increase in chylomicrons and chylomicron remnants. Due to the nature of the apo E2 binding defect, the increased dietary cholesterol is not taken up efficiently into the liver, and the relatively sensitive feedback mechanism of reduced hepatic de novo cholesterol synthesis upon hepatic uptake of dietary cholesterol that appears to function in free-living outpatients (McNamara et al. 1987), becomes partially uncoupled. In attempts to normalize the

level of plasma cholesterol which can be detected by the hepatocyte, de novo cholesterol synthesis is decreased, but due to a decreased sensitivity in this feedback mechanism, this decrease is not significant enough to compensate for the increased cholesterol absorption, and elevated plasma cholesterol levels result (Kestin et al. 1989).

This possible mechanism is supported by several studies in various areas. Generally, it is estimated that cholesterol absorption efficiency ranges from 30-60% (Grundy 1978). Kesaniemi et al. (1987) have proposed a relationship between the absorption efficiency of cholesterol and apo E phenotype. Apo E2 homo- and heterozygotes were shown to have the lowest plasma cholesterol levels, lowest absorption efficiency and corresponding increased cholesterol synthesis rates, as compared to other apo E phenotypes. Accordingly, enhanced efficiency of cholesterol absorption in type III HLP subjects would be expected to cause increased plasma cholesterol levels and decreased synthesis, although not significant by virtue of decreased dietary cholesterol hepatic uptake, as compared to apo E2 non-hyperlipidemics.

The viability of a combined defect of an insensitive feedback mechanism to dietary cholesterol in addition to increased absorption in type III HLP subjects, is supported by other studies demonstrating that although most individuals do not respond to increases in dietary cholesterol with increased plasma levels, in some "hyper-responsive" individuals, de novo cholesterol synthesis is not suppressed, indicating imperfect regulation of the feedback inhibition of endogenous cholesterol synthesis (McNamara et al. 1987, Grundy et al. 1988b). It is possible that some hypercholesterolemics are more diet-sensitive than the general population (Kestin et al. 1989). It is also possible that hyper-responders to dietary cholesterol may also show hypersensitivity to saturated fats (Beynen et al. 1988), therefore an increased response to dietary saturated fats, may also exist in Type III HLP subjects.

If the mechanism for the absolute increase in plasma cholesterol levels in type III HLP subjects is related to increased cholesterol absorption, the lipoproteins one would expect to be elevated in this disease are those responsible for transporting dietary fat to the liver, chylomicrons. In type III HLP fasting plasma is characterized by chylomicron remnants or β

VLDL which are increased in number, as compared to the residual β-VLDL observed in fasting plasma of apo E2 non-hyperlipidemic individuals with primary dysbetalipoproteinemia (Mahley et al. 1984b).

Finally, if dietary cholesterol and absorption are factors in the increased levels of cholesterol observed in type III HLP subjects, this disorder would be expected to respond to dietary treatment. Of all the familial hyperlipoproteinemias, treatment of type III HLP is the most successful (Brown et al. 1983b). Reductions in cholesterol and saturated fat, and increases in P:S ratio have been shown to be effective in the dietary management of this disorder (Brown et al. 1983b, Mahley et al. 1984b, and Brewer et al. 1983).

Although no significant differences were found between type III HLPs and controls, the type III HLPs tended to synthesize cholesterol at a lower rate and in lower absolute amounts as compared to the controls in the fed state, while this trend was reversed in the fasted state. A possible explanation for these observations may relate to the activity of HMG CoA reductase in response to differential overall cholesterol concentration in the cell during the fed and fasted states. In the fed state, the absolute increase in plasma cholesterol levels observed in type III HLPs in response to increased intestinal absorption as compared to controls, may fully saturate the defective apo E2 mediated chylomicron remnant uptake mechanism, therefore increasing the uptake of exogenous cholesterol into the hepatocyte relative to controls, causing a relatively greater cellular cholesterol concentration and therefore increased feedback inhibition of cholesterogenesis as compared to controls, possibly through reduction in HMG CoA reductase activity. In the fasted state, presentation of dietary cholesterol to the hepatocyte is diminished, therefore overall cellular cholesterol concentration in type III HLPs relative to the fed state is decreased. In response to the greater relative change in cellular cholesterol concentration from the fed to the fasted state in type III HLPs as compared to controls, the activity of HMG CoA reductase may be upregulated to a greater extent in type III HLPs in the fasted state, resulting in an increased cholesterol synthesis relative to controls.

5.4 EFFECT OF FEEDING STATE ON CHOLESTEROL FSR AND CHOLESTEROL NET SYNTHESIS

The second main objective of the study was to determine whether cholesterol synthesis is the same in the fed as compared to the fasted state in the apo E2 control and type III HLP subjects tested. The statistically significant main effect of feeding state on cholesterol FSR and cholesterol net synthesis, demonstrated that cholesterol FSR and cholesterol net synthesis did not remain constant across feeding states, but decreased significantly during the short-term fasted state; lack of a statistically significant interaction between feeding state and subject group for free cholesterol net synthesis, demonstrated that this relationship was constant in all subjects, regardless of group.

Several animal studies have demonstrated a modulation of hepatic de novo lipid synthesis by feeding status. In rats, fasting for 24-48 hours markedly reduced cholesterol synthesis, as measured in vitro by cholesterol synthesis from ^{14}C-labelled acetate in liver homogenates (Scaife et al. 1957). In vivo studies in the rat have demonstrated a 91% reduction in hepatic cholesterol synthesis by tritiated water methodology after fasting for 48 hours (Jeske et al. 1980).

A recent study in human subjects (Jones et al. 1988) employed the deuterium incorporation methodology to evaluate cholesterol fractional synthetic rate in the M1 cholesterol pool during a 24 hour fasting period followed by a 24 hour feeding period. Cholesterol FSR was significantly lower during fasting compared to feeding during the nocturnal periods of each feeding state. This study is not directly comparable to the present study as the main effect of overall fed state as compared to fasted state was not reported.

The mechanisms responsible for the feeding state modulation of cholesterol synthesis remain to be determined, yet several possibilities may be considered.

Firstly, endocrine alterations which occur in feeding and fasting may contribute to the lowered rates of cholesterogenesis observed in the fasted state, through effects on the activity of the rate-limiting enzyme in cholesterol biosynthesis, HMG CoA reductase. HMG CoA reductase activity has been shown to be altered by reversible phosphorylation (Geelen et al. 1980). It has

further been demonstrated that the hormones glucagon and insulin can modulate the phosphorylation of HMG CoA reductase and hence its activity in vitro (Geelen et al. 1980), although the specific mechanisms remain to be defined. In various in vitro systems, insulin and glucagon produced stimulation and inhibition of hepatic cholesterol synthesis, respectively (Geelen et al. 1980). Studies in animals lacking insulin have demonstrated an in vivo drop in HMG CoA reductase activity and cholesterol synthesis, which may be reversed upon treatment with insulin (Kraemer et al. 1986). The reciprocal release of insulin/glucagon in response to elevated/depleted blood glucose levels of the fed/fasted state may effect phosphorylation/dephosphorylation and corresponding activation/deactivation of HMG-CoA reductase resulting in stimulation/inhibition of de novo cholesterol synthesis.

Secondly, the availability of substrate through diet for use by HMG CoA reductase in cholesterol synthesis may play a role (Grundy et al. 1978). In the fed state anabolism prevails, and the availability of acetyl CoA, a base unit in the formation of cholesterol, is high as a result of glycolysis, and cholesterol synthesis proceeds. After a period of fasting, the glycolytic, lipogenic state is replaced by mechanisms which stimulate gluconeogenesis and lipolysis, resulting in drop in the acetyl CoA pool from decreased contributions by glycolysis. Activation of hormone sensitive lipase and β-oxidation of fatty acids to meet energy needs then occurs, with a resultant production of acetyl CoA. Now in a state of energy conservation, a preferential partitioning of acetyl CoA into energy production via the citric acid cycle may occur, rather than expenditure of this resource in anabolic processes such as cholesterol synthesis (Jones et al. 1988).

5.5 EVALUATION OF DEUTERIUM INCORPORATION METHODOLOGY

5.5.1 Metabolic Considerations

The mean negative FSR value determined in the fasting state for the control group and also for some of the type III HLP individuals suggests that the actual fractional synthetic rates in these individuals may have been underestimated due to: 1) loss of deuterium labelled de novo

synthesized free cholesterol from the M1 (central) total cholesterol pool; and/or 2) dilution of the deuterium labelled de novo synthesized free cholesterol in the M1 total cholesterol pool by unlabelled free cholesterol.

Loss of labelled free cholesterol from the M1 pool during the sampling period may arise from actual removal from the pool as free cholesterol in bile or catabolism to bile acids (Grundy 1978). Also, transformation of free cholesterol within the M1 pool via intracellular and intravascular esterification of free cholesterol to cholesterol ester (Norum et al. 1983) may account for loss of some of the deuterium label during the sampling period (Jones et al. 1990). Therefore measurement of total cholesterol deuterium enrichment may make the FSR calculation more numerically correct.

Three possible sources of unlabelled free cholesterol may be considered. Firstly, de-esterification of cholesterol within the total cholesterol M1 central pool could contribute a portion of unlabelled free cholesterol (Norum et al. 1983). Secondly, transfer of free cholesterol from the slowly equilibrating side pools 2 and 3 may occur during the sampling interval (Goodman et al. 1973). A third possible source for unlabelled free cholesterol which may cause dilution of labelled cholesterol during the fed state, is entrance of free dietary cholesterol into the M1 pool. Even if absorption were identical in all subjects, the differing amounts of dietary cholesterol provided in their experimental diets may have differentially diluted the labelled free cholesterol.

The finding of a non-negative mean FSR value in the fasted state for type III HLPs may indicate that FSR values are just proportionately higher in these individuals in this feeding state, or that less dilution of the central pool free cholesterol is occurring in these individuals due to altered rates of de-esterification, for example, acting only in these individuals. To evaluate more precisely the mechanisms contributing to the negative cholesterol FSR values, the amount of unlabelled free cholesterol influx from side pools may be estimated perhaps by determining deuterium enrichments of free cholesterol when the effect of free cholesterol entrance from the diet is removed by fasting, and de novo free cholesterol synthesis is shut down in the M1 pool through use of HMG-CoA reductase inhibiting agents (Krukemyer et al. 1987).

5.5.2 Methodologic Considerations

The assumption that endogenous cholesterol synthesis takes place only in pool 1 in the three pool model of body cholesterol turnover, and therefore the accuracy of sampling only from the plasma component of the rapidly exchangeable cholesterol pool to measure whole body cholesterol synthesis, has been questioned by reports of studies with baboons suggesting contributions of side pool 3 to cholesterol synthesis (Dell et al. 1985). Whether total synthesis is partitioned in a similar manner in humans as in baboons remains to be determined.

As deuterium enrichment of plasma water during the 48 hour course of the experimental trial was determined in one blood sample only at hour 24, it was generally assumed that this represented similar enrichments during the rest of the time period. This assumption of minimal fluctuations appears valid as Jones et al. (1988) reported variations in plateau enrichments of plasma water of only 5.5% over a 12-48 hour time span.

Minimal dilution of plasma water deuterium enrichments may have been encountered in individuals who consumed some of the unlabelled calorie free, caffeine free beverage available on the fasting day. As this beverage was not well liked and was only consumed by a few of the male subjects in limited quantities (approximately 100-500 ml), overestimation of FSR in this time period would be expected to be minimal.

Finally, the time period over which the assumptions of the deuterium incorporation methodology remain valid and therefore give the most accurate estimates of cholesterol FSR, must be verified. Appropriate time must be given for equilibration of deuterium atoms in the deuterium oxide bolus dose with the protium atoms of the body water pool and NADPH. As the time period over which sampling is carried out extends, however, precursors for acetyl CoA synthesis (glucose and fatty acids) may begin to become significantly labelled with deuterium, potentially increasing the deuterium:carbon incorporation ratio, and exaggerating true FSR (Dietschy et al. 1984).

5.6 CONCLUSIONS

Cholesterol fractional synthetic rate was determined in apo E2 type III hyperlipoproteinemic and apo E2 non-hyperlipidemic subjects in both fed and fasted states. Measurement of cholesterol FSR and cholesterol net synthesis in both fed and fasted states indicated a significant reduction in de novo cholesterol synthesis during fasting state in both hyperlipidemic and non-hyperlipidemic individuals. Possible mechanisms for this response may include hormonal regulation of the rate limiting step in cholesterogenesis through effects on HMG CoA reductase activity and/or preferential partitioning of cholesterol precursors for energy production rather than anabolism.

No significant difference in the rate of de novo cholesterol synthesis was observed between the two subject groups when all subjects were included. A significant effect of group on cholesterol FSR, and a significant interaction between group and feeding state were observed when subject EK, taking medication which possibly affected lipid levels, was excluded. These significant effects were lost when individual M1 total cholesterol pool sizes were considered. Calculation of absolute amounts of cholesterol synthesized per day through multiplication of cholesterol FSR values by M1 pool size revealed no significant differences in cholesterol net synthesis between groups, when all subjects were included, or when subject EK was excluded. This demonstrates the importance of assessing individual M1 free cholesterol pool size from which the labelled de novo synthesized free cholesterol is sampled, to prevent over- or underestimation of actual FSR.

The increased plasma cholesterol levels characteristic of type III hyperlipoproteinemia were therefore not shown to be a result of elevated cholesterol biosynthesis. Further investigation as to the factor(s) associated with the expression of type III HLP in apo E2 individuals are required. Possible mechanisms may include an increased absorption efficiency of dietary cholesterol combined with insensitivity of the negative feedback control of hepatic endogenous cholesterol synthesis in response to exogenous cholesterol, and therefore implications for more strict dietary control in these subjects as opposed to the population at large. Studies evaluating

plasma lipid levels, endogenous cholesterol synthesis and fractional absorption of dietary cholesterol are required in apo E2 non-hyperlipidemic and type III hyperlipidemic individuals.

APPENDIX ONE INFORMATIONAL LETTER SENT TO TYPE III HYPERLIPOPROTEINEMICS

Dear _____

The physicians of the Shaughnessy Lipid Clinic, and Dr. Peter Jones and Shani Dendy of the Division of Human Nutrition at the University of British Columbia, are currently conducting a research project measuring cholesterol production in different individuals. The specific objective of the study is to determine whether the elevated cholesterol levels observed in individuals with Type III Hyperlipoproteinemia are a result of altered rates of cholesterol production by the liver. A unique, non-hazardous technique for measuring cholesterol will be employed.

Patients identified with Type III Hyperlipoproteinemia, are now being selected by the research project coordinators as potential volunteers for this study. As an eligible candidate, we invite your participation.

The study will take place at the University of British Columbia, over a three day period, most likely on a Friday, Saturday, and Sunday. Volunteers will remain at UBC for the full day on Saturday only. During the study, you will be asked to donate small samples of blood and consume normal meals provided by the investigators. One hundred dollars remuneration will be given upon completion of the study to compensate you for your time and travel expenses.

It is anticipated that this research will provide us with valuable information relating to the cause of the elevated cholesterol levels observed in your type of lipid disorder. This may help to improve treatment of this disease.

The testing will commence in the Fall of 1988. Please indicate your interest to be included in this research project by checking the appropriate response on the enclosed form and returning it to us by mail. A stamped, self addressed envelope is enclosed for your convenience.

Sincerely,

encl:

APPENDIX TWO SUBJECT INFORMATION SHEET FOR INDIVIDUALS SCREENED FOR TYPE III HLP TEST GROUP

CHOLESTEROL SYNTHESIS RESEARCH PROJECT
SUBJECT INFORMATION SHEET

Name_____ Age_____

Address_____

Phone (home)_____ (work)_____

Occupation/Place of Employment_____

Social Insurance #_____

Ethnic Background_____

Height_____ Weight_____

Siblings:

NAME AGE ADDRESS PHONE #

_____

Please answer the following questions. If your answer to any question is "yes", please give details.

Do you have any food allergies?

_____

Do you have any food restrictions &/or strong food preferences?

_____

Do you take any medication on a regular basis?

_____

_____ Yes, I would like to participate in the 3 day research project.

Month(s) available _____ Days available _____

No, I am not interested in participating in the 3 day research project.

ALL INFORMATION WILL BE KEPT STRICTLY CONFIDENTIAL

_____

APPENDIX THREE SUBJECT INFORMATION SHEET FOR INDIVIDUALS SCREENED FOR CONTROL GROUP

CHOLESTEROL SYNTHESIS RESEARCH PROJECT
SUBJECT INFORMATION SHEET

Name_____ Age_____

Address_____

Phone (home)_____ (work)_____

Occupation/Place of Employment_____

Social Insurance #_____

Ethnic Background_____

Height_____ Weight_____

Please answer the following questions. If your answer to any question is "yes", please give details.

Do you have any food allergies?

_____

_____

Do you have any food restrictions &/or strong food preferences?

_____

_____

Do you take any medication on a regular basis?

_____

_____

Have you ever been diagnosed as having a problem with you lipid (fat) metabolism? (e.g. high cholesterol or high fat levels?

_____

_____

Availability:

Month(s)_____ Days_____

_____

APPENDIX FOUR CONSENT BY SUBJECT OF RESEARCH PROTOCOL

CHOLESTEROL SYNTHESIS RESEARCH PROJECT
CONSENT FORM

Protocol #:_____ Subject Name:_____
Research Protocol: Cholesterol Synthesis in Type III Hyperlipoproteinemic and Non-Hyperlipidemic Individuals.

Research Directors: Shani Dendy
Dr. Peter Jones
Dr. Jiri Frohlich

I, _____, the undersigned, hereby consent to participate as a subject in the above-named research project conducted by the University of British Columbia. The nature of the procedure or treatment, its risks and/or benefits, and possible alternatives, follow:

I. Nature and Duration of Procedure:

The objective of the study is to compare the synthesis of cholesterol in individuals with normal lipid levels and individuals with type III hyperlipoproteinemia. This study involves your eating a prepared diet for 1 out of 3 days. This diet will be fed at a level which should maintain your normal body weight. On the second day you will fast for the entire day. You will be given drinking water containing a stable tracer. You will be required to remain in the testing facility from 8 am to 8 pm on day 2. Blood samples of 28 ml will be collected on 5 occasions during the three day study.

II. Potential Risks and/or Benefits:

There is no known hazard associated with the use of the stable labelled tags in the procedure. There are no risks of the procedure other than that normally associated with blood-taking.

The substance of the project and procedures associated with it have been fully explained to me, and all experimental procedures have been identified. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that I may withdraw my consent at any time. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained. Confidentiality of the records concerning my involvement in this project will be maintained in an appropriate manner. I understand that I will receive $100.00 upon completion of the study. If I decide to withdraw before completion of the study, I will receive an appropriate prorated fraction of this amount.

I acknowledge receiving a copy of this consent form and all appropriate attachments.

Doctor:_____
Witness:_____
Date:_____ Time _____ am/pm

_____
Signature of Subject
If relative or legal representative signs, please indicate relationship or other authority.

APPENDIX FIVE DESCRIPTION OF RESEARCH PROJECT PROTOCOL

CHOLESTEROL SYNTHESIS RESEARCH PROJECT
DESCRIPTION OF RESEARCH PROJECT PROTOCOL

Research Directors:
Shani Dendy, Dr. Peter Jones, Dr. Jiri Frohlich
Division of Human Nutrition / University Hospital, Shaughnessy Site Lipid Clinic
The University of British Columbia

THE RESEARCH PROJECT WILL CONSIST OF TWO PHASES:

PHASE I: INITIAL SUBJECT SCREENING (Fall, 1988)

- To identify appropriate test and control subjects for the experimental trial
- ALL volunteers will complete a Subject Information Sheet
- SOME volunteers will donate a small sample (28 ml = 2 tbsp) of blood
- NOT ALL volunteers screened will be asked to participate in the experimental trial

PHASE II: EXPERIMENTAL TRIAL (Fall, 1988 and Spring, 1989)

The experimental trial will take place over a period of 3 consecutive days, at the University of British Columbia (UBC) Metabolic Laboratory/Kitchen, most likely on a Friday, Saturday and Sunday.

A. TWO WEEKS BEFORE THE EXPERIMENTAL TRIAL:

1. Subjects will meet at the UBC Metabolic Laboratory/Kitchen for an orientation meeting

   - height, weight, and daily activity information will be obtained
   - food record instructions will be given

B. DURING THE EXPERIMENTAL TRIAL:

1. Subjects will consume only the test meals provided by the Research Directors

   - Day 0 (day before study begins): subjects will fast after consuming the evening meal
   - Day 1: 3 complete meals will be provided
   - Day 2: FAST from evening meal on Day 1 until the breakfast meal on Day 3
   - Day 3: breakfast snack provided

2. Subjects will be given a priming bolus dose of deuterium oxide (D_2O) on Day 1 and drinking water that contains deuterium on Days 1 and 2

3. Subjects will donate 5 small samples of blood (28 ml each = 2 tbsp)

   - Day 1: Blood Sample #1 = before consuming deuterium oxide (D_2O) bolus dose
     Blood Sample #2 = HOUR 12 post D_2O bolus dose
   - Day 2: Blood Sample #3 = HOUR 24 post D_2O bolus dose
     Blood Sample #4 = HOUR 36 post D_2O bolus dose
   - Day 3: Blood Sample #5 = HOUR 48 post D_2O bolus dose

4. Subjects will report to the UBC Metabolic Laboratory/Kitchen at the following times:

   - Day 1: 7:00 am-8:30 am and 7:00 pm-8:30 pm
   - Day 2: 8:00 am-9:00 pm (all day)
   - Day 3: 8:00 am-9:00 am

APPENDIX SIX FOOD RECORD INSTRUCTIONS

CHOLESTEROL SYNTHESIS RESEARCH PROJECT
FOOD RECORD INSTRUCTIONS

PLEASE READ CAREFULLY:

1. Write down EVERYTHING you eat and drink each day. Be sure to include all SNACKS and ALCOHOL. Record immediately after each meal and snack to ensure accuracy.

2. Write down HOW MUCH you eat and drink each day:

   A. Use VOLUME measures (cup, tbsp, tsp, or ml) for cereals, cooked rice and pasta, vegetables, canned fruit, peanut butter, mayonnaise, salad dressing, butter, margarine, sauces, gravies, soups, sugar, jam, beverages, etc.

   B. Use COOKED WEIGHTS (ounces or grams) for meat, fish, and poultry.

      Note: the weight of meat, poultry and fish decreases by about 25% during cooking.
      Examples:
      4 oz. raw beef shrinks to 3 oz. cooked beef
      6 oz. raw cod shrinks to 4.5 oz. cooked cod

   C. Use SIZE for raw fruits, muffins, crackers, cakes, pies, cookies, desserts, etc. Give dimensions.
      Example:
      1 oatmeal cookie, 1" in diameter

   D. Be specific about the TYPE OF FOOD, BRAND NAME IF APPLICABLE, HOW THE FOOD WAS PREPARED, AND CONTENT OF MIXED DISHES.

   E. For combination items, list each item separately.
      Example:
      a cheese cheeseburger would be described as: bun, cooked ground beef, processed cheese, butter, relish, etc.

   F. IF THE FOOD IS PREPARED BY SOMEONE OTHER THAN YOURSELF, please try to estimate the portion size and describe the contents of the dish that is served to you.

   G. Don't forget the EXTRAS!
      Examples:
      sugar on cereal or in coffee
      dressing on salad
      candy, soft drinks, alcohol

EXAMPLE:

TIME	FOOD ITEM DESCRIPTION	AMOUNT	LOCATION
Breakfast	2% milk	1/2 cup	Smitty's Restaurant
	whole wheat bread	2 slices	
	margarine	2 tsp	
	strawberry jam	2 tsp	
	omelette:		
	eggs	2 large	
	cheese	1/2 oz.	
	orange juice	6 oz	

APPENDIX SEVEN DIET FED TO CONTROL AND TYPE III HLP SUBJECTS ON FEEDING DAY OF EXPERIMENTAL TRIAL

Meal	Food Item	Quantity[1]	
Breakfast:	whole wheat bread	60	g
	2% milk	260	ml
	soft sunflower margarine	15	g
	Kellogg's bran flakes	55	g
	seedless raisins	25	g
	apple juice	145	ml
	omelette:		
	brick cheese	35	g
	egg whites	1.5	whites
	egg yolk	0.5	yolk
	butter	6.5	g
	soft sunflower margarine	4.5	g
Lunch:	whole wheat bread	50	g
	soft sunflower margarine	10	g
	dip:		
	1.55% yogurt	25	g
	Miracle Whip	15	g
	macaroni and cheese:		
	cooked macaroni	160	g
	evaporated 2% milk	100	ml
	cheddar cheese	35	g
	soft sunflower margarine	13	g
	egg	0.5	egg
	chopped onion	60	g
	carrot sticks	60	g
	celery sticks	60	g
	fresh pear	1	6.4 cm x 8.9 cm
Dinner:	whole wheat bread	30	g
	soft sunflower margarine	10	g
	cooked chicken thigh (no skin)	150	g
	hollandaise sauce:		
	egg yolk	0.38	yolk
	lemon juice	3.75	ml
	butter	10	g
	cooked rice	150	g
	frozen peas	75	g
	frozen carrots	75	g
	vanilla ice milk	75	g
	Hershey's chocolate syrup	35	g
	Cool Whip	15	ml

[1]diet contains 3000 kcal and food item quantities were modified to meet the estimated daily caloric requirements for each subject.

APPENDIX EIGHT ESTIMATED DAILY CALORIC REQUIREMENTS OF CONTROL AND TYPE III HLP SUBJECTS ADMINISTERED ON FEEDING DAY OF EXPERIMENTAL TRIAL

Subject by Group	Body Surface Area[1]	Basal Metabolic Rate[2]	Reported Activity	Activity Factor[3]	Daily Caloric Requirement[4]
	(meters^2)	(kcal/24 hrs)			(kcal/24 hrs)
Controls:					
CD	1.90	1730	-run, 3 miles,5x/wk -weights, light, 3x/wk -hockey, .5hr, 2x/wk -WORKING	1.7	2914
MKa	1.84	1670	-bike, walk, 4x/wk -WORKING	1.7	2839
JG	1.91	1660	-swim, 15 min, 2x/wk -bike, 10 min, 2x/wk -RETIRED	1.5	2490
DD	1.84	1596	-YMCA aerobics, 45 min,5x/wk -WORKING	1.7	2713
EM	1.86	1620	-walk, periodically -SEMI-RETIRED	1.5	2430
RS	1.99	1730	-swim, 1 hr, 4x/wk -walk, 4 km/wk -RETIRED	1.5	2595
HD	1.66	1370	-walk, 1 mile, 2x/wk -WORKING	1.5	2055
MW	1.64	1343	-walk, 10 blks/day -RETIRED	1.5	2015
Mean (±SEM)					2506.4 (117.9)
Type III HLPs:					
NN	1.85	1675	-bike, 45 min/day -sit-ups, push-ups/day -WORKING	1.7	2847
WJ	2.25	1800	-bike, 100km/wk -row, 15 min/day -weights, sit-ups, 10 min/day -WORKING	1.7	3060
ES	1.77	1540	-RETIRED	1.5	2310
JB	1.88	1630	-WORKING	1.5	2445
AT	1.90	1645	-walk, 1.5 hrs, 2x/wk -sit-ups, 500/day -RETIRED	1.5	2468
MKu	1.64	1320	-bike, 10 min/day -walk, 1hr/day -swim, .5 hr/day -RETIRED	1.5	1980
DP	1.69	1360	-walk, 3 miles/day -WORKING	1.5	2040
EK	1.48	1210	-walk, 2 miles/wk -RETIRED	1.5	1815
Mean (±SEM)					2370.6 (151.9)[5]

[1]obtained from Mayo Clinic Nomogram using ideal weight and height; [2]obtained from Mayo Clinic Nomogram using surface area, age and sex; [3]determined from references Bogert et al. 1973 and Bell et al. 1985; [4]basal metabolic rate x activity factor; [5]$p = 0.49$

APPENDIX NINE ESTIMATED TOTAL BODY WATER CONTENT OF CONTROL AND TYPE III HLP SUBJECTS AND CORRESPONDING DEUTERIUM OXIDE BOLUS DOSE AND DEUTERIUM LABELLED DRINKING WATER ADMINISTERED DURING EXPERIMENTAL TRIAL

Subject by Group	Weight[1]	Total Body Water[2]	D_2O Bolus Dose[3] Theor.	Exp.	Deuterium Labelled Drinking Water Fed	Fasted
	(kg)	(kg)	(g)		(g D_2O/l H_2O)	
Controls:						
CD	83.2	49.9	34.9	34.96332	1.4	0.7
MKa	79.5	47.7	33.4	33.43536	1.4	0.7
JG	78.3	47.0	32.9	32.92700	1.4	0.7
DD	79.6	47.8	33.4	33.43000	1.4	0.7
EM	75.5	45.3	31.7	31.72262	1.4	0.7
RS	74.9	44.9	31.5	31.51710	1.4	0.7
HD	57.7	34.6	24.2	24.23900	1.4	0.7
MW	77.6	46.6	32.6	32.63749	1.4	0.7
Type III HLPs:						
NN	84.2	50.5	35.4	35.41171	1.4	0.7
WJ	91.3	54.8	38.3	38.32930	1.4	0.7
ES	86.0	51.6	31.0	31.01200	1.2[3]	0.6[3]
JB	84.2	50.5	35.4	35.40389	1.4	0.7
AT	84.7	50.8	35.6	35.60550	1.4	0.7
MKu	71.3	42.8	29.9	29.91295	1.4	0.7
DP	109.3	65.6	39.4	39.43600	1.2	0.6
EK	54.9	32.9	23.0	23.02661	1.4	0.7

[1]obtained at orientation meeting prior to experimental trial; [2]60% of body weight; [3]0.7 g D_2O/kg total body water, however 0.6 g D_2O/kg total body water given when deuterium supply limited; [3]due to limited deuterium supply

APPENDIX TEN RESULTS OF APO E PHENOTYPE AND PLASMA LIPID CONCENTRATION ANALYSES IN SUBJECTS SCREENED FOR CONTROL GROUP

Subject by Phenotype	Total Cholesterol	Triglyceride	E3:2 Ratio	Age	Sex
	(mmol/l)	(mmol/l)		(years)	
E2/2					
CD	4.12	1.19	-	35	M
E3/2					
RD	-	-	0.99	28	M
SO	4.09	0.82	0.97	26	F
KJ	4.11	0.82	1.14	38	F
MB	6.25	2.52	0.97	45	M
BT	5.20	2.54	0.86	38	M
LE	5.35	1.29	0.98	63	M
AD	6.15	1.12	0.94	57	F
ML	6.09	1.54	1.11	56	F
DT	6.38	1.95	0.70	28	F
BS	6.01	0.94	1.18	42	M
HD	6.35	1.11	0.97	52	F
GB	5.19	1.53	1.06	68	M
MW	4.76	2.25	0.82	73	F
ED	7.46	1.68	1.11	66	F
AM	5.75	1.81	1.11	69	F
JG	4.89	1.04	0.91	68	M
DD	5.66	1.08	0.83	53	M
EM	6.07	1.38	0.97	62	M
JO-R	4.52	1.23	0.91	61	F
JJ	5.80	2.56	0.79	68	M
JC	4.99	1.60	1.08	71	M
RS	4.87	2.11	1.03	67	M
M-LS	8.36	3.34	0.84	60	F
E3/3					
WW	4.27	0.55	1.49	37	F
AT	5.28	1.03	1.26	32	M
RR	5.14	1.67	1.22	62	M
E4/3					
MS	3.43	0.85	-	40	F
EE	5.41	1.33	-	42	F
WM	4.60	0.82	-	40	M
DS	4.49	1.17	-	36	M
MC	4.70	4.56	-	53	F
TF	5.88	1.77	-	40	M
MT	5.82	1.99	-	61	F
DR	6.59	1.33	-	60	F
BR	4.96	1.38	-	66	F

APPENDIX ELEVEN BODY WEIGHT FLUCTUATIONS OF CONTROL AND TYPE III HLP SUBJECTS THROUGHOUT EXPERIMENTAL TRIAL

Subject by Group	Day 1 (Feeding)	Day 2 (Fasting)	Day 3 (Fasting)	Weight Change Feeding[1]	CV	Total[2]	CV
		(kg)			(%)		(%)
Controls:							
CD	81.5	82.5	81.0	1.0	0.86	-0.5	0.94
MKa	81.6	81.8	80.8	0.2	0.17	-0.8	0.65
JG	77.4	76.8	75.8	-0.6	0.55	-1.6	1.05
DD	80.1	80.0	78.9	-0.1	0.09	-1.2	0.84
EM	74.8	74.1	73.4	-0.7	0.67	-1.4	0.95
RS	73.8	74.5	72.3	0.7	0.67	-1.5	1.53
HD	59.0	58.5	58.0	-0.5	0.60	-1.0	0.86
MW	77.3	77.2	75.8	-0.1	0.09	-1.5	1.09
Mean	75.7	75.7	74.5	0.0	0.46	-1.2	0.99
±SEM	2.6	2.7	2.6	0.2	0.11	0.1	0.09
Type III HLPs:							
NN	82.6	82.6	81.3	0.0	0.00	-1.3	0.91
WJ	92.5	90.1	89.6	-2.4	1.86	-2.9	1.71
ES	86.0	86.4	84.9	0.4	0.33	-1.1	0.91
JB	83.3	82.6	81.3	-0.7	0.60	-2.0	1.23
AT	85.1	-	83.5	-	-	-1.6	1.34
MKu	68.4	68.3	66.4	-0.1	0.10	-2.0	1.67
DP	108.3	108.0	106.0	-0.3	0.20	-2.3	1.16
EK	54.6	54.1	53.1	-0.5	0.65	-1.5	1.42
Mean	82.6	81.7	80.8	-0.5	0.53	-1.8	1.29
±SEM	5.6	6.4	5.5	0.3	0.24	0.2	0.11
	p=0.28	p=0.38	p=0.32	p=0.23	p=0.78	p=0.02	p=0.05

[1]Day 2-Day 1; [2]Day 3-Day 1

APPENDIX TWELVE PLASMA WATER DEUTERIUM ENRICHMENT IN BLOOD SAMPLE DRAWN FROM CONTROL AND TYPE III HLP SUBJECTS DURING EXPERIMENTAL TRIAL

Subject by Group	Plasma water $^2H/^1H$ relative to SMOW[1] Baseline[2]	24 hr[3]	corrected 24 hr[4]
	(parts per thousand [‰])		
Controls:			
CD	-100	700.2 (7.0)	4775.9
MKa	-100	647.5 (5.1)	4460.0
JG	-100	680.6 (0.2)	4658.3
DD	-100	542.3	3828.8
EM	-100	595.2 (6.2)	4145.9
RS	-100	562.7 (3.6)	3950.9
HD	-100	649.1	4469.6
MW	-100	820.5	5498.0
Type III HLPs:			
NN	-100	662.9 (0.1)	4552.1
WJ	-100	686.6 (9.3)	4694.6
ES	-100	680.6 (2.5)	4658.3
JB	-100	702.7 (8.1)	4791.2
AT	-100	675.4 (0.7)	4627.4
MKu	-100	770.5 (7.2)	5198.0
DP	-100	777.6 (47.9)	5240.6
EK	-100	749.5 (20.4)	5071.7

[1]Standard Mean Ocean Water; [2]theoretical value based on Vancouver tap water deuterium enrichment; [3]mean (SD) of duplicate samples except when single sample only available; [4]correction for dilution of plasma with Vancouver tap water, and deuterium enrichment of the diluent (sample calculation below)

Corrected plasma water deuterium enrichment sample calculation:

Subject NN:

-plasma dilution: 1 part plasma:5 parts Vancouver tap water
-plasma deuterium enrichment= 662.9 ‰
-Vancouver tap water deuterium enrichment= -100 ‰
-baseline plasma deuterium enrichment= -100 ‰
-to equate H2O content of plasma (95% H2O, 5% solids) and diluent (100% water, 0% solids), 0.95 correction factor applied to diluent

=(undiluted plasma ‰) - (Vancouver tap water ‰) - (baseline plasma ‰)
=(662.9 ‰ x 6) - ([-100 ‰ x 5] x 0.95) - (-100 ‰)
=4552.1

APPENDIX THIRTEEN PLASMA FREE CHOLESTEROL DEUTERIUM ENRICHMENT IN BLOOD SAMPLES DRAWN FROM CONTROL AND TYPE III HLP SUBJECTS DURING EXPERIMENTAL TRIAL

Subject by Group	Plasma free cholesterol $^2H/^1H$ relative to SMOW[1]		
	0 hr[2]	24 hr	48 hr
	(parts per thousand [‰])		
Controls:			
CD	-290.6 (4.7)	-174.4 (10.5)	-194.6 (1.0)
MKa	-283.2 (2.4)	-133.5 (1.2)	-165.9 (1.9)
JG	-293.0 (5.9)	-142.9 (1.1)	-95.9 (0.1)
DD	-297.0 (1.1)	-170.6 (8.3)	-173.4 (6.6)
EM	-304.8 (5.0)	-123.5 (2.6)	-173.2 (0.2)
RS	-280.0 (16.4)	-118.5 (2.4)	-124.0 (3.3)
HD	-306.0 (1.8)	-137.2 (0.3)	-149.6 (0.6)
MW	-247.4 (87.4)	-20.0 (28.7)	42.6 (2.5)
Type III HLPs:			
NN	-292.4 (26.4)	-200.2 (2.9)	-201.4[3]
WJ	-282.0 (28.9)	-140.5 (0.1)	-157.3 (1.9)
ES	-300.9 (2.9)	-190.2 (1.5)	-182.9 (3.2)
JB	-290.7 (14.1)	-176.8 (2.2)	-150.9 (2.3)
AT	-303.2	-155.8 (2.5)	-175.1 (3.0)
MKu	-295.6 (3.4)	-235.1 (2.5)	-227.7 (9.8)
DP	-113.1 (4.9)	-19.9 (9.3)	32.5 (28.0)
EK	-292.1 (15.5)	2.1 (28.5)	37.9 (2.2)

[1]Standard Mean Ocean Water; [2]numbers are mean (SD) of triplicate samples; [3]duplicate samples identical

Cholesterol FSR sample calculation:

Subject NN, 0-24 hr interval:

-cholesterol deuterium enrichment at hour 0= -292.4 ‰
-cholesterol deuterium enrichment at hour 24 = -200.2 ‰
-plasma water deuterium enrichment= 4552.1 ‰

$$\text{FSR (per day)} = \frac{[-200.2\ ‰ - (-292.4)]}{4552.1\ ‰ \times 0.81\ ^2H/C \times 27C/46H}$$

$$= 0.042 \text{ per day}$$

APPENDIX FOURTEEN PLASMA FREE CHOLESTEROL DEUTERIUM ENRICHMENT BLOOD SAMPLES DRAWN FROM CONTROL AND TYPE III HLP SUBJECTS AT 12 HOUR INTERVALS DURING EXPERIMENTAL TRIAL

Subject by Group	Plasma free cholesterol $^2H/^1H$ relative to SMOW[1]				
	0 hr[2]	12 hr	24 hr	36 hr	48 hr
	(parts per thousand [o/oo])				
Controls:					
CD	-290.6 (4.7)	-251.2 (2.8)	-174.4 (10.5)	-186.2 (8.8)	-194.6 (1.0)
MKa	-283.2 (2.4)	-211.0 (3.0)	-133.5 (1.2)	-160.6 (1.1)	-165.9 (1.9)
JG	-293.0 (5.9)	-216.8 (2.7)	-142.9 (1.1)	-96.4 (0.1)	-95.9 (0.1)
DD	-297.0 (1.1)	-241.0 (0.3)	-170.6 (8.3)	-193.1 (3.5)	-173.4 (6.6)
EM	-304.8 (5.0)	-219.5 (0.4)	-123.5 (2.6)	-164.4 (3.9)	-173.2 (0.2)
RS	-280.0 (16.4)	-191.1 (1.0)	-118.5 (2.4)	-117.4 (1.0)	-124.0 (3.3)
HD	-306.0 (1.8)	-226.8 (9.7)	-137.2 (0.3)	-148.3 (1.5)	-149.6 (0.6)
MW	-247.4 (87.4)	-64.0 (3.8)	-20.0 (28.7)	50.3 (5.8)	42.6 (2.5)
Type III HLPs:					
NN	-292.4 (26.4)	-266.4 (4.9)	-200.2 (2.9)	-201.6 (3.8)	-201.4[3]
WJ	-282.0 (28.9)	-232.1 (0.7)	-140.5 (0.1)	-160.0 (1.7)	-157.3 (1.9)
ES	-300.9 (2.9)	-241.0 (16.6)	-190.2 (1.5)	-191.03 (1.7)	-182.9 (3.2)
JB	-290.7 (14.1)	-205.2 (26.4)	-176.8 (2.2)	-186.1 (2.3)	-150.9 (2.3)
AT	-303.2	-208.6 (51.1)	-155.8 (2.5)	-173.3 (1.6)	-175.1 (3.0)
MKu	-295.6 (3.4)	-281.7 (5.2)	-235.1 (2.5)	-236.2 (7.3)	-227.7 (9.8)
DP	-113.1 (4.9)	-85.4 (1.2)	-19.9 (9.3)	41.7 (8.8)	32.5 (28.0)
EK	-292.1 (15.5)	-155.3 (5.5)	2.1 (28.5)	5.5 (5.5)	37.9 (2.2)

[1]Standard Mean Ocean Water; [2]numbers are mean (SD) of triplicate samples; [3]duplicate samples identical

APPENDIX FIFTEEN CHOLESTEROL FRACTIONAL SYNTHETIC RATE IN CONTROL AND TYPE III HLP SUBJECTS OVER 12 HOUR TIME INTERVALS DURING EXPERIMENTAL TRIAL

Subject by Group	Fed State 0-12 hr	Fed State 12-24 hr	Fed State 0-24 hr	Fasted State 24-36 hr	Fasted State 36-48 hr	Fasted State 24-48 hr	0-48 hr
			(FSR[1] [per day])				
Controls:							
CD	0.035	0.067	0.051	-0.010	-0.007	-0.009	0.021
Mka	0.068	0.073	0.070	-0.025	-0.005	-0.015	0.028
JG	0.068	0.066	0.067	0.042	0.0004	0.021	0.044
DD	0.061	0.077	0.069	-0.025	0.022	-0.002	0.034
EM	0.086	0.097	0.092	-0.041	-0.009	-0.025	0.033
RS	0.094	0.077	0.086	0.001	-0.007	-0.003	0.041
HD	0.074	0.084	0.079	-0.010	-0.001	-0.006	0.037
MW	0.140	0.034	0.087	0.054	-0.006	0.024	0.055
Mean	0.078	0.072	0.075	-0.002	-0.002	-0.002	0.037
±SEM	0.011	0.006	0.005	0.012	0.004	0.006	0.004
Type III HLPs:							
NN	0.024	0.061	0.042	-0.001	0.0002	-0.001	0.021
WJ	0.045	0.082	0.063	-0.017	0.002	-0.008	0.028
ES	0.054	0.046	0.050	-0.001	0.007	0.003	0.027
JB	0.075	0.025	0.050	-0.008	0.031	0.011	0.031
AT	0.086	0.048	0.067	-0.016	-0.002	-0.009	0.029
MKu	0.011	0.038	0.024	-0.001	0.007	0.003	0.014
DP	0.022	0.052	0.037	0.049	-0.007	0.021	0.029
EK	0.113	0.130	0.121	0.003	0.027	0.015	0.068
Mean	0.054	0.060	0.057	0.001	0.008	0.004	0.031
±SEM	0.013	0.012	0.010	0.007	0.005	0.004	0.006
All Subjects:							
Mean	0.066	0.066	0.066	-0.0004	0.003	0.001	0.034
±SEM	0.009	0.007	0.006	0.007	0.003	0.004	0.003

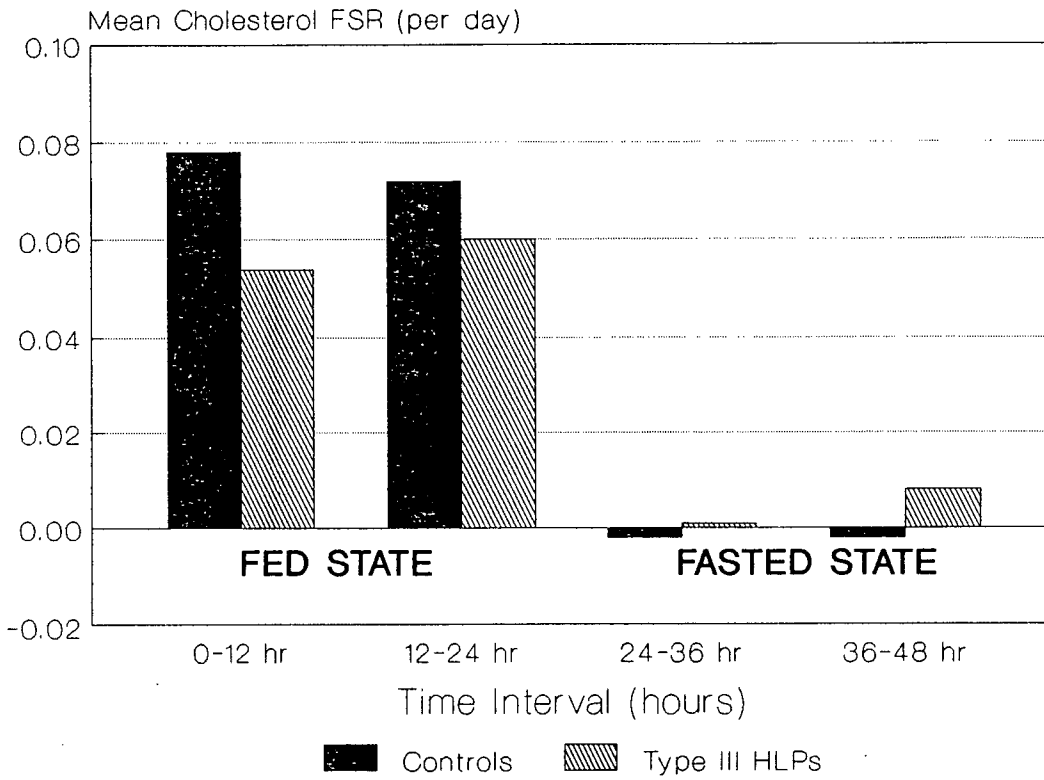
[1]Fractional Synthetic Rate

APPENDIX SIXTEEN INDIVIDUAL FREE CHOLESTEROL NET SYNTHESIS PER DAY BASED ON INDIVIDUAL M1 (CENTRAL) TOTAL CHOLESTEROL POOL SIZE IN CONTROL AND TYPE III HLP SUBJECTS OVER 12 HOUR TIME INTERVALS DURING EXPERIMENTAL TRIAL

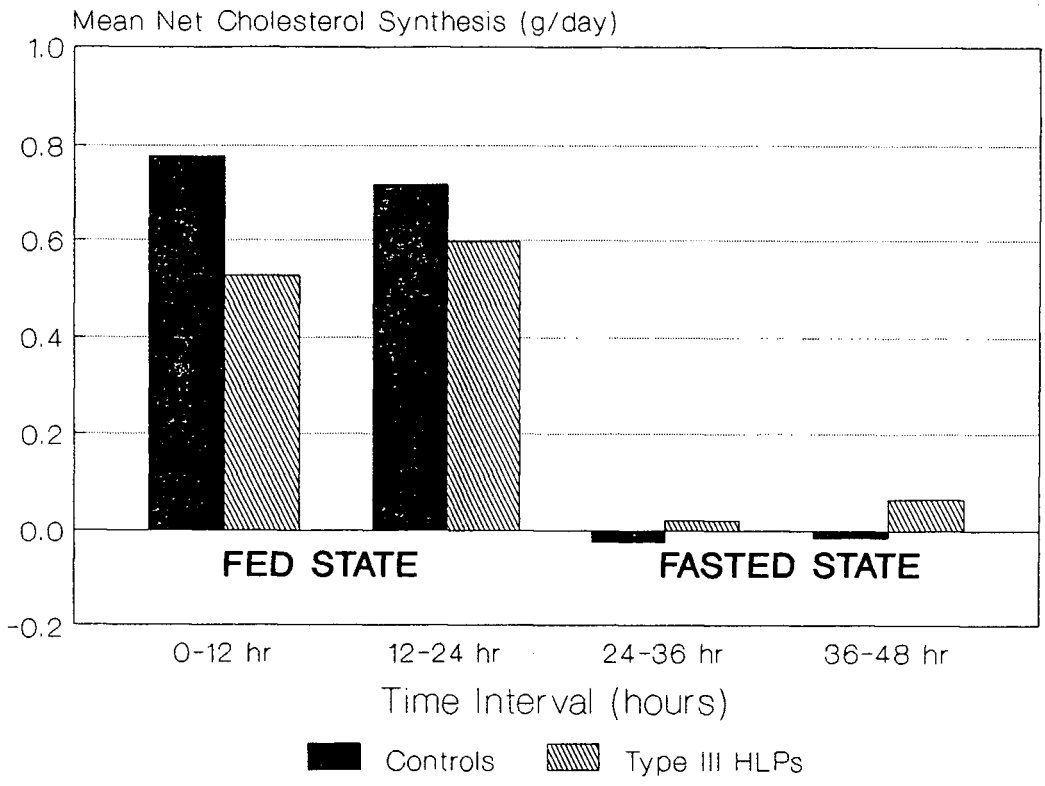
Subject by Group	M1 Pool[1]	Fed State 0-12 hr	Fed State 12-24 hr	Fed State 0-24 hr	Fasted State 24-36 hr	Fasted State 36-48 hr	Fasted State 24-48 hr	0-48 hr
		(g free cholesterol synthesized per day)[2]						
Controls:								
CD	25.1	0.351	0.673	0.512	-0.100	-0.070	-0.090	0.211
MKa	25.3	0.688	0.739	0.708	-0.253	-0.051	-0.152	0.283
JG	24.7	0.672	0.652	0.662	0.415	0.004	0.207	0.435
DD	27.0	0.659	0.832	0.745	-0.270	0.238	-0.022	0.367
EM	26.5	0.912	1.028	0.975	-0.435	-0.095	-0.265	0.350
RS	24.7	0.929	0.761	0.850	0.010	-0.069	-0.030	0.405
HD	21.6	0.639	0.726	0.683	-0.086	-0.009	-0.052	0.320
MW	24.6	1.378	0.335	0.856	0.531	-0.059	0.236	0.541
Mean	24.9	0.778	0.718	0.749	-0.024	-0.014	-0.021	0.364
±SEM	0.6	0.106	0.069	0.050	0.119	0.038	0.060	0.035
Type III HLPs:								
NN	27.5	0.264	0.671	0.462	-0.011	0.002	-0.011	0.231
WJ	30.0	0.540	0.984	0.756	-0.204	0.024	-0.096	0.336
ES	26.8	0.579	0.493	0.536	-0.011	0.075	0.032	0.289
JB	25.3	0.759	0.253	0.506	-0.081	0.314	0.111	0.314
AT	28.3	0.974	0.543	0.758	-0.181	-0.023	-0.102	0.328
MKu	21.6	0.095	0.328	0.207	-0.009	0.060	0.026	0.121
DP	33.2	0.292	0.691	0.491	0.651	-0.093	0.279	0.385
EK	15.8	0.714	0.822	0.765	0.019	0.171	0.095	0.430
Mean (all subj.)	26.1	0.527	0.598	0.560	0.022	0.066	0.042	0.304
±SEM	1.9	0.104	0.086	0.068	0.095	0.045	0.044	0.034
Mean (no subj. EK)	27.5	0.500	0.566	0.531	0.022	0.051	0.034	0.286
±SEM	1.4	0.116	0.093	0.071	0.109	0.049	0.050	0.033
All Subjects:								
Mean	25.5	0.653	0.658	0.655	-0.001	0.026	0.010	0.334
±SEM	1.0	0.079	0.056	0.048	0.074	0.030	0.037	0.025

[1]see Table 7;[2]free cholesterol net synthesis per day (g)=size of M1 total cholesterol pool (g) x cholesterol FSR corresponding to time interval x 0.4. Factor 0.4 used as it is estimated that approximately 40% of the M1 (central) total cholesterol pool is free cholesterol (Jones et al. 1988).

APPENDIX SEVENTEEN CHOLESTEROL FRACTIONAL SYNTHETIC RATE IN CONTROL AND TYPE III HLP SUBJECTS OVER 12 HOUR TIME INTERVALS DURING EXPERIMENTAL TRIAL



APPENDIX EIGHTEEN INDIVIDUAL FREE CHOLESTEROL NET SYNTHESIS PER DAY BASED ON INDIVIDUAL M1 (CENTRAL) TOTAL CHOLESTEROL POOL SIZE IN CONTROL AND TYPE III HLP SUBJECTS OVER 12 HOUR TIME INTERVALS DURING EXPERIMENTAL TRIAL



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